ONLINE METHODS

HCVcc. Plasmids containing the full-length HCV JFH-1 genome (pJFH1)[36], full-length HCV JFH-1 genome with a G451R mutation in the E2 glycoprotein (pJFH-1^{G451R})[37] and the eight intergenotypic clones (described in ref. 21) were XbaI linearized and transcribed using MEGAscript T7 (Ambion), and 10 μg *in vitro*–transcribed RNA was electroporated (BioRad) into Huh7 cells[38]. We generated HCVcc viral stocks by infecting naive Huh7 cells at an MOI of 0.01 FFU per cell with medium from Huh7 cells electroporated with *in vitro*–transcribed RNA from pJFH-1–based vectors, as previously described[38].

Treatments and analysis. Huh7 cultures were established as previously described[38]. We performed RNA silencing experiments by reverse transfection (Lipofectamine RNAiMAX, Invitrogen) of siRNAs into Huh7 cells. Transfected cells were infected with equal titers of HCVpp or VSVGpp or HCVcc at an MOI of 0.05 FFU per cell) at the indicated times after transfection (**Fig. 1c–e**). For antibody experiments, we treated cells with 36 μg ml^{-1} of antibody before and during infection with HCVcc at an MOI of 0.05 FFU per cell. For ezetimibe inhibition experiments, cells were vehicle-treated or treated with increasing concentrations of ezetimibe (Sequoia Research Products) before infection, during the time of virus inoculation and/or after virus inoculation with HCVcc at an MOI of 0.1–1.0 FFU per cell. The ezetimibe concentrations of 3.125–30 μM (that is, 1.5–12.28 μg per ml culture medium) used in this study are consistent with previously published reports[19,20,39] and are additionally in line with patient daily intake concentrations of 10 mg d^{-1} (that is, 2.0–3.3 μg per ml of serum). For RT-qPCR analysis, we isolated total cellular RNA from triplicate culture wells after infection or transfection. For HCV E2–positive foci analysis, we fixed infected cells with 4% (wt/vol) paraformaldehyde 72 h after infection, and immunocytochemical staining for HCV E2 was performed. See **Supplementary Methods** for further details.

HCV infection in chimeric mice. All mouse studies were conducted with protocols approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Male uPA-SCID mice transplanted with human hepatocytes (BD Biosciences[35]) were purchased from PhenixBio[40]. Mice were treated daily with 10 mg per kg body weight ezetimibe via oral gavage of a 0.02 mg ml^{-1} solution of ezetimibe resuspended in corn oil (100 μl 20g^{-1}) for a total of 3 weeks, with treatment initiation beginning 2 weeks, 1 week or 2 d before infection[16]. Control mice were treated via oral gavage with corn oil alone (100 μl per 20 g body weight). A total of four to seven mice were included in each group. On day 0, we intravenously inoculated mice with serum from HCV-infected humans containing 1.0×10^5 copies of HCV genotype 1b. Mouse serum samples were obtained for HCV RNA or human albumin determination by RT-qPCR and Alb-II Kit (Eiken Chemical), respectively.

Statistical analyses. Data are presented as the means ± s.d. We determined significant differences by one-way ANOVA followed by Tukey's post-hoc *t* test (GraphPad Prism software). To compare categorical variables we used a two-tailed Fisher's exact test (SPSS). In all cases, a P value <0.05 was considered statistically significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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HEPATOLOGY

Interleukin-28B single nucleotide polymorphism of donors and recipients can predict viral response to pegylated interferon/ribavirin therapy in patients with recurrent hepatitis C after living donor liver transplantation

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Key words
core, hepatitis C virus, interferon sensitivity-determining region, interleukin-28B, liver transplantation.

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Abstract

Background and Aim: Interleukin-28B (*IL28B*) single nucleotide polymorphism (SNP) influences viral response (VR) to interferon (IFN) therapy in patients with hepatitis C. We studied the relationship between VR and the *IL28B* polymorphism (rs8099917) in patients on long-term pegylated IFN plus ribavirin (PEGIFN/RBV) therapy for recurrent hepatitis C after living-donor liver transplantation (LDLT).

Methods: Thirty-five patients with recurrent hepatitis C after LDLT were treated with PEGIFN/RBV. We evaluated the effect of *IL28B* SNP on the outcome in 20 patients infected with hepatitis C virus genotype 1 who completed IFN therapy.

Results: The sustained VR (SVR) rate was 54% (19/35) for all patients; 46% (13/28) for genotype 1. The SVR rate of donors' TT group (major genotype) was higher than that of donors' TG + GG group (minor genotype) (73% *vs* 20%), while that of recipients' TT group was similar to that of recipients' TG + GG group (64% *vs* 50%). With regard to the combined effect of donors' and recipients' *IL28B* SNP, the SVR rates of TT : TT (donors' : recipients'), TT : TG + GG, TG + GG : any group were 81%, 50%, and 20%, respectively. The VR rate of TT : TT, TT : TG + GG and TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, 20%, and those at the end of treatment were 100%, 50%, 20%, respectively. The multivariate analysis identified *IL28B* of donors : recipients (TT : TT) as the only independent determinant of SVR (odds ratio 15.0, $P = 0.035$).

Conclusion: Measurement of donors' and recipients' *IL28B* SNP can predict the response to PEGIFN/RBV therapy, and the donors' *IL28B* SNP might be a more significant predictor than that of the recipients.

Introduction

Hepatitis C virus (HCV) has infected 170 million people worldwide, and such infection sometimes progresses to liver cirrhosis and/or hepatocellular carcinoma.[1] The current treatment for patients infected with HCV genotype 1 (HCV-1) is the combination of pegylated interferon-α and ribavirin (PEGIFN/RBV) for 48 weeks.[2] However, this treatment results in sustained viral response (SVR) in only approximately 50% of patients with HCV-1 infection.

In a recent genome-wide association study, a single nucleotide polymorphism (SNP) upstream of the interleukin (IL)-28B (*IL28B*) gene on chromosome 19, coding for IFN-λ-3, was found to be strongly associated with SVR rate in treatment-adherent HCV-1 patients.[3–8] The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas a T nucleotide was found to be associated with a fair response to treatment (major allele) in Japanese patients.

HCV-related end-stage liver disease is currently the leading indication for liver transplantation (LT). However, the outcome of LT for patients with HCV-related liver disease has been less satisfactory than those with HCV-negative liver disease.[9–15] HCV recurrence is universal after LT with accelerated progression of liver fibrosis. Approximately 20–25% of HCV-positive

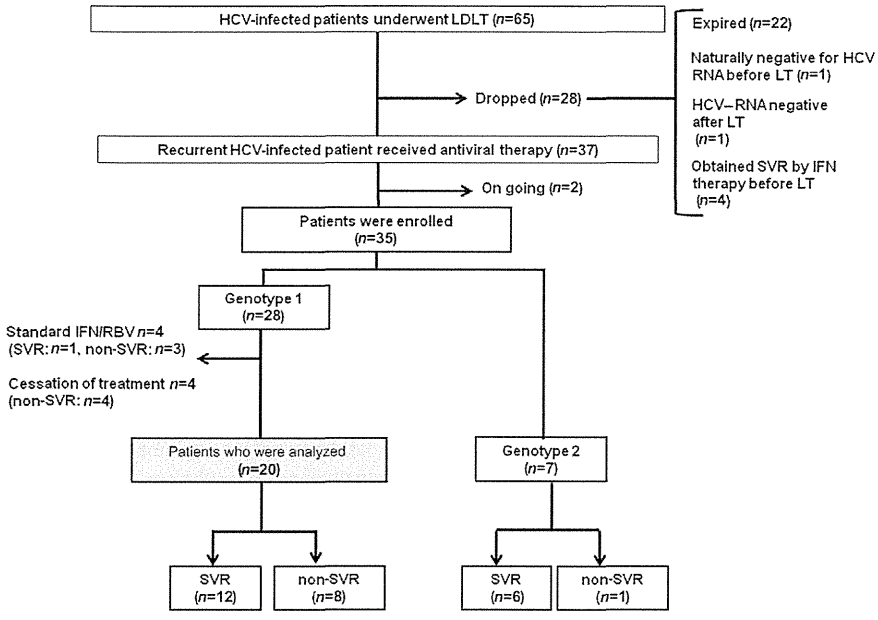

Figure 1 Flow diagram of patient recruitment. HCV, hepatitis C virus; IFN, interferon; LDLT, living-donor liver transplantation; LT, liver transplantation; RBV, ribavirin; SVR, sustained viral response.

patients develop cirrhosis within 5 years after LT, and approximately 50% within 10 years.[13,16,17] LT recipients with recurrent HCV are treated with a combination of PEGIFN/RBV for 48 weeks. However, eradication with IFN therapy after LT is hampered by the use of immunosuppressive agents, anemia, frequent side-effects, and the need to discontinue or reduce therapy. The outcome of PEGIFN/RBV antiviral therapy after LT is poor, with the SVR rate ranging from 10% to 30% for HCV-1-infected patients.[18–24]

However, Fukuhara *et al.*[8] reported that in patients with recurrent HCV infection after LT, combination analyses of SNP of *IL28B* in both the donor and recipient tissues and mutations in HCV–RNA allow the prediction of SVR to PEGIFN/RBV therapy.

We reported previously the effectiveness of the treatment of recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy).[25] Others also reported SVR rates of 34% and 50% under the same treatment, respectively.[26,27]

In the present study, we analyzed the viral response to long-term PEGIFN/RBV therapy in patients according to the major and minor genotypes of the polymorphic *IL28B* gene.

Methods

Patients. Sixty-five patients underwent living-donor LT (LDLT) for HCV-related end-stage liver disease between 2000 and January 2011. Among them, 22 patients died before the start of therapy, one was naturally negative for HCV–RNA before LT, one did not become positive for HCV–RNA after LDLT, and four obtained SVR by IFN therapy before LT, thus leaving 37 patients treated with IFN therapy at our institution. Of these, two patients are currently continuing antiviral therapy. A total of 35 patients were enrolled in this retrospective study.

There were 28 patients with HCV-1, and seven with HCV-2. The data of eight of the 28 patients with HCV-1 were excluded from the analysis due to the use of standard IFN/RBV in four patients, and cessation due to side-effects in four patients. Thus, the study included 20 patients with HCV-1 (Fig. 1).

Protocol of antiviral therapy. Patients received PEGIFN-α-2b subcutaneously once weekly combined with RBV (200 mg/day). The dose of the latter was increased to 800 mg/day in a stepwise manner, according to individual tolerance within the first 12 weeks of therapy. The combination PEGIFN/RBV therapy was continued for more than 48 weeks after the disappearance of serum HCV–RNA. At the end of the active treatment, patients were followed for another 24 weeks without treatment. In patients who remained positive for HCV–RNA in spite of treatment for more than 48 weeks, PEGIFN was switched to PEGIFN-α-2a, and treatment was continued as described earlier.

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the local ethics committees of all participating centers. Written, informed consent was obtained from all participating patients.

Assessment of therapy efficacy. HCV–RNA levels were measured using one of several reverse transcription–polymerase chain reaction (RT–PCR)-based methods (*Taq*Man RT–PCR test) at weeks 4, 8, and 12, and thereafter every 4 weeks of treatment, and at 24 weeks after the cessation of therapy.

SNP genotyping and quality control. Because the two reported significant *IL28B* SNP (rs8099917 and rs12979860) are in strong linkage disequilibrium, we examined only rs8099917 in this study. Some samples obtained from patients with HCV-1 were determined using the Illumina HumanHap610-Quad Genotyping BeadChip (San Diego, CA, USA), whereas the remaining samples were genotyped using the Invader assay (Third Wave Technologies, Madison, WI, USA), as described previously.[28,29]

Table 1 Characteristics of 20 patients with recurrent hepatitis C genotype 1 after living-donor liver transplantation

Age (years)†	58 (44–70)
Sex (male/female)	15/5
Body mass index (kg/m²)†	24.3 (18.8–42.2)
Viral load at therapy (LogIU/mL)†	6.6 (4.9–7.8)
Time from transplantation to therapy (months)†	4 (1–41)
No. mutations in the ISDR (0–1/2–5)	12/8
HCV core70 region (mutant/wild)	12/8
HCV core 91 region (mutant/wild)	10/10
Donors' *IL28B* genotype TT/TG + GG	15/5
Recipients' *IL28B* genotype TT/TG + GG	14/6
Combination of donors' and recipients' *IL28B* genotype (TT : TT/TT : TG + GG/TG + GG : TT/TG + GG : TG + GG)	11/4/3/2
Immunosuppression (tacrolimus/cyclosporine)	16/4
Adherence to PEGIFN ≥ 70/< 70 (%)†	11/9
Adherence to RBV ≥ 50/< 50 (%)†	8/12

†Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

Analysis of the nucleotide sequences of the core and non-structural 5A regions. The amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region and mutation at the IFN sensitivity-determining region were analyzed in the non-structural 5A region of HCV by the direct sequencing method, as described previously by our group.[25,30,31] Samples after LT were used.

Statistical analysis. Non-parametric tests (χ^2-test and Fisher's exact probability tests) were used to compare the characteristics of the groups. Univariate logistic regression analysis was used to determine those factors that significantly contributed to early viral dynamics. The odds ratios and 95% confidence intervals were also calculated. All P-values < 0.05 using two-tailed tests were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) in the univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Statistical analyses were performed using PASW 18 statistical software (SPSS, Chicago, IL, USA).

Results

Patient characteristics. Table 1 shows the baseline characteristics of the 20 patients with recurrent hepatitis C after LT who completed PEGIFN/RBV treatment. The median age of the patients (15 males and 5 females) was 58 years, and the median body mass index was 24.3. The median latency between transplantation and the initiation of antiviral therapy was 4 months. The median pretreatment serum HCV–RNA viral load was 6.6 LogIU/mL. The *IL28B* genotype (rs8099917) of the donors was TT in 15 patients, and TG + GG in five patients, whereas that of the recipients was TT in 14, and TG + GG in six. Immunosuppressive therapy included tacrolimus in 16, and cyclosporine in four.

Efficacy and tolerance of IFN therapy and side-effects. Figure 1 shows the effects of IFN therapy according to genotype. The SVR rate was 54.2% (19/35) for all patients. Among the patients infected with HCV-1, one of eight patients who were treated with mono-IFN/RBV or ceased treatment had SVR. Twelve of 20 patients with HCV-1 who completed IFN therapy achieved SVR. Thus, the SVR rate was 46.4% (13/28) for those with HCV-1, and 85.7% (6/7) with HCV-2. In patients with HCV-1, four ceased IFN therapy due to adverse effects. These included general fatigue in one, rejection in two, and cerebral hemorrhage in one patient.

Relationship between IL28B and viral response in patients infected with HCV genotype 1. Data or eight of 28 patients with HCV-1 were excluded from the analysis due to standard-IFN plus RBV in four patients, and the cessation of IFN therapy due to adverse effects in four patients. Thus, the data of 20 patients with HCV-1 were available for the analysis of *IL28B*.

In the donors, the SVR rate of the TT group (73.3% [$n = 11/15$]) was higher than that of the TG + GG group (20% [$n = 1/5$], $P = 0.053$, Fig. 2a). In the recipients, the SVR rate of the TT group (64.2% [$n = 9/14$]) was similar to that of the TG + GG group (50% [$n = 3/6$]) (Fig. 2b). The SVR rate of the TT : TT group (donors' *IL28B* : recipients' *IL28B*) was 81.8% ($n = 9/11$), which was higher than the SVR rate of the TT : TG + GG group (50% [$n = 2/4$], Fig. 2c). The SVR rate of the TG + GG : any group (donors' *IL28B* : recipients' *IL28B* of either TT or TG + GG) was 20% ($n = 1/5$), which was lowest among the three groups. There was significant difference between the SVR of the TT : TT group and TG + GG : any group ($P = 0.036$). We also analyzed the viral response (VR) rate according to the combination of donors' and recipients' *IL28B*. The VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. The VR rate of the TT : TT group was 63.6% ($n = 7/11$), which was higher than the VR rate of the TG + GG : any group (0% [$n = 0/5$]) at 24 weeks. The VR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the VR rate of the TG + GG : any group (20% [$n = 1/5$]) at the end of treatment. The SVR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the SVR rate of the TG + GG : any group (20%, $n = 1/5$) at 24 weeks at the end of treatment (Fig. 3).

Analysis of factors associated with SVR in HCV-1 patients with recurrent hepatitis C. The univariate analysis identified three parameters that correlated with SVR either significantly or marginally: the combination of donors' and recipients' *IL28B* (TT : TT $P = 0.037$), donors' *IL28B* (TT genotype; $P = 0.053$), and adherence to RBV therapy (≥ 50; $P = 0.076$, Table 2). The combination of donors' and recipients' *IL28B* (TT : TT genotype) and adherence to RBV (> 50; $P = 0.076$) were entered into the multiple logistic regression analysis to identify significant independent predictive factors. The multivariate analysis identified the combination of donors' and recipients' *IL28B* (TT : TT) as the only significant and independent factor that influenced the SVR: (odds ratio: 15.0, 95% CI: 1.2–185.1, $P = 0.035$).

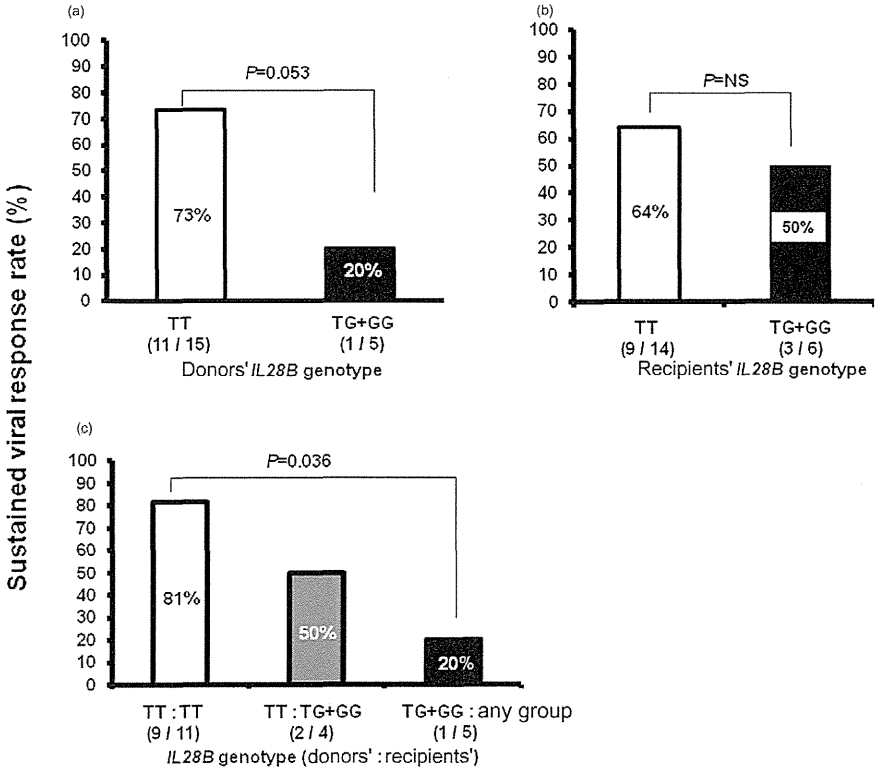

Figure 2 Sustained viral response rates according to (a) donors' interleukin-28B (*IL28B*), (b) recipients' *IL28B*, and (c) donors' and recipients' *IL28B* in patients infected with hepatitis C virus genotype 1. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). NS, not significant.

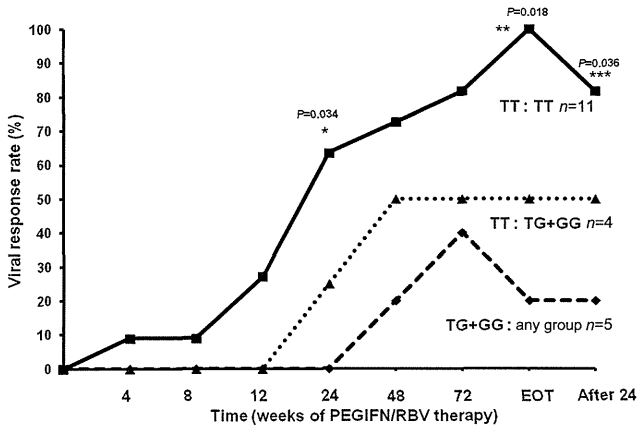

Figure 3 Viral response rates according to donors' and recipients' interleukin-28B (*IL28B*) genotyping. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). *Viral rate (VR) of the TT : TT group was 63.6% ($n = 7/11$), which was higher than the VR rate of the TG + GG : any group (0%, $n = 0/5$) at 24 weeks. **VR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the VR rate of the TG + GG : any group (20%, $n = 1/5$) at the end of treatment (EOT). ***Sustained VR (SVR) rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the SVR rate of the TG + GG : any group (20%, $n = 1/5$) at 24 weeks at the EOT. PEGIFN, pegylated interferon; RBV, ribavirin.

Discussion

The SVR rate has improved since the introduction of PEGIFN/RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with a history of HCV-1 infection is 30–50%.[21–24,26,27] These results are much better than those reported in the 1990s and early 2000s; however, more than half of recipients still suffer from recurrent chronic hepatitis C.

Although many studies have determined the predictive factors of the viral response for PEGIFN/RBV among patients with chronic hepatitis C, recent molecular biological analyses and genome-wide analyses of the human genome have identified genetic variations of *IL28B* and amino-acid substitution of HCV core 70 as the most significant predictive factors for IFN response.[3–5,32,33] *IL28B* encodes a cytokine distantly related to type I IFN and the IL-10 family. It has been reported that the expression level of the *IL28* gene in peripheral blood mononuclear cells is significantly lower in individuals with minor alleles than in individuals with major alleles.[5]

Several studies have determined the predictive factors for the viral response to PEGIFN/RBV in patients with recurrent post-LT hepatitis C viral infection, and recent molecular and genome wide analyses of the human genome have demonstrated that genetic variation of *IL28B* is the most significant predictive factor of the response to IFN.[8,34–37] In the present study, we examined whether the same factors can also predict the response to PEGIFN/RBV in LT recipients. Several groups have reported that recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent hepatitis C after LT.[8,36,37] Furthermore, others

Table 2 Univariate analysis of factors associated with sustained viralresponse (SVR) during interferon therapy in genotype 1 patients with recurrent hepatitis C

	SVR ($n = 12$)	Non-SVR ($n = 8$)	*P*-value
Age (years)†	60 (44–69)	57 (47–65)	0.48
Sex (male/female)	10/2	5/3	0.3
Body mass index (kg/m^2)†	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.4
Viral load at therapy (LogIU/mL)†	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.52
Time from transplantation to therapy (months)†	4 (1–41)	3 (1–6)	1.7
No. mutations in the ISDR (0–1/2–5)	7/5	5/3	1.0
HCV core70 region (mutant/wild)	7/5	5/3	1.0
HCV core 91 region (mutant/wild)	7/5	3/5	0.6
Donors' *IL28B* genotype TT/TG + GG	11/1	4/4	0.053
Recipients' *IL28B* genotype TT/TG + GG	9/3	5/3	0.6
Donors' and recipients' *IL28B* genotype TT : TT/others	9/3	2/6	0.037
Immunosuppression (tacrolimus/cyclosporine)	9/3	7/1	1.0
Adherence to PEGIFN ≥ 70/< 70 (%)†	8/4	3/5	0.3
Adherence to RBV ≥ 50/< 50 (%)†	7/5	1/7	0.076

†Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT,[34] and that recipients' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C.[35,36]

The results of the present study indicate that both donors' and recipients' *IL28B* influence the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C. Both recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in recurrent hepatitis C after LT; however it is not clear whether the recipients' or donors' *IL28B* influenced the SVR to PEGIFN/RBV.

However, the donors' *IL28B* might have influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C more than the recipients' *IL28B*. This conclusion is based on the following results: although the SVR rate of the TT group (64.2%) was similar to that of the TG + GG group (50%), according to the recipients' *IL28B*, the SVR rate of the TT group (73.3%) was higher than that of the TG + GG group (20%), according to the donors' *IL28B*. Furthermore, the VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. That is, the time to VR of the TG + GG : any group was the latest among the three groups. Lange *et al.* reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT.[34] In this regard, Hiraga *et al.*[38] reported that IFN-stimulated gene expression levels in mice livers measured at 2 weeks after IFN treatment were significantly higher in mice transplanted with donor human hepatocytes (*IL28B*; TT) than from donor (*IL28B*; TG + GG) mice. Furthermore, previous studies reported that the expression level of IFN- λ-3, coded for the *IL28B* gene, was higher in hepatocytes than hematopoietic cells.[39]

However, we demonstrated the feasibility of treatment of LT recipients with PEGIFN/RBV until HCV–RNA reached undetectable levels, followed by the continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy). In fact, the SVR rate (50%) of the recipients' *IL28B* TG + GG group was higher than that reported by others[8] (SVR rate: 11%). Furthermore, the SVR rate (81%) of the combination of donors' and recipients' *IL28B* (TT : TT) group was higher than that reported by Fukuhara *et al.*[8] (SVR rate: 56%). However, the SVR rate of the donors' *IL28B* TG + GG group (SVR rate: 20%) was similar to that reported by Fukuhara *et al.*[8] (SVR rate: 9%). We believe that the treatment of LT recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by the continuation of treatment for at least 48 weeks, is not useful for donors with *IL28B* TG + GG.

In Japan, LDLT is more common than orthotopic LT. In finding a suitable donor, it is better to select a donor with TT of the *IL28B* gene than a TG or GG donor. In conclusion, our results demonstrated the suitability of donors with the TT *IL28B* genotype, and that long-term PEGIFN/RBV therapy seems useful for recipients of LDLT who develop recurrent hepatitis C after transplantation.

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IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy

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Background & Aims: Common genetic variation within the IL28 locus has been found to influence the effect of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. Expression of *IL28* in peripheral blood cells has been reported to be higher in patients with *IL28* SNP genotypes associated with favorable response.

Methods: We analyzed 52 liver and 114 blood samples obtained from patients with HCV genotype 1b. We used reverse transcription-real time polymerase chain reaction to analyze expression levels of *IL28* and several interferon stimulated genes (ISGs), including *MxA*, double stranded RNA dependent protein kinase (*PKR*), 2′–5′ oligo-nucleotide synthetase (*OAS1*), *ISG15*, and *SOCS1*.

Results: Interestingly, expression of *IL28* was significantly lower in patients with the response-favorable rs8099917 TT genotype compared to those with TG or GG genotypes (p <0.005). In hepatic cells, expression of *MxA*, *PKR*, *OAS1*, and *ISG15* were also significantly lower in rs8099917 TT patients (p <0.001, p = 0.005, p = 0.001, p <0.001, respectively), whereas in peripheral blood mononuclear cells ISG expression levels did not differ significantly. Among patients treated with peg-interferon plus ribavirin therapy, liver mRNA levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly or marginally lower in responders who became negative for HCV RNA (p = 0.001, 0.004, 0.014, 0.051, and 0.015, respectively).

Conclusions: Expression levels of ISGs are differentially regulated in the liver and peripheral blood. The mechanism underlying the expression levels of IL28 and ISGs and the correlation with the effect of the therapy should be further investigated.

Introduction

Chronic hepatitis C virus infection often results in the development of chronic hepatitis, which leads to cirrhosis and hepatocellular carcinoma [1,2]. Currently, patients with chronic HCV infection are treated with a combination of pegylated interferon and ribavirin [3,4]. The eradication rate of the virus has been reported to be about 50% in patients treated with the standard 48 week therapy [4–6]. Although the eradication rate of the virus has been slightly improved by extending the treatment period to 72 weeks, there are many patients who fail to eradicate the virus [7]. Furthermore, many patients fail to complete the therapy because of severe side effects.

Many predictive factors have been reported so far that affect response to combination therapy. Viral factors, such as substitutions at core amino acids 70 and 91 [8,9], or within the interferon sensitivity determining region (ISDR) [10,11] or the interferon and ribavirin response determining region (IRRDR) [12] have been reported.

Among host factors, many single nucleotide polymorphisms (SNPs) associated with outcome of therapy have been identified. They include SNPs in interferon-alpha pathway genes [13] and interferon induced genes [14], within the promoters of the *MxA* [15] and osteopontin [16] genes, and within an intron of *MAPK APK3* [17].

Recently, Ge *et al.* [18] identified SNPs located 5′ to the *IL28B* gene that affect response to combination therapy. Furthermore, two other research groups also independently reported that these SNPs are associated with the effectiveness of combination therapy [19,20]. More recently, Thomas *et al.* reported that the SNP allele related to favorable therapy response is also associated with spontaneous clearance of HCV [21]. They reported that the allele related to HCV clearance is the major allele in the majority of Asian and European countries.

Keywords: IL28; Liver biopsy; ISG15; MxA; Single nucleotide polymorphism.
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Abbreviations: HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; IRRDR, interferon and ribavirin response determining region; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NVR, non-viral responder; OAS1, 2′–5′ oligoadenylate synthetase 1 ; PKR, double stranded RNA dependent protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

IL28A, *IL28B*, and *IL29* gene products belong to the interferon lambda family [22,23]. These cytokines are interferons functionally, but have been reported to be structurally related to the IL-10 family [24]. IL29 has been reported to reduce the replication levels of the HCV replicon [25] as well as hepatitis B virus [26]. IL29 has also been reported to reduce the replication of HCV cooperatively with interferon alpha and gamma [27]. These observations suggest that higher expression levels of interferon lambda should be observed in the liver and should correspond with a favorable response to therapy. However, no report has analyzed the expression levels of these cytokines and levels of ISG expression in the liver. In this study, we investigated mRNA expression levels of *IL28*, IL28 receptor, and several ISGs using biopsy samples obtained from patients with chronic hepatitis C and analyzed the relationship between the *IL28* genotype and the effect of combination therapy.

Materials and methods

Patients

We analyzed liver specimens from 52 patients who underwent liver biopsies at Hiroshima University Hospital between December 2002 and November 2008 and who were treated with a peg-interferon plus ribavirin combination for chronic hepatitis C genotype 1b at the same or other hospitals. Clinical characteristics of patients are shown in Table 1. Patients received weekly injections of peg-interferon-alpha-2b for 48 weeks with the dosage adjusted by body weight (60 μg for 35–45 kg, 80 μg for 46–60 kg, 100 μg for 61–75 kg, 120 μg for 76–90 kg, and 150 μg for 91–120 kg). Ribavirin was administered orally with the dosage based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels were reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. The response to therapy categories are defined as follows: sustained viral responders (SVR) were negative for HCV RNA 24 weeks after cessation of therapy; relapsers were negative for HCV RNA only transiently during and after the therapy; and non-viral responders (NVR) never became negative for HCV RNA. Liver biopsy specimens, which were obtained in routine clinical practice in an amount beyond what was needed for pathological diagnosis, were kept frozen at −80 °C until analysis. Liver samples obtained by surgical operation from patients who received resection for hepatocellular carcinoma were also kept frozen. Fibrosis stage and activity were diagnosed according to the criteria of Desmet *et al.* [28].

Although we attempted to analyze blood samples from the same patients who provided liver specimens, more than half of these patients were not treated at Hiroshima University Hospital. Accordingly, we collected blood samples from 114 genotype 1b patients who visited Hiroshima University Hospital from November 2009 to March 2010 to analyze ISG mRNA levels. We excluded patients who were under treatment with therapies including interferon or immunosuppressants. Patients who had eliminated HCV with therapy were also excluded. Clinical characteristics of patients who contributed blood samples for ISG analysis are shown Table 1.

All patients provided written informed consent to participate in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the ethical committee of Hiroshima University and RIKEN.

Genotyping

We genotyped SNPs rs8099917 and rs12979860 from 52 patients using either the Invader assay or the Taqman assay. In the Invader assay, allele-specific oligonucleotide pairs and invasive probes were designed and supplied by Third Wave Technologies (WI). FRET probes were labeled with FAM or VIC corresponding to alleles. The 10 μl reaction volume consisted of 0.5 μl of signal buffer, 0.5 μl of FRET probes, 0.5 μl of structure-specific cleavage enzyme, 1 μl of allele-specific probe mix, and 2 μl of PCR product diluted 1:10. Samples were incubated at 95 °C for 5 min and then at 63 °C for 15 min in an ABI PRISM 7700 (Applied Biosystems), and then fluorescence data were collected. Signal intensity was calculated as the ratio of FAM or VIC to ROX, an internal reference. Genotypes were determined visually in the dye components view of the SDS software.

In the TaqMan assay, we carried out PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, CA), 1 ng DNA, 0.2 μM of each primer, and 40 nM of probe provided by Applied Biosystems in 3-μl reactions. Each 384-well plate contained 376 samples of an unknown genotype and 8 no-DNA control samples. Thermal cycle conditions were 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 92 °C for 15 s, and 58 °C for 1 min. Thermal cycling was done on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems), and then fluorescence data were collected and the genotypes were determined using the SDS software [29,30].

We calculated linkage disequilibrium using the LD method in the genetics library in the R 2.11 statistics package (http://www.r-project.org) and found high linkage disequilibrium between rs8099917 and rs12979860 ($r^2 = 0.99$ and $D' = 1$).

Table 1. Characteristics of the two cohorts of patients analyzed for ISG expression levels. All patients were infected with HCV genotype 1b.

	Treated patients	WBC patients
Characteristic	(n = 52)	(n = 114)
Age [median (range)]	56 (31-77)	63 (30-88)
Sex (Male/Female)	29/23	63/51
ALT [median (range)] IU/L	47 (13-246)	62 (15-259)
γ-GTP [median (range)] IU/L	47 (15-708)	53 (10-469)
Fibrosis (F1/F2/F3/F4)	20/18/4/10	23/28/17/11
Activity (A1/A2/A3)	13/30/9	14/48/14
Virus titer [median (range)] kIU/L	850 (15-6500)	850 (0.5-8200)
Core 70[a] (Wild/Mutant/ND)	27/19/6	40/21/53
Core 91[a] (Wild/Mutant/ND)	24/22/6	34/27/53
ISDR[b] substitutions (0/1/>2/ND)	14/16/12/10	37/12/9/56
rs8099917 allele (TT/TG/GG)	30/17/5	88/33/1
Outcome of therapy (SVR/relapser/NVR)[c]	25/19/8	not applicable

Outcome of therapy	TT	TG	GG
SVR	20	5	0
Relapser	4	8	2
NVR	1	4	3
Total	42	24	6

[a]Hepatitis C virus core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants. ND, not determined.
[b]Interferon sensitivity determining region: the number of substitutions relative to the ISDR of the reference sequence [31].
[c]SVR, sustained viral responder; NVR, non-viral responder.

Quantitative analysis of mRNA of ISGs

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of each RNA sample was reverse transcribed with ReverseTra Ace (TOYOBO Co. Ltd., Japan) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the mRNA for *IL28*, *MxA*, 2′–5′ oligoadenylate synthetase1 (*OAS1*), double stranded RNA dependent protein kinase (*PKR*), *ISG15*, and *SOCS1* with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). As it was difficult to measure *IL28A* and *IL28B* mRNA separately, we measured *IL28A* plus *IL28B* mRNA and expressed *IL28* mRNA. Amplification and detection were performed using an ABI PRISM 7300 (Applied Biosystems). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of MxA protein in peripheral mononuclear cells

The MxA protein level in whole blood sample was measured using an ELISA system (MxA ELISA Kit, Kyowa Medex, Tokyo, Japan). Briefly, lysing solution was added to blood samples and the lysate was applied to ELISA plates coated with

Research Article

a MAb (KM1135, Kyowa Medex, Tokyo, Japan). After 2 h of incubation, the plates were washed, and a different peroxidase-labeled MAb (KM1124, Kyowa Medex) was added. After 1 h of incubation and washing, substrate was added. Chemiluminescence was detected using Multiskan MS (Labsystems Version 8.0, Helsinki, Finland). The sensitivity of MxA in this ELISA system was 3.2 ng/ml.

Analysis of amino acid sequences in the core and ISDR region

PCR amplification and nucleotide and amino acid sequence analysis of core and ISDR were performed as reported previously [31] with a slight modification. Briefly, HCV RNA was extracted from 100 μl serum samples by SepaGene RV-R (Sanko Junyaku Co., Tokyo, Japan) and dissolved in 20 μl of H_2O. The RNA was then reverse-transcribed with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The resultant cDNA was then amplified by nested PCR. PCR was performed in 25 μl of the reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer, and 1.25 U of LA Taq (Takara Bio Inc., Otsu, Japan) with a buffer supplied by the manufacturer. One microliter of 10 × -diluted products from the first PCR was used as a template for the second PCR. The PCR primer sequences are listed in Table 2. The PCR protocol involved initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing of primers for 1 min at 57 °C and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 7 min. The amplified DNA fragments were separated onto a 2% agarose gel and purified with the QIAquick gel extraction Kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).

The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype 1b HCV-J (GenBank Accession No.: D90208) [32]. Amino acids at positions 70 and 91 of the core region identical to the reference sequence (arginine and leucine, respectively), were considered as wild type. The number of amino acid substitutions in the ISDR was determined as in Enomoto *et al.* [11,12].

Statistical analysis

Statistical analysis was performed using R version 2.11 or PASW Statistics 18 (SPSS Inc., IL). Categorical data were analyzed using Fisher exact tests, and continuous data were analyzed using the non-parametric Mann–Whitney *U* test. Given the large number of possible predictors, we used multiple logistic regression with variable selection to identify a model with the most important predictors for virological response. To identify independent predictive factors, variables that were significant at the 0.05 level in univariate non-parametric tests were considered as candidate factors for multiple logistic regression analysis. Multicollinearity among predictor variables were examined using hierarchical clustering based on Spearman rank. The model was reduced using forward/backward stepwise selection using the stepAIC function in R, and then boostrap validation was performed using the rms library (formerly called the Design library). Partial residual plot and leverage plots were examined to identify outliers and assess model assumptions. The rms calibrate function was used to calculate R^2 shrinkage, and log odds were corrected for over-optimism using penalized maximum likelihood [33].

Table 2. Primers used in this study.

HCV core protein	
outer forward	5'-GCC ATA GTG GTC TGC GGA AC-3'
outer reverse	5'-GGA GCA GTC CTT CGT GAC ATG-3'
inner forward	5'-GCT AGC CGA GTA GTG TT-3'
inner reverse	5'-GGA GCA GTC CTT CGT GAC ATG-3'
HCV NS5A ISDR[a]	
outer forward	5'-TTC CAC TAC GTG ACG GGC AT-3'
outer reverse	5'-CCC GTC CAT GTG TAG GAC AT-3'
inner forward	5'-GGG TCA CAG CTC CCA TGT GAG CC-3'
inner reverse	5'-GAG GGT TGT AAT CCG GGC GTG C-3'

[a]Interferon sensitivity determining region.

Results

IL28B SNP genotype and mRNA expression levels of ISGs in liver samples

We genotyped two SNPs (rs8099917 and rs12979860) in the *IL28B* locus, which have been reported to affect the outcome of the therapy, and compared them with mRNA expression levels in ISGs. Because of linkage disequilibrium, the results are the same for both SNPs, and thus only results for rs8099917 are presented. Other SNPs in this locus for the association with therapy outcome were several orders of magnitude less significant (data not shown). Expression levels of *IL28* mRNA in blood cells have been reported to be significantly higher in the patients homozygous for the response favorable allele (rs8099917 TT or rs12979860 CC) in peripheral blood [19,20]. However, our results showed that expression levels of *IL28* mRNA in the liver were significantly lower in rs8099917 TT patients (Table 3). Furthermore, hepatic mRNA levels of each of the major anti-viral ISGs, i.e., *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly lower in rs8099917 TT patients (Table 3). In contrast, expression levels of *SOCS1*, which functions as a repressor of interferon signaling, did not differ significantly between the two groups of patients (Table 3).

IL28B SNP genotype and mRNA expression levels of ISGs in peripheral blood

We examined mRNA expression levels in blood cells. In contrast to liver expression levels, mRNA expression levels of *IL28* and other ISGs were not statistically different between the two groups of patients (Table 3). *IL28B* mRNA levels, as well as four of the five ISGs, were only slightly higher in rs8099917 TT patients (Table 3).

MxA protein levels in peripheral mononuclear cells

We examined the levels of MxA protein in the peripheral mononuclear cells of 43 patients with genotype 1b chronic hepatitis C who were treated with combination therapy. In this case, consistent with previous reports [19,20], the protein levels of MxA were marginally higher in patients homozygous for the major allele (Fig. 3). Furthermore, MxA protein levels in these patients were significantly higher two days after the beginning of therapy (Fig. 1).

IL28 locus genotypes and the effect of combination therapy

Fifty-two patients with chronic hepatitis C genotype 1b were treated with combination therapy. Numbers of SVR, relapser, and NVR patients were 25 (48%), 19 (37%) and 8 (15%), respectively. Responses to therapy by rs8099917 genotype are noted in Table 1. SVR was most frequent in rs8099917 TT patients.

Effect of the combination therapy and mRNA expression levels

As shown in Fig. 2, when patients were divided into VR (SVR + relapser) and NVR categories, expression levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly higher in NVR patients (Fig. 2). There was no significant difference in *SOCS1* mRNA expression between the two groups of patients. Similarly, when patients were classified as SVR and non-SVR (relapser and

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Table 3. Effect of rs8099917 genotype on ISG expression in hepatic and peripheral blood cells.

ISG		TT		GT/GG	*p*
Liver					
IL28	0.00044	(0.00012-0.005)	0.012	(3E-04-0.023)	0.00493
IL28RA	0.0013	(0.00084-0.0019)	0.0015	(0.0011-0.0019)	0.39
MxA	0.0034	(0.0011-0.0094)	0.02	(0.0084-0.06)	8.04E-05
PKR	0.25	(0.022-0.45)	0.77	(0.26-1.1)	0.00493
OAS1	0.18	(0.10-0.31)	0.54	(0.22-1.1)	0.00106
ISG15	0.29	(0.14-0.59)	2	(0.87-3.9)	8.65E-07
SOCS1	0.0016	(0.0011-0.0024)	0.0017	(0.0012-0.0030)	0.707
Peripheral blood					
IL28	0.00078	(0.00045-0.0010)	0.00062	(0.00032-0.001)	0.31
IL28RA	0.016	(0.011-0.023)	0.015	(0.011-0.02)	0.34
MxA	0.011	(0.0043-0.029)	0.011	(0.0036-0.053)	0.9
PKR	0.18	(0.12-0.3)	0.18	(0.10-0.27)	0.386
OAS1	1.9	(0.75-3.4)	1.3	(0.85-2.3)	0.242
ISG15	3	(1.2-7.7)	2.7	(1.7-4.9)	0.59
SOCS1	0.022	(0.014-0.032)	0.019	(0.014-0.027)	0.292

The median and interquartile range are shown for the TT and GT/TT genotypes for SNP rs8099917 in the hepatic cells (upper) and in peripheral blood mononuclear cells (lower). Results of Mann–Whitney *U* test for effect of genotype on ISG expression levels are shown.

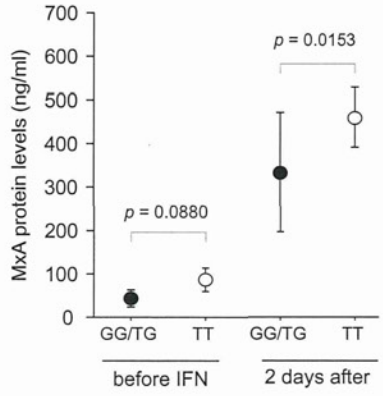

Fig. 1. MxA protein levels in peripheral white blood cells before and two days after the beginning of therapy. Points are classified by rs8099917 genotype (GG/TG vs. TT).

NVR), expression levels were also higher in non-SVR patients than SVR for all ISGs except *SOCS1*, although statistical significance was seen only for *Mx1* and *ISG15* ($p = 0.033$ and 0.031, respectively) (data not shown).

IL28 locus genotypes and amino acid sequences of core and ISDR

As amino acid mutations in the core protein and ISDR region have been reported to be associated with the effect of combination therapy, we examined the relationship between *IL28* genotype and amino acid substitutions within the ISDR and at core amino acids 70 and 91. mRNA expression of the genes examined tended to be higher in patients with core amino acid 70 and 91 mutants and ISDR mutants, and expression levels of *IL28* ($p = 0.035$), *MxA* ($p = 0.031$), and *SOCS1* ($p = 0.018$) were significantly higher in patients with amino acid 91 substitutions (Fig. 3).

Factors associated with the effect of combination therapy

We examined combinations of factors associated with the effect of combination therapy for patients with genotype 1b. Gene expression levels among ISGs were correlated (Fig. 4). To identify factors that contribute independently to virological response, we performed multiple logistic regression analysis using ISG expression levels as well as *IL28B* genotypes and the number of viral substitutions for patients with HCV genotype 1b (Table 4). Following forward/backward stepwise selection based on AIC score, only *ISG15*, *MxA*, *IL28*, and *OAS1* remained in the model, and only *MxA* was significant at the 0.05 level. Age, sex, and other patient and viral factors were not significant.

Discussion

The association of *IL28* locus polymorphisms and response to peg-interferon and ribavirin combination therapy has been reported independently by three groups of researchers [18–20]. Two of the three studies have reported that expression of IL28 in peripheral leukocytes was higher in patients homozygous for the favorable allele [19,20]. It seems reasonable that higher levels of IL28 combined with administration of peg-interferon and ribavirin is related to better response to the therapy. In fact, an additive effect of lambda interferon and alpha interferon has been reported [27]. Accordingly, we assumed that expression levels should be also higher in the liver in such patients.

Interestingly, however, the expression levels of *IL28* were significantly lower in rs8099917 TT patients (Table 3). Expression

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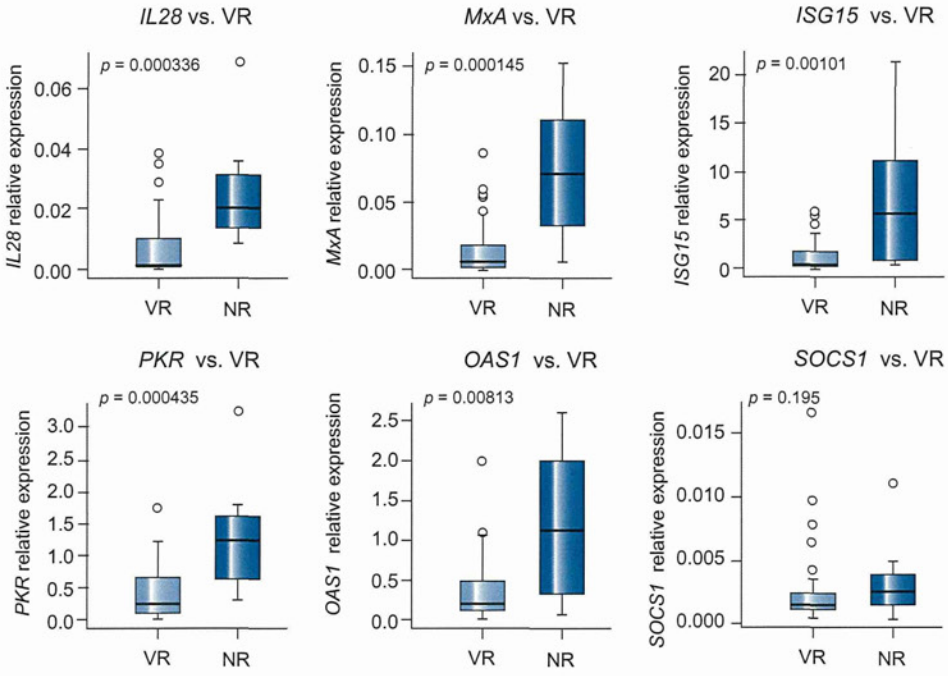

Fig. 2. Intrahepatic expression of *IL28* and interferon stimulated genes by response to therapy. Figures under each panel show the classification of patients with HCV genotype 1b by response to therapy: VR, sustained viral responder and relapser; NR, nonviral responder.

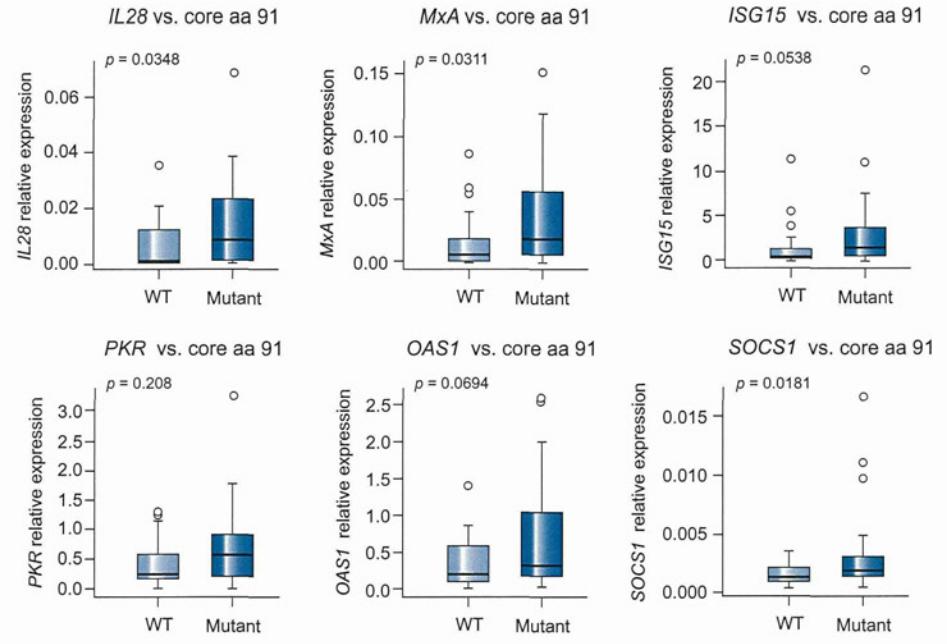

Fig. 3. Association between viral factors and ISG expression. Patients with a substitution at HCV core protein amino acid (aa) 91, which is associated with poorer response to treatment, showed significantly or marginally significantly increased expression of several ISGs involved in establishment of the antiviral state as well as decreased expression of one ISG involved in the suppression of interferon signaling.

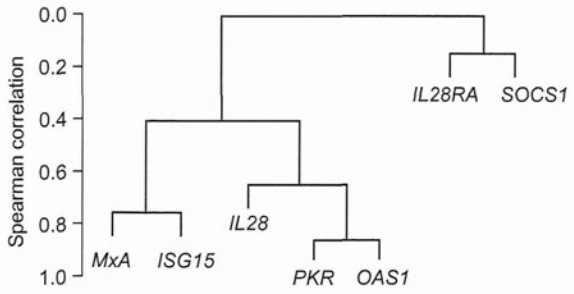

Fig. 4. Spearman correlation among predictor variables. Hierarchical clustering identified groups of genes with similar expression patterns.

levels of each of the ISGs involved in the establishment of antiviral defense (*MxA*, *PKR*, *OAS* and *ISG15*) were also lower in rs8099917 TT patients. We infer from these results that expression levels of *IL28* and other ISGs are regulated differently in the liver compared to peripheral blood cells. The finding that ISG expression levels were lower in patients homozygous for the major allele associated with a favorable response is consistent with Sarasin-Filipowicz *et al.* [34], who showed that lower ISG expression levels in the liver are associated with positive response to therapy, and vice versa. Feedback mechanisms that down-regulate the response to interferon administered during therapy might negatively affect the response to therapy in HCV infected liver cells. Our result showed that only the expression level of *SOCS1* did not differ among patients with rs8099917 genotype, which implies that the expression level of *SOCS1* relative to ISGs is higher in rs8099917 TT patients. Such relatively higher expression levels of inhibitory genes may contribute to the poor response to the therapy.

The relationship between *IL28B* polymorphisms and *IL28B* expression level remains unknown. Another SNP in strong linkage disequilibrium with the SNPs analyzed in this study resides in a possible promoter region of the *IL28B* gene [18], which might, therefore, affect mRNA expression levels, but different expression levels of ISG mRNA between liver and peripheral mononuclear cells cannot be explained simply by a single SNP in the promoter region. Further study is necessary to address this issue.

As it has been reported that amino acid substitutions in the core protein and the ISDR are associated with different responses to therapy [8–11], we attempted to uncover a relationship between core aa70 and 91 substitutions and expression levels of ISGs. Previously we found that core aa70 wild type viruses accumulated in rs8099917 TT patients, and several studies have reported poor response to therapy in the case of aa70 and aa91 substitutions [8]. Consistent with these results, in this study we found an association between elevated ISG expression and core aa91 substitutions, both of which are associated with poor response to therapy.

Multivariate analysis in this study reflected the tiered relationships among the predictors. The *IL28* rs8099917 genotype term was highly significant when analyzed alone, but it was

Table 4. Factors associated with virological response in patients with HCV genotype 1b (sustained viral response or transient/relapse response).

Variable	Univariate tests			Multiple logistic regression			
	n	OR	*p*	n	OR	(95% CI)	*p*
Age	52	1.13	0.4573				
Sex	52	0.604	0.2777				
rs8099917 (TT vs TG/GG)	52	3.68	0.0072				
Fibrosis stage	52	1.67	0.5672				
Activity	52	0.495	0.5788				
ALT	47	0.597	0.1845				
Gamma-GTP	47	0.539	0.0881				
Core aa70 (WT vs mutant)	46	1.24	0.7002				
Core aa91 (WT vs mutant)	46	1.43	0.4513				
ISDR (0 vs ≥1)	42	1.12	1.0000				
Titer	44	1.2	0.6377	52	0.297	(0.0794-1.11)	0.0706
IL28	52	0.273	0.0003				
IL28RA	51	0.792	0.3381	52	0.186	(0.047-0.736)	0.0165
MxA	52	0.255	0.0001	52	0.38	(0.124-1.16)	0.0892
ISG15	52	0.44	0.0010				
PKR	52	0.186	0.0004				
OAS1	52	0.372	0.0081	52	9.14	(0.974-85.7)	0.0528
SOCS1	52	0.87	0.1954	-	-	-	-

Univariate tests (Fisher exact and Mann–Whitney *U* tests) and multiple logistic regression analysis were used to examine the association between viral response and *IL28B* rs8099917 genotype, ISG gene expression, age and sex. Following multiple logistic regression *IL28*, *MxA*, and *OAS1* expression remained significant at the 0.05 level. Odds ratios for multiple logistic regression were adjusted using penalized maximum likelihood.

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not significant when *IL28* and ISG mRNA expression levels were included in the model, suggesting that whatever the mechanism of action reflected by this polymorphism, it may directly or indirectly affect expression of the *IL28B* gene and downstream ISGs. Similarly, *MxA* and *ISG15* clustered together by Spearman rank correlation (Fig. 4), making it unlikely that both would remain significant in a multivariate model, and in this case the ISG with the stronger univariate effect (*MxA*) was selected.

Conclusions

In summary we found that the expression levels of ISGs in hepatic cells are inversely related with *IL28* SNP genotype relative to peripheral mononuclear cells. Analysis of the mechanism underlying different expression levels among *IL28* genotypes, especially differential regulation of anti-viral ISGs and *SOCS1*, should be important in understanding the mechanism behind variations in response to therapy and give us an insight into ways to develop more effective therapeutic regimens.

Financial support

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Conflict of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Acknowledgments

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Impact of Viral Amino Acid Substitutions and Host Interleukin-28B Polymorphism on Replication and Susceptibility to Interferon of Hepatitis C Virus

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Amino acid (aa) substitutions of core 70 and 91 and in the NS5A (nonstructural protein 5A) interferon sensitivity determining region (ISDR) as well as genetic polymorphisms in the host interleukin-28B (IL28B) locus affect the outcome of interferon (IFN)-based therapies for patients with chronic hepatitis C. The combination of these factors and the quasispecies nature of the virus complicate understanding of the underlying mechanism. Using infectious hepatitis C virus (HCV) genotype 1b clone HCV-KT9, we introduced substitutions at both core aa70 (Arg to Gln) and aa91 (Leu to Met). We also introduced four and nine ISDR aa substitutions into core mutant HCV-KT9. Using human hepatocyte chimeric mice with different IL28B genotypes, we examined the infectivity, replication ability, and susceptibility to IFN of these clones. Although aa substitutions in the ISDR significantly impaired infectivity and replication ability of the virus, core aa70 and 91 substitutions did not. The effect of IFN treatment was similar in core wild-type and mutant viruses. Interestingly, virus titer was significantly higher in mice with the favorable IL28B allele (rs8099917 TT and rs12979860 CC) in the transplanted hepatocytes than in mice with hepatocytes from rs8099917 TG and rs12979860 TT donors ($P < 0.001$). However, the effect of IFN was significantly greater, and intrahepatic expression levels of IFN-stimulated genes were significantly higher in mice with the favorable IL28B allele. *Conclusion*: Our data suggest that HCV replication levels and response to IFN are affected by human hepatocyte IL28B single-nucleotide polymorphism genotype and mutations in the ISDR. The mechanism underlying the clinically observed association of wild-type core protein in eradication-favorable host cells should be investigated further. (HEPATOLOGY 2011;54:764-771)

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.[1,2] Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection, and the most effective currently available therapy is combination therapy with pegylated (PEG)-IFN and ribavirin (RBV).[3-5] Among HCV genotypes, genotype 1 is the most resistant to

Abbreviations: aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL28B, interleukin-28B; ISDR, interferon-sensitivity–determining region; ISG, interferon-stimulated gene; MxA, myxovirus resistance protein A; NVR, nonvirological response; OAS, oligoadenylate synthetase; PBS, phosphate-buffered saline; PEG, pegylated; PKR, RNA-dependent protein kinase; RBV, ribavirin; RT-PCR, reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SNP, single-nucleotide polymorphism; SVR, sustained virological response; uPA, urokinase-type plasminogen activator.

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IFN therapy.[6] The limited success of combination therapy for genotype 1 HCV infection is because of the low response rate during therapy and high relapse rate after therapy.[7]

Recent studies have identified both viral and host factors predictive of IFN therapy. Among the viral factors, amino acid (aa) substitutions in the IFN-sensitivity–determining region (ISDR) (nucleotides 2209-2248 or aa positions 237-276 within the NS5A region) are associated with sustained virological response (SVR) after IFN treatment in HCV genotype 1b patients.[8,9] Akuta et al. reported that substitution of aa70 or 91 in the HCV core region are independent predictors of SVR and nonvirological response (NVR).[10-12] Recently, we[13] and another group[14] also reported that wild-type HCV core aa70 and two or more aa substitutions in the ISDR are effective predictors of SVR in patients with HCV genotype 1b.

Among host factors associated with SVR, many common genetic polymorphisms in the human genome have been identified, including single-nucleotide polymorphisms (SNPs).[15-19] More recently, an association between several linked SNPs in the interleukin-28B (IL28B) locus and the effect of combination therapy has been reported.[20-22]

We recently reported that the core aa wild type is significantly more likely to be found in patients with the eradication-favorable IL28B SNP genotype.[23-25] The underlying mechanism of this association as well as the reason for the differential response to therapy by viruses with core aa substitutions are unknown. This is partly because of the presence of HCV quasispecies in human serum samples and the difficulty of performing infection experiments in a small animal model.

The severe combined immunodeficient (SCID) urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice able to develop HCV viremia after injection of serum samples positive for the virus.[26] We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs, such as IFN-alpha and NS3-4A protease inhibitor.[27,28] We have further improved the replacement levels of the human hepatocytes in this mouse model,[29] which enabled us to perform infection experiments more easily because highly repopulated mice (defined as human serum albumin [HSA] levels well above 1 mg/mL) successfully develop viremia more often than poorly repopulated mice.[30] Using this mouse model, we developed a reverse genetics system for HCV.[31,32] This system is useful for studying characteristics of HCV strains with various substitutions of interest, because the effects of quasispecies can be minimized. Furthermore, as there is no adaptive immune system in this mouse model, we are able to examine the replication of HCV and the effect of therapy while avoiding the influence of the immunological response. In the present study, we investigated effects of viral and host factors on HCV infectivity, replication ability, and IFN susceptibility using genetically engineered genotype 1b HCV-infected mice that underwent transplantation with hepatocytes having eradication-favorable or eradication-unfavorable IL28B SNP genotypes.

Table 1. Characteristics of Donors for Transplanted Human Hepatocytes

Donor	A	B	C	D
Sex	Female	Male	Female	Male
Age	10	2	5	2
Ethnic group	Caucasian	Caucasian	African American	Hispanic
rs8099917	TG	TT	TG	TT
rs8109886	AA	CC	AA	CC
rs12979860	TT	CC	TT	CC
rs11882871	GG	AA	GG	AA
rs73930703	TT	CC	TT	CC
rs8107030	AG	AA	AG	AA
rs28416813	GG	CC	GG	CC
rs8103142	CC	TT	CC	TT
rs11881222	GG	AA	GG	AA
rs4803217	AA	CC	AA	CC

Materials and Methods

Animal Treatment. Generation of the $uPA^{+/+}$/$SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously.[29] All animal protocols described in this study were performed in accord with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of HSA, which serve as useful markers of the extent of repopulation, were measured as previously described.[29] Mice underwent transplantation with frozen human hepatocytes obtained from four different human donors (Table 1). Genotyping of IL28B SNPs of human hepatocytes was performed using the Invader assay as described previously.[33,34] We used 1000 IU/g/day of IFN-alpha (Dainippon Sumitomo Pharma Co., Tokyo, Japan) for 2 weeks. This dosage was selected based on a previous report showing that this regimen reduced mouse serum

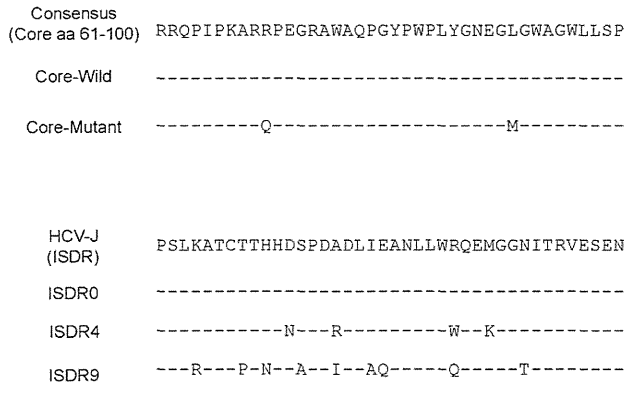

Fig. 1. The aa sequences of infectious genotype 1b HCV clones, Core-Wild, Core-Mutant (substitutions at aa70 and aa91), and ISDR variants (with 0, 4, and 9 substitutions).

HCV RNA levels by 0.5-2 log copies/mL during therapy.[31]

HCV RNA Transcription and Inoculation into Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9, that was obtained from a Japanese patient with severe acute hepatitis (GenBank accession no. AB435162).[32] Ten micrograms of plasmid DNA, linearized by digestion with *Xba*I (Promega, Madison, WI), was transcribed in a 100-μL reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and then analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μL of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.[32] The HCV-KT9 clone has aa substitutions at aa70 and 91 (arginine to glutamine and leucine to methionine, respectively) in the core region (Core-Mutant), compared to the consensus sequence,[10-12] and no aa substitutions in the ISDR (ISDR0),[8] relative to the prototype sequence (HCV-J).[35] Using the original HCV-KT9 clone, we created two additional HCV clones having wild-type core aa70 and 91 (Core-Wild) and four (ISDR4) and nine (ISDR9) aa substitutions in the ISDR, respectively (Fig. 1). To introduce the aa substitutions, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Human Serum Samples. Human serum samples containing a high titer of genotype 1b HCV (2.2×10^6 copies/mL) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. Core 70 and 91 aas were Gln and Leu, respectively, and only one aa substitution was present in the ISDR. The study protocol involving human subjects conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

Quantitation of HCV RNA and IFN-stimulated gene-expression levels. RNA was extracted from mice serum and liver samples by Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan), dissolved in 8.8 μL of ribonuclease-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in 20 μL of reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostics, Tokyo, Japan) were performed as previously described.[32] Quantitation of IFN-stimulated genes (ISGs) (myxovirus resistance protein A [MxA], oligoadenylate synthetase [OAS], and RNA-dependent protein kinase [PKR]) was performed using real-time PCR Master Mix (Toyobo, Kyoto, Japan) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. ISG messenger RNA expression levels were expressed relative to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis. The HCV infectious ratio of chimeric mice was assessed using the chi-square test. Mice serum HCV RNA titers, HSA concentrations, and ISG expression levels were compared using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

Results

Influence of aa Substitutions in the HCV Core Region and ISDR on HCV Infectivity and Replication Ability. We investigated the influence of aa substitutions in the core region and ISDR on HCV infectivity and replication ability in mice that underwent transplantation with human hepatocytes obtained from donor A (Table 1). Each 30 μg of *in vitro*–transcribed RNA was inoculated into the livers of mice. Six weeks after inoculation, serum HCV RNA titers increased above the detectable limit (1000 copies/mL) in 11 of 12 (92%) mice infected with Core-Wild-ISDR0 and in 14 of 16 (88%) mice with Core-Mutant-ISDR0 (Fig. 2A). HCV RNA titers in Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice increased to the same levels (Fig. 2B). In contrast, serum HCV

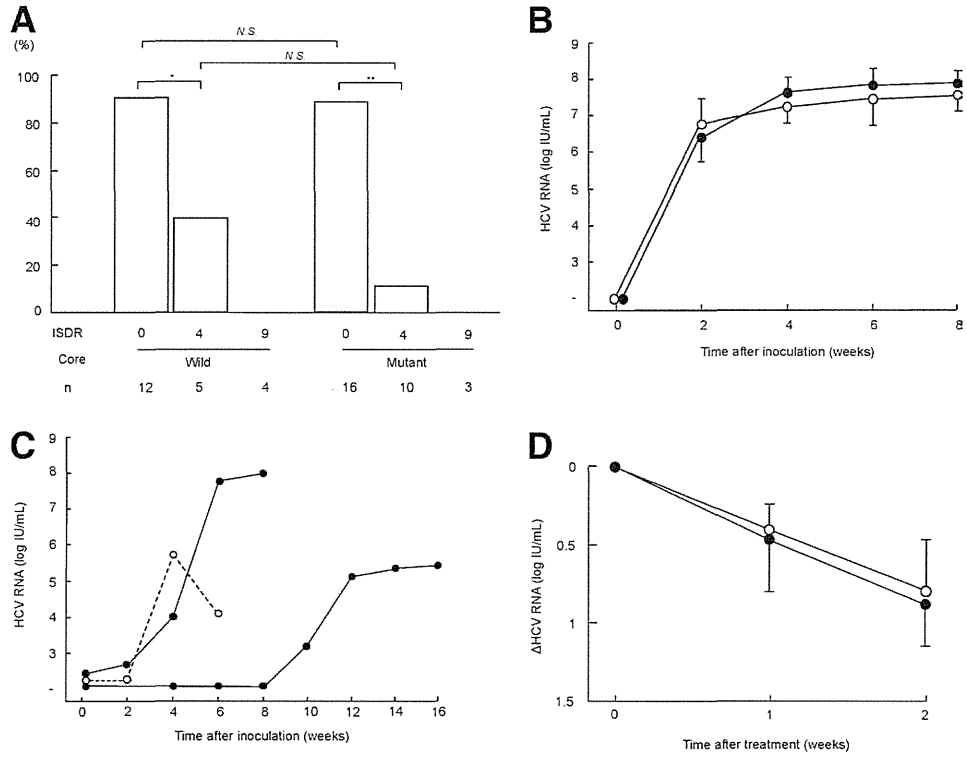

Fig. 2. Infectivity and replication ability of HCV clones. Mice that underwent transplantation with hepatocytes obtained from donor A were inoculated with 30 μg of *in vitro*-transcribed RNAs of indicated clones. (A) Proportion of HCV-infected mice. Infection was defined as serum HCV RNA titer above the detection limit (1000 copies/mL) 6 weeks after inoculation. aa sequences of the core (Wild or Mutant) and number of substitutions in the ISDR are noted below the graph. (B) Time course of serum HCV RNA levels in mice inoculated with either Core-Wild-ISDR0 (closed circles, n = 11) or Core-Mutant-ISDR0 (open circles, n = 14) HCV clones. Data are represented as mean ± standard deviation. (C) Time course of serum HCV RNA levels in two Core-Wild-ISDR4-infected mice (closed circles) and a Core-Mutant-ISDR4-infected mouse (open circles). Serum HCV RNA levels were measured until the mice died. (D) Core-Wild-ISDR0- (closed circles, n = 8) and Core-Mutant-ISDR0 (open circles, n = 4)-infected mice were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Mice serum HCV RNA titers were measured at the indicated times. $*P < 0.05$, $**P < 0.01$; NS, not significant.

RNA titer increased above the detection limit in only two of five (40%) Core-Wild-ISDR4 mice and in only 1 of 10 (10%) Core-Mutant-ISDR4 mice, and the titers in these mice were lower than in mice with ISDR0 (Fig. 2C). HCV RNA titers failed to increase above the detection limit in mice with Core-Wild-ISDR9 and Core-Mutant-ISDR9 (Fig. 2A).

Influence of Core aa Substitutions on the Effect of IFN. To investigate the influence of aa substitutions in the core region on the effect of IFN, Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in a 0.84 ± 0.3 log IU/mL reduction of HCV RNA titer in Core-Wild-ISDR0–infected mice and a 0.79 ± 0.34 log IU/mL reduction in Core-Mutant-ISDR0–infected mice (Fig. 2D).

We also investigated the influence of aa substitutions in the core region on the effect of IFN plus RBV combination therapy. Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha and 20 mg/kg of RBV daily for 2 weeks. The treatment resulted in similar HCV RNA reductions in all treated mice. However, as with IFN monotherapy, there were no significant differences in HCV reductions among mice with different aa substitutions in the core region (data not shown). The dose of ribavirin used was relatively small, however, because of the drug's toxicity in mice.

HCV Infectivity, Replication Levels, and IFN Susceptibility by Core aa Substitutions and Genetic Variation in the IL28B Locus. We investigated the influence of IL28B genotypes on HCV infectivity, replication ability, and IFN susceptibility. *In vitro*–transcribed RNA (30 μg) was inoculated into the livers of mice with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) or donor B (rs8099917 TT and rs12979860 CC). Eight weeks after inoculation, serum HCV RNA titers increased above the detection limit in 22 of 25 (88%) mice with hepatocytes from donor A and in 20 of 23 (87%) mice with hepatocytes from donor B (Fig. 3A). Serum HCV RNA levels were

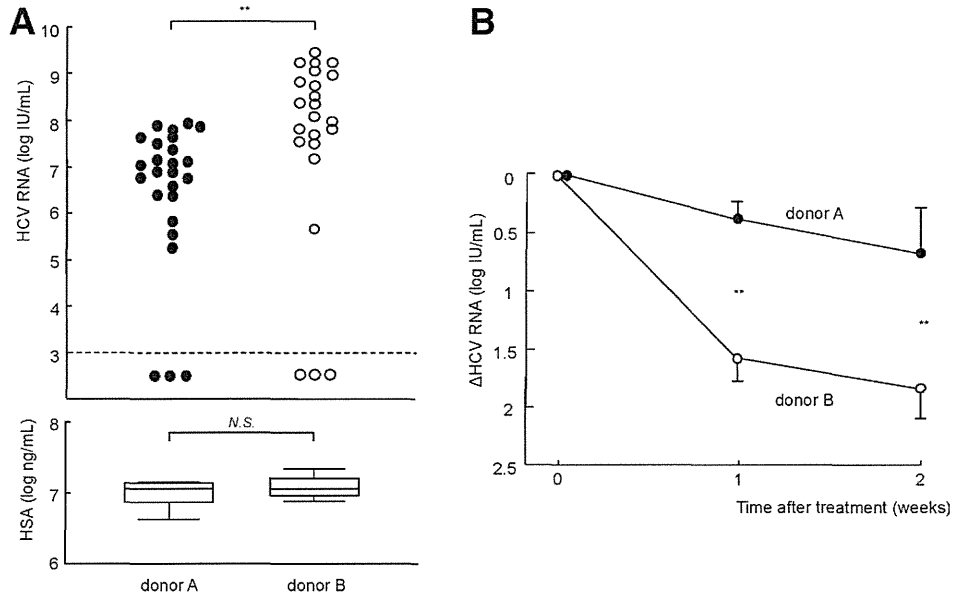

Fig. 3. HCV infectivity, replication ability, and IFN susceptibility in HCV-KT9-injected mice. Mice that underwent transplantation with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) (closed circles, n = 25) or B (rs8099917 TT and rs12979860 CC) (open circles, n = 23) were intrahepatically inoculated with RNA transcribed from either Core-Wild-ISDR0 or Core-Mutant-ISDR0 clones. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (B) HCV-infected mice with hepatocytes from donor A (closed circles, n = 12) or B (open circles, n = 8) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. Data are represented as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$; NS, not significant.

significantly higher in mice with hepatocytes from donor B than from donor A ($P < 0.001$). HCV-infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in 0.65 ± 0.38 and 1.84 ± 0.23 log IU/mL reductions in HCV RNA titer in mice with hepatocytes from donors A and B, respectively ($P < 0.01$) (Fig. 3B). Interestingly, despite the higher serum HCV RNA levels, reduction levels of HCV were higher in mice that underwent transplantation with hepatocytes obtained from donor B than in mice that underwent transplantation with hepatocytes obtained from donor A.

To confirm an association between IL28B SNP genotype and HCV RNA titer, we compared HCV RNA titers using mice with hepatocytes from an additional pair of donors with the favorable (donor C) and unfavorable (donor D) SNP genotypes. To determine whether results obtained by clonal infection would be comparable to results obtained using the more natural serum injection, which should have contained more complex viral species, mice were injected with genotype 1b HCV obtained from a human patient with core and ISDR substitutions, as described above. Mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) or donor D (rs8099917 TT and rs12979860 CC) were inoculated intravenously with 10^5 copies of HCV. Eight weeks after inoculation, serum HCV RNA titer increased above the detection limit in 13 of 14 (93%) mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) and in 12 of 12 (100%) mice with hepatocytes from donor D (rs8099917 TT and rs12979860 CC) (Fig. 4A). With results similar to those found for the mice inoculated with transcribed HCV RNA, serum HCV RNA levels were significantly higher in mice with hepatocytes from donor D than from donor C ($P < 0.001$), and the effect of IFN was also greater in donor D mice than in donor C mice (Fig. 4B); however, statistical significance using these donors was only achieved at week 1, probably resulting from fluctuation of HCV RNA titers and the small number of animals analyzed.

Expression Levels of ISGs in Mouse Livers. ISG expression levels in mice livers were measured after 2 weeks of IFN treatment (Fig. 4B). MxA, OAS, and PKR levels were significantly higher in mice with human hepatocytes from donor D than from donor C (Fig. 4C).

Discussion

In this study, we investigated the effect of substitutions at core protein aa70 and 91 and within the