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.

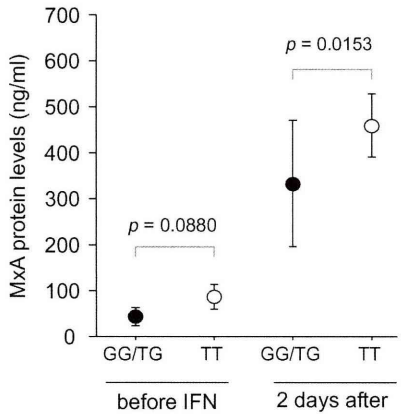

Fig. 1. MxA protein levels in peripheral white blood cells before and two days after the beginning of therapy. Points are classified by rs8099917 genotype (GG/TG vs. TT).

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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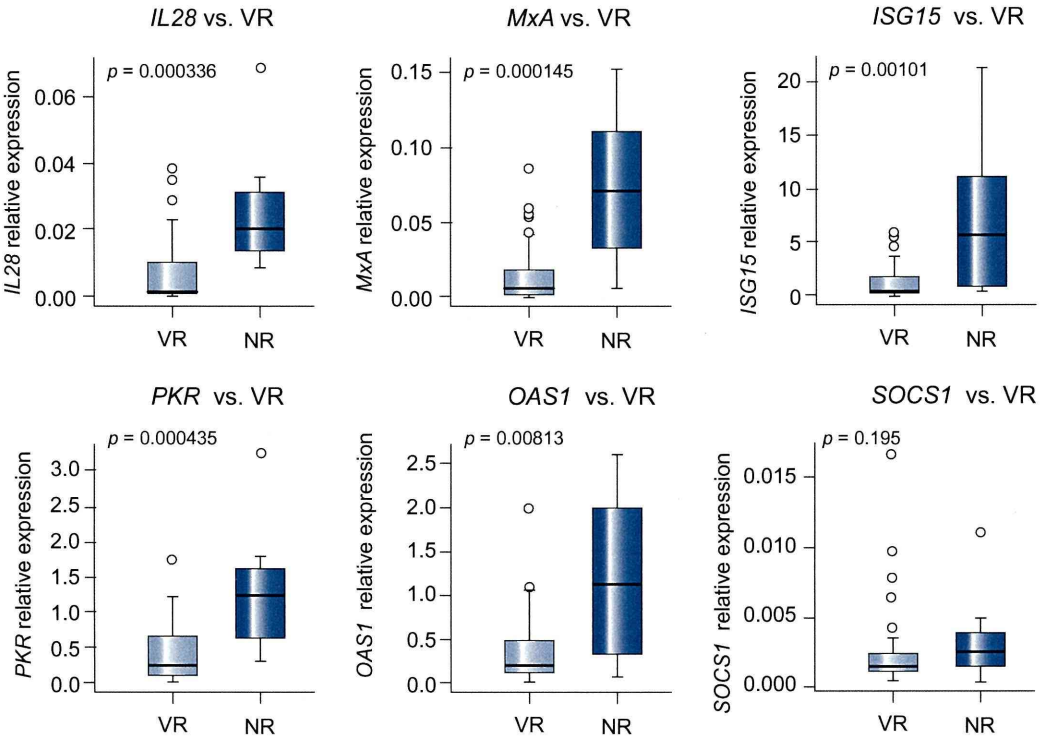

Fig. 2. Intrahepatic expression of *IL28* and interferon stimulated genes by response to therapy. Figures under each panel show the classification of patients with HCV genotype 1b by response to therapy: VR, sustained viral responder and relapser; NR, nonviral responder.

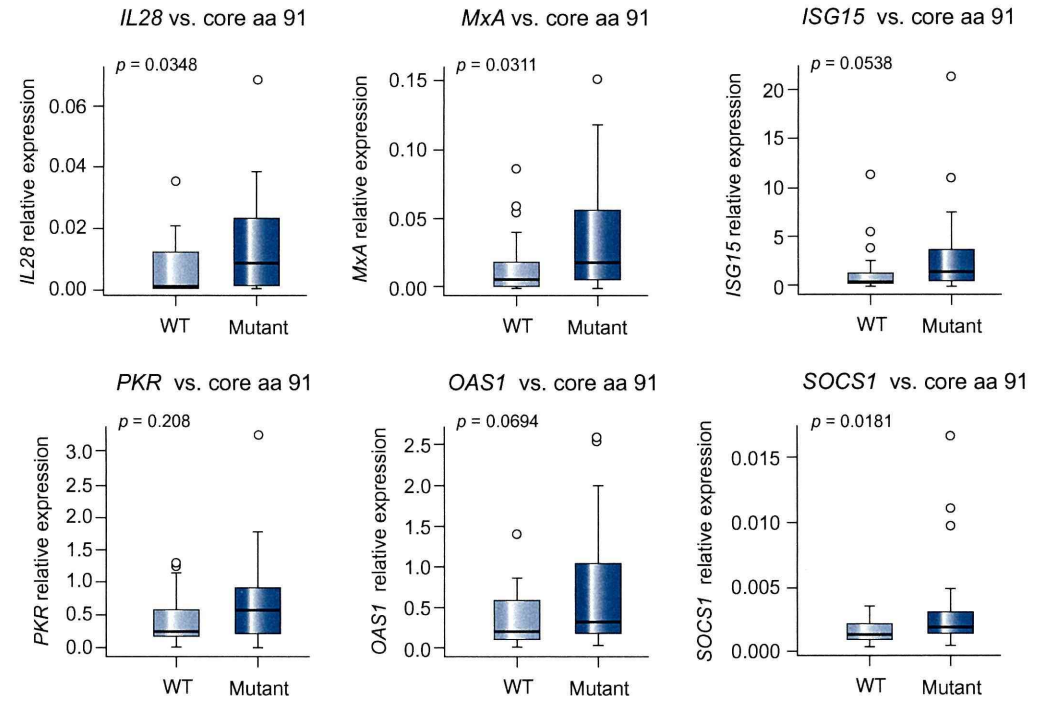

Fig. 3. Association between viral factors and ISG expression. Patients with a substitution at HCV core protein amino acid (aa) 91, which is associated with poorer response to treatment, showed significantly or marginally significantly increased expression of several ISGs involved in establishment of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

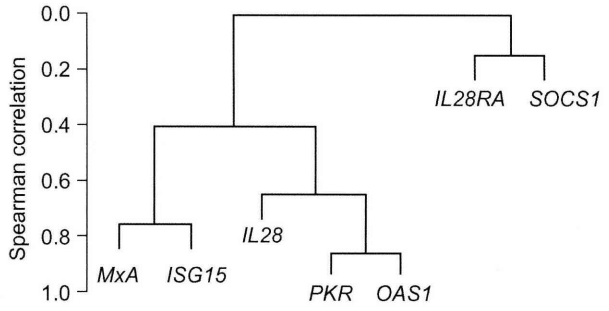

Fig. 4. Spearman correlation among predictor variables. Hierarchical clustering identified groups of genes with similar expression patterns.

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.

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.

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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

Nobuhiko Hiraga,[1,2] Hiromi Abe,[1,2] Michio Imamura,[1,2] Masataka Tsuge,[1,2] Shoichi Takahashi,[1,2] C. Nelson Hayes,[1,2] Hidenori Ochi,[2,3] Chise Tateno,[2,4] Katsutoshi Yoshizato,[2,4] Yusuke Nakamura,[5] Naoyuki Kamatani,[6] and Kazuaki Chayama[1,2,3]

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.

From the [1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; [2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan; [3]Laboratory for Digestive Diseases, RIKEN Center for Genomic Medicine, Hiroshima, Japan; [4]PhoenixBio Co., Ltd., Higashi-Hiroshima, Japan; [5]Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan; and [6]Laboratory for Statistics, RIKEN Center for Genomic Medicine, Yokohama, Japan.

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Address reprint requests to: Kazuaki Chayama, M.D., Ph.D., Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate school of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp; fax: +81-82-255-6220.

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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

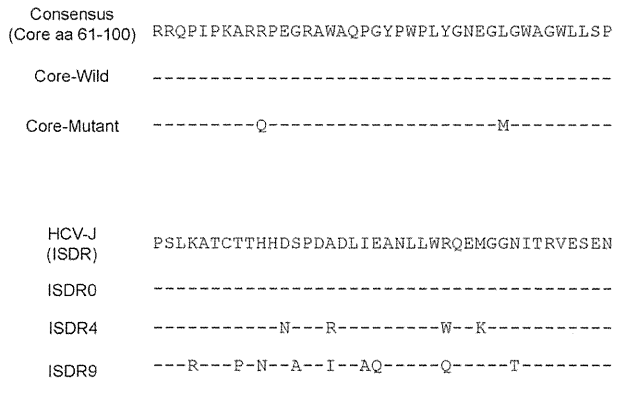

Fig. 1. The aa sequences of infectious genotype 1b HCV clones, Core-Wild, Core-Mutant (substitutions at aa70 and aa91), and ISDR variants (with 0, 4, and 9 substitutions).

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

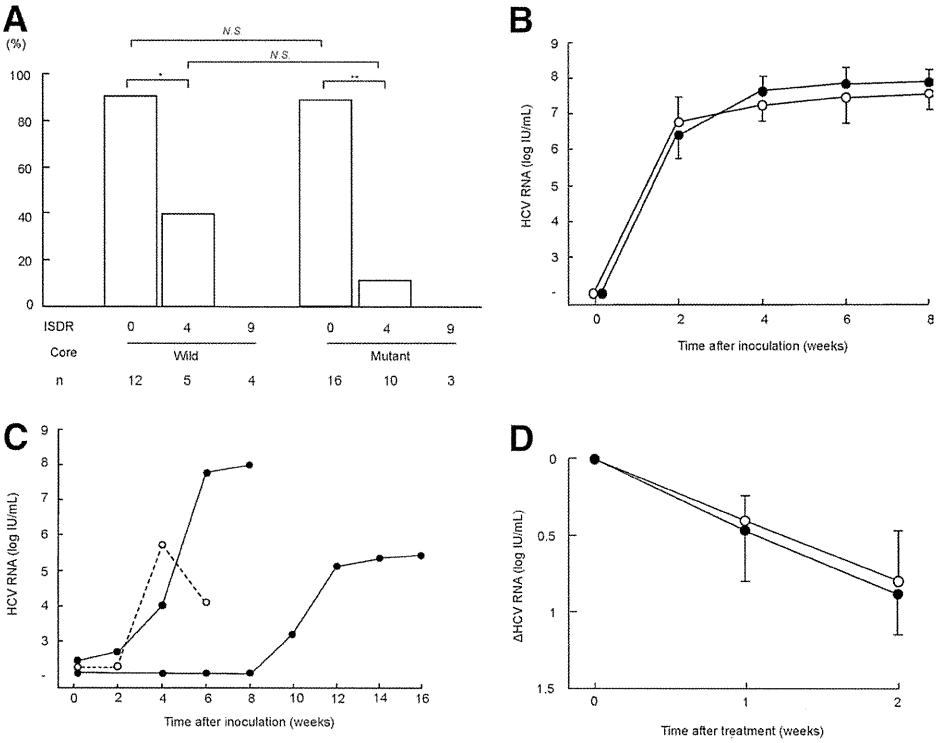

Fig. 2. Infectivity and replication ability of HCV clones. Mice that underwent transplantation with hepatocytes obtained from donor A were inoculated with 30 μg of *in vitro*-transcribed RNAs of indicated clones. (A) Proportion of HCV-infected mice. Infection was defined as serum HCV RNA titer above 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

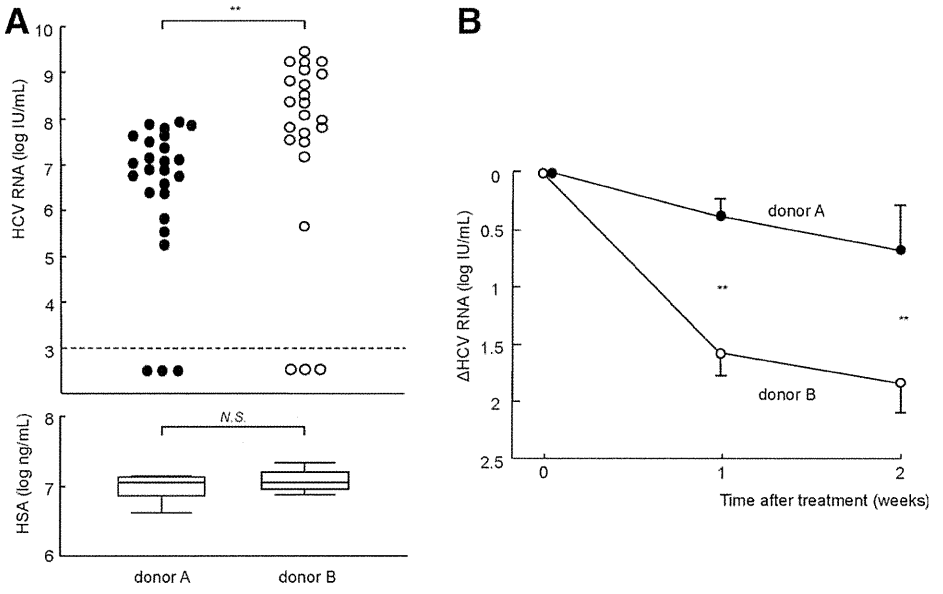

Fig. 3. HCV infectivity, replication ability, and IFN susceptibility in HCV-KT9-injected mice. Mice that underwent transplantation with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) (closed circles, n = 25) or B (rs8099917 TT and rs12979860 CC) (open circles, n = 23) were intrahepatically inoculated with RNA transcribed from either Core-Wild-ISDRO or Core-Mutant-ISDRO 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

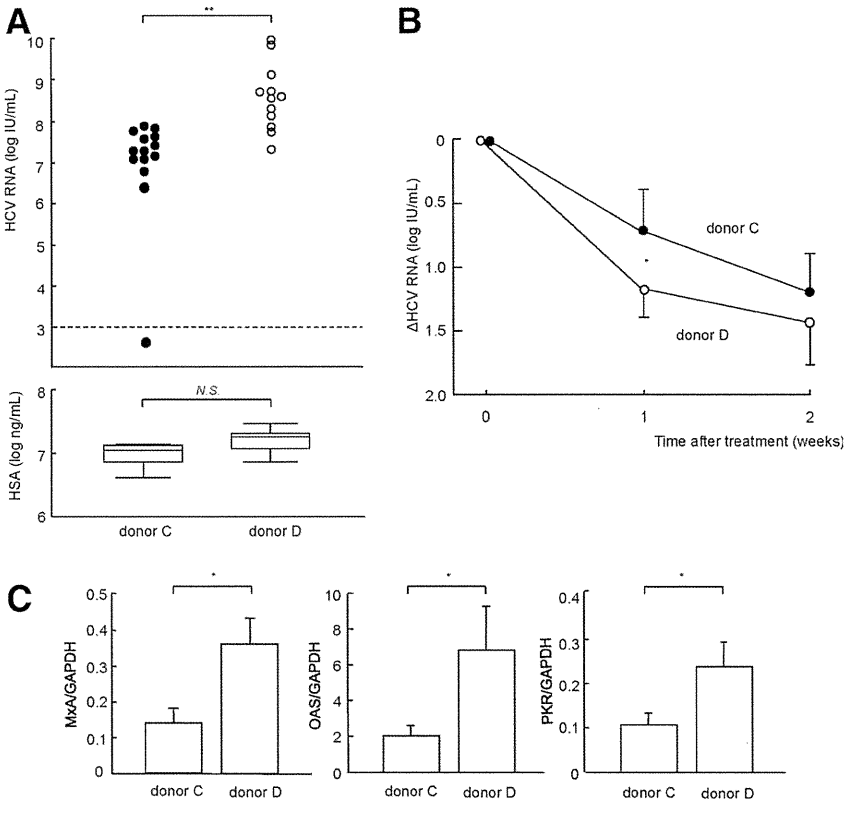

Fig. 4. HCV infectivity, replication ability, and IFN susceptibility in HCV-infected mice. Mice that underwent transplantation with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) (closes circles, n = 14) or D (rs8099917 TT and rs12979860 CC) (open circles, n = 12) were intravenously injected with HCV-infected patient serum samples. (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. HCV-infected mice with hepatocytes from donor C (closed circles, n = 5) or D (open circles, n = 4) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. (B) Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. (C) Intrahepatic ISG expression levels in the IFN-treated mice with donor C (n = 4) or D (n = 3) were measured and expressed relative to GAPDH messenger RNA. Data are reported as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

ISDR, which have been reported to be associated with the outcome of IFN plus ribavirin combination therapy.[8-14] Clones with core aa70 and 91 substitutions showed comparable infection and replication abilities, whereas clones with substitutions in the ISDR showed reduced infectivity and replication rates. It has been reported that patients infected with HCV strains with multiple substitutions in the ISDR have lower viral titers than those with wild-type ISDR, and that these patients respond well to IFN therapy.[8,9] We showed, in this study, that infectivity and replication ability of HCV are apparently impaired in ISDR mutants (Fig. 2A,C). This may explain, at least partially, the better effect of IFN therapy in patients with multiple ISDR mutations. However, why aa substitutions in this particular region are associated with the effect of IFN still remains to be elucidated. In contrast, aa substitutions in the core, which more profoundly affect the outcome of combination therapy,[10-13] did not influence the infectivity and replication ability of the virus (Fig. 2A,B). This suggests that aa substitutions in this region affect response to therapy in a way that is independent of the replication level of the virus. A recent report by Eng et al.[36] showed that a mutation in core aa91 results in the production of minicore protein, which might alter the effect of IFN. The presence of minicore protein and its effect on IFN therapy should be further investigated using the chimeric mouse model.

In contrast to these viral substitutions, host IL28B genotype significantly affected viral replication levels (Figs. 3A and 4A). Curiously, replication levels of the virus are higher in mice with human hepatocytes from donors with rs8099917 TT and rs12979860 CC genotypes, even though these genotypes are associated with successful response to the therapy.[20-22] This result is consistent with clinical observation of higher viral loads in patients with the rs12979860 CC genotype.[20] The favorable IL28B genotype is associated not only with successful response to IFN treatment, but also to spontaneous clearance of the virus.[37,38] However, the incidence of HCV infection was similar in mice with hepatocytes from donors with rs8099917 TT and rs8099917 TG (Figs. 3A and 4A), suggesting that spontaneous clearance was rare. The fact that our animal model was immunodeficient suggests that spontaneous clearance of HCV might require the involvement of the adaptive immune system. The wild-type core protein, aa70, is reported to be found more often in patients with the rs8099917 TT genotype,[23,24] even though patients with this genotype are more likely to be able to eradicate the virus without therapy during

the natural course of infection.[37,38] These data suggest that core aa70 wild-type virus can be eradicated more easily in the natural course of infection, especially in patients with rs8099917 TT or rs12979860 CC genotypes; but once the infection is established, core aa70 wild type replicates more effectively than core aa70 mutant strains.

The effect of IFN on reduction of the virus did not differ between core aa70 wild-type and mutant strains, which showed similar replication levels (Fig. 2D). This is in contrast to clinical observations that the effect of therapy on viral reduction is more prominent in patients with wild-type core protein.[13,25] One of the differences between the mouse model and human patients is term of infection. Long-term HCV infection results in alteration of lipid metabolism and accumulation of lipids in hepatocytes.[39] Patients with fatty change of the liver often fail to respond to therapy.[40] We observed no severe fatty change in mouse livers, suggesting that such long-term change might be absent in this mouse model (data not shown).

On the other hand, the effect of IFN was significantly greater in mice with hepatocytes with the eradication-favorable IL28B genotype (rs8099917 TT and rs12979860 CC) (Figs. 3B and 4B), despite the higher replication rate of the virus. This suggests that the IL28B genotype affects the outcome of therapy based on a different mechanism than viral replication. Because of strong linkage disequilibrium, genotypes of the SNPs around the two IL28B landmark SNPs (rs8099917 and rs12979860) were identical between donors A and C as well as between B and D (data not shown). Further study using human hepatocytes with various IL28B SNP genotypes will identify a primary SNP that directly affects the outcome of therapy. Response to IFN was associated with higher expression levels of ISGs, including MxA, OAS, and PKR (Fig. 4C). This is in agreement with previous studies showing that SVR is associated with stronger induction of ISG expression.[41] However, we observed no statistically significant differences in ISG expression levels from the IL28B SNP genotype before therapy (data not shown). This may result from lower ISG expression levels before therapy and the relatively small number of mice examined. Because there is no adaptive immune system in this mouse model, such differences primarily involve individual hepatocytes, although whether the presence of immune cells enhances this difference should be investigated further.

In summary, we demonstrated that viral infectivity and replication ability are associated with hepatocyte IL28B genotype and are not associated with viral substitutions in the core protein or ISDR. Understanding the mechanism underlying the higher, more prolonged expression of antiviral genes in response-favorable hepatocytes will help us to develop improved therapeutic regimens to eradicate HCV more effectively.

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IL28B polymorphism may guide pegylated interferon plus ribavirin therapy even after curative treatment for hepatitis C virus-related hepatocellular carcinoma

T. Kawaoka,[1,2] H. Aikata,[1] S. Takaki,[1] A. Hiramatsu,[1] K. Waki,[1] N. Hiraga,[1] D. Miki,[1,2] M. Tsuge,[1] M. Imamura,[1] Y. Kawakami,[1] S. Takahashi,[1] H. Ochi,[1,2] H. Tashiro,[3] H. Ohdan[3] and K. Chayama[1,2] [1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Minami-ku, Hiroshima University, Hiroshima, Japan;* [2]*Laboratory for Digestive Diseases, Centre for Genomic Medicine, RIKEN (The Institute of Physical and Chemical Research), Minami-ku, Hiroshima, Japan; and* [3]*Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan*

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SUMMARY. The present study was designed to determine the predictive factors for the viral response to pegylated interferon-alpha plus ribavirin combination therapy (PEGIFN/RBV) administered after curative treatment for hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC). The study group was 78 patients treated between January 2005 and January 2009. The sustained viral response (SVR) rate was 25.8% (15/58) in patients infected with HCV-genotype 1 and 55.0% (11/20) in those with genotype 2. Among the 78 patients, 32 (41.0%) could not complete the treatment protocol, and this was because of HCC recurrence in 17 (53%) of them. Multivariate analysis identified partial early viral response (pEVR) as the only independent determinant of SVR [odds ratio (OR) 14.73, $P = 0.013$] for patients with genotype 1. Multivariate analysis identified male gender (OR 8.72, $P = 0.001$) and interleukin-28B (IL-28B) genotype (rs8099917) TT (OR 7.93, $P = 0.007$) as independent predictors of pEVR. Multivariate analysis also identified IL-28B genotype GG+TG (OR 14.1, $P = 0.021$) and α-fetoprotein >30 (OR 5.4, $P = 0.031$) as independent predictors of null response. Patients with SVR showed a better survival rate than those without SVR ($P = 0.034$). The second HCC recurrence rate tended to be lower in patients with SVR than in those without SVR ($P = 0.054$). With regard to the prognosis of patients with SVR, it is desirable to achieve SVR with interferon therapy even when administered after HCC treatment. IL-28B genotype is a potentially useful marker for the response to PEGIFN/RBV therapy administered after curative treatment of HCV-related HCC.

Keywords: curative treatment, hepatitis C virus, hepatocellular carcinoma, interleukin-28B, pegylated interferon-alpha plus ribavirin combination therapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Chronic infection with hepatitis C virus (HCV) has been associated with hepatocarcinogenesis [1–3]. Recent advances in imaging and treatment modalities have brought about some improvement in the prognosis of patients with HCV-related HCC, but the overall outcome remains unsatisfactory; the 5-year survival rate is only 50–70%, even after curative treatment such as hepatic resection or local ablation [4]. The reasons for this unfavourable prognosis are considered to include high intrahepatic tumour recurrence rates and sustained hepatic damage, both resulting from HCV infection [5]. Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumour recurrence within 1 year is 20–40%, rising to about 80% by 5 years [4,6,7].

Intrahepatic recurrence of HCC may result from intrahepatic metastasis originating from the primary HCC or from ongoing multicentric carcinogenesis related to chronic HCV infection. The background HCV-related hepatic damage may

Abbreviations: AFP, α-fetoprotein; cEVR, complete early viral response; HCV, hepatitis C virus; IFN, interferon; IL-28B, interleukin-28B; NR, null response; PEGIFN, pegylated interferon; PEGIFN/RBV, pegylated interferon-alpha plus ribavirin combination therapy; pEVR, partial early viral response; RBV, ribavirin; SNP, single-nucleotide polymorphism; SVR, sustained viral response; TR, transient viral response.

Correspondence: Hiroshi Aikata, MD, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: aikata@hiroshima-u.ac.jp

also compromise hepatic functional reserve and worsen clinical outcome. Thus, the prevention of HCC recurrence as well as preservation of liver function constitutes high priorities for the improvement of prognosis of patients with HCV-related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective in reducing serum alanine transaminase (ALT) activity and in eradicating HCV [8,9] and thus IFN could be of value in minimizing hepatic necrosis, inflammation and fibrosis, as well as reducing the incidence of HCC. Several recent studies have reported that IFN therapy, applied even after curative treatment for HCV-related HCC, could prevent HCC recurrence and improve survival [10–21].

The recent introduction of pegylated interferon-alpha plus ribavirin combination therapy (PEGIFN/RBV) has improved the treatment efficacy [22,23]. Recent studies have highlighted the relationship between various single-nucleotide polymorphisms (SNPs) in the IL-28 locus and the effect of PEGIFN/RBV in patients infected with HCV [24–29]. Further, the results of few recent studies suggest that PEGIFN/RBV could prevent HCC recurrence and improve survival even when used after curative treatment of HCV-related HCC [30,31]. To our knowledge, however, there are no studies on the factors that could predict a sustained viral response (SVR) to PEGIFN/RBV after treatment of HCC (e.g. IL-28B as a host factor). The present study was designed to determine the predictive factors of viral response to PEGIFN/RBV in patients with HCV treated for HCC.

MATERIAL AND METHODS

Patients

The study subjects were 78 patients treated with PEGIFN/RBV after curative intent treatment (hepatic resection or radiofrequency ablation) for HCV-related HCC between January 2005 and January 2009 in this retrospective cohort study. Tumour staging was defined based on the Liver Cancer Study Group of Japan/Tumour-Node-Metastasis staging system of the Liver Cancer Study Group of Japan (LCSGJ): stage I [fulfilling three intrahepatic conditions: solitary, <2 cm, no vessel invasion, $n = 28$ (36%)], stage II [two of the three intrahepatic conditions, $n = 27$ (35%)], stage III [one of the three intrahepatic conditions, $n = 23$ (29%)], stage IVa (none of the three intrahepatic conditions, with no distant metastases or any intrahepatic conditions with lymph node metastases) and stage IVb (any intrahepatic condition with distant metastases) [stage IV, $n = 0$ (0%)] [32]. The median duration was 7 months (range, 1–60) from curative intent treatment for HCC to the start of PEGIFN/RBV therapy.

Antiviral treatment protocol

Each patient received 1.5 μg/kg body weight (BW) pegylated interferon-alpha (PEGIFN) (Peg-Intron; Schering-Plough, Segrate, Italy) subcutaneously (s.c.) once weekly, together with ribavirin (RBV) (Rebetol; Schering-Plough). The RBV dose was adjusted according to BW to 600 mg for patients <60 kg BW, 800 mg for >60 but ≤80 kg BW and 1000 mg for >80 kg BW, based on the drug information for RBV supplied by the manufacturer. The above durations and dosages are those approved by the Japanese Ministry of Health, Labour and Welfare.

The daily dose of RBV was reduced by 200 mg when haemoglobin (Hb) fell below 10 g/dL, acute fall in Hb concentration followed by stabilization at more than 3 g/dL from baseline, or appearance of clinical symptoms of anaemia (e.g. fatigue, pallor, palpitation, dyspnoea on efforts and fatigue) associated with a decrease in Hb of >2 g/dL from baseline. Once the RBV dose was reduced, it was maintained at that level throughout the rest of study. The protocol also included withdrawal of RBV when Hb fell below 8.5 g/dL or when patients manifested more severe anaemia including orthostatic hypotension. After the end of the treatment, the patients were followed up for 24 more weeks without treatment. The treatment term was 48 weeks for patients infected with HCV genotype 1 and 24 weeks for those with genotype 2.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Ethics Committees of all participating centres. Written informed consent was obtained from each patient. At each visit, information on possible side effects was obtained by questioning the patients in a structured manner about specific, commonly observed and expected side effects of the study medication, such as flu-like symptoms, fatigue, nausea, vomiting, diarrhoea, dizziness, depression and hair loss.

Single-nucleotide polymorphism genotyping and quality control

Because the two reported significant IL-28 SNPs (rs8099917 and rs12979860) are in strong linkage disequilibrium, we analysed only rs8099917 in this study. Some samples obtained from patients with HCV were determined using the Illumina HumanHap610-Quad Genotyping BeadChip, whereas the remaining samples were genotyped using the Invader assay, as described previously [33,34].

Analysis of nucleotide sequence of the core and NS5A region

The amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region and mutation at the interferon sensitivity-determining region (ISDR) in the nonstructural 5A (NS5A) region of HCV were analysed by the direct sequencing method as described previously by our group [35–37].

Assessment of viral response

Serum HCV RNA was determined at baseline, after 4, 8, 12, 16 and 20 weeks of treatment, at the end of treatment and

at the end of the 24-week drug-free follow-up period. HCV RNA was assessed by qualitative reverse transcription polymerase chain reaction (TaqMan RT-PCR). SVR represented a negative HCV RNA at 24-week follow-up without treatment after the end of active treatment. Transient viral response (TR) was defined as positive HCV RNA at 24-week follow-up after a negative HCV RNA at the end of active treatment. Complete early viral response (cEVR) was defined as negative HCV RNA at week 12 of active treatment. Partial early viral response (pEVR) was defined as HCV RNA ≥ 2 log10 drop from baseline at week 12 of active treatment. Null response (NR) was defined as HCV RNA that never dropped by ≥2 log10 from baseline at week 12 of active treatment.

Histopathological stage was assessed before treatment and determined based on the histological scoring system of Desmet *et al.* [38].

Assessment of hepatocellular carcinoma recurrence

The concentrations of serum tumour markers α-fetoprotein (AFP) and des-γ-carboxy prothrombin were measured once a month after hepatic resection or radiofrequency ablation. Follow-up US was performed every 3 months; and CT or MR imaging was performed every 6 months. IFN therapy was discontinued upon suspicion of HCC recurrence.

Statistical analysis

Nonparametric tests (chi-square test and Fisher's exact probability test) were used to compare the clinical and laboratory parameters of the two groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to early viral dynamics. The odds ratio and 95% confidence intervals (95% CI) 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 ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors.

Cumulative survival and recurrence rates were calculated from the initial date of hepatic resection or radiofrequency ablation and assessed by the Kaplan–Meier life-table method, with differences evaluated by the log rank test. All statistical analyses were performed using PASW 18 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

Table 1 shows the baseline characteristics of the patients treated with PEGIFN/RBV after hepatic resection or radiofrequency ablation for HCC. The median age of the patients (55 men and 23 women) was 66 years. The median body mass index was 22.4 kg/m^2. The median pretreatment serum HCV RNA viral load was 6.0 log IU/mL. Most patients were infected with HCV genotype 1 ($n = 58$) followed by genotype 2 ($n = 20$). IL-28B genotype (rs8099917) was TT ($n = 51$), GG+TG ($n = 25$) and no date ($n = 2$).

Table 1 The baseline characteristics of the all 78 patients treated with PEGIFN/RBV

	$n = 78$
Gender (male/female)	55/23
Age (years)*	66 (48–83)
Body mass index (kg/m^2)*	22.4 (15.6–40.1)
IL28B genotype (TT/GG+TG/ND)	51/25/2
White blood Cell (×10^3/μL)*	4.2 (2.4–7.5)
Haemoglobin (g/dL)*	13.3 (8.7–18.1)
Platelet count (×10^4/mm^3)*	11.1 (3.9–20.5)
T-bilirubin (mg/dL)*	0.7 (0.2–2.8)
Alanine aminotransferase (IU/L)*	44 (8–189)
Prothrombin time activity (%)*	87 (58–121)
Albumin (g/dL)*	4.0 (2.7–5.2)
γ-glutamyl transpeptidase (IU/L)*	45 (12–371)
HbA1c (%)*	5.3 (3.9–10.8)
Indocyanine green retention rate (%)*	15.4 (3.5–45.4)
Fibrosis stage (F1-3/F4/ND)	20/19/39
Genotype (1/2)	58/20
HCV viral load (Log IU/mL)*	6.0 (2.1–7.2)
Tumour stage (I/II/III/IV)†	28/27/23/0
α-Fetoprotein (ng/mL)*	11 (0.5–286)
Des-γ-carboxy prothrombin (mAU/mL)*	29 (10–4550)
Tumour size (mm)*	21 (7–110)
Number of tumour*	1 (1–4)
Hepatic resection/radiofrequency ablation	28/50

ND, not done; HCV, hepatitis C virus; PEGIFN/RBV, pegylated interferon-alpha plus ribavirin combination therapy. *Data are median and (range). †Tumour staging was defined based on the Liver Cancer Study Group of Japan/Tumor-Node-Metastasis staging system of the Liver Cancer Study Group of Japan.

Efficacy and tolerance of therapy and adverse events

Figure 1 shows the effects of PEGIFN/RBV treatment according to genotype. The SVR rate was 33.3% (26/78) for all patients. The PRGIFN/RBV treatment protocol could not be completed by 32 (41%) patients; 17 (53%) of the 32 developed HCC recurrence. In 58 patients with genotype 1, PEGIFN and RBV were discontinued in 29% (17/58) patients because of HCC recurrence and because of other reasons in another 9 (15.5%) [general fatigue ($n = 3$), cancer of the

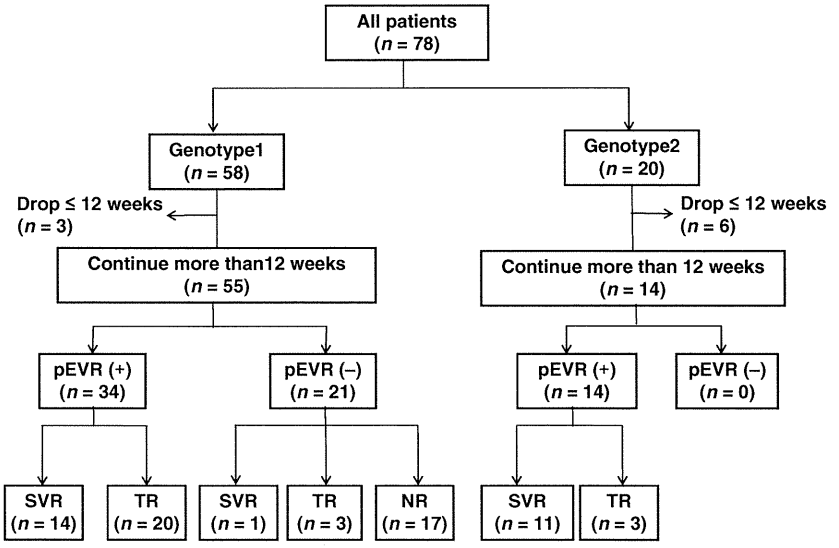

Fig. 1 Flow diagram showing the course of Peg-related interferon plus ribavirin therapy after curative treatment for hepatitis C virus (HCV)-related hepatocellular carcinoma. According to HCV genotype, 78 patients treated with pegylated interferon-alpha plus ribavirin combination therapy were divided into three groups, namely the sustained virological response, transient response and null response. *n*, number of patients.

throat (n = 1), vomiting (n = 1), itching (n = 1), pulmonary haemorrhage (n = 1), jumpiness (n = 1), sarcoidosis (n = 1)] within 48 weeks. Thus, PEGIFN and RBV treatment could be achieved in 55% (32/58) of the patients. Furthermore, 95% (55/58) of the patients continued the treatment for more than 12 weeks. Among 55 patients, 34 achieved pEVR, including 21 patients achieved cEVR, 14 achieved SVR and 20 showed TR. In the other 21 patients who did not achieve pEVR, one patient achieved SVR and three patients showed TR while 17 patients showed NR. Thus, the SVR rate was 25.8% (15/58) for patients infected with HCV genotype 1.

Among the 20 patients infected with genotype 2, 6 discontinued treatment because of side effects [general fatigue (n = 3), thrombocytopenia (n = 1), diabetes mellitus (n = 1), bleeding from oesophageal varices (n = 1)] within 12 weeks. The remaining 14 (70%) patients completed the treatment protocol. All 14 patients achieved pEVR, including 11 who showed SVR and three achieved TR. Thus, the SVR rate was 55.0% (11/20) for patients infected with genotype 2 (Fig. 1).

Relationship between IL-28B and viral response in patients infected with hepatitis C virus genotype 1

In patients infected with HCV genotype 1, number of patients with TT genotype of IL-28B was 44 (TT group) and GG+TG was 14 (GG+TG group). The SVR rate of the TT group [34.3% (n = 14/41)] was higher than that of the TG+GG group [7% (n = 1/14), P = 0.08, Fig. 2A]. The pEVR rate of TT group [73.1% (n = 30/41)] was also significantly higher than that of the TG+GG group [28.5% (n = 4/14), P = 0.009, Fig. 2B]. The NR rate of the TT group [19.5% (n = 8/41)] was significantly lower than that of the TG+GG group [64.2% (n = 9/14), P = 0.005, Fig. 2C].

Determinants of sustained viral response in patients infected with hepatitis C virus genotype 1

Next, we analysed the factors that determine SVR using data of 55 patients infected with HCV genotype 1 who continued PEGIFN/RBV therapy for more than 12 weeks (Table 2). Univariate analysis identified five parameters that correlated with SVR: pEVR (P = 0.004), viral load (<6.0 g/dL; P = 0.008), completion of therapy (P = 0.06), IL-28B genotype (TT genotype; P = 0.08) and gender (man; P = 0.043). Multivariate analysis identified pEVR as the only significant and independent factor that influenced the SVR: (odds ratio, 14.73, 95%CI 1.7–123.2, P = 0.013).

Determinants of partial early viral response in patients infected with hepatitis C virus genotype 1

Next, we analysed the factors that determine pEVR using data of 55 patients infected with HCV genotype 1 who continued PEGIFN/RBV treatment for >12 weeks. Univariate analysis identified three parameters that correlated with pEVR: IL-28B genotype (TT genotype; P = 0.009), gender (man; P = 0.005) and viral load (<6.0 g/dL; P = 0.068) (Table 3). Multivariate analysis identified two parameters that independently influenced the pEVR: gender (male; odds ratio 8.72, 95%CI 2.1–41.6, P = 0.001) and IL-28B genotype (TT genotype; odds ratio 7.93, 95%CI 1.7–36.0, P = 0.007, Table 4). Mutations of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region were not significantly different between the pEVR and non-pEVR groups among patients infected with HCV genotype 1b in our study.

Determinants of null response in patients infected with hepatitis C virus genotype 1

Next, we analysed the factors that determine the NR in patients infected with HCV genotype 1 (n = 55). Univariate

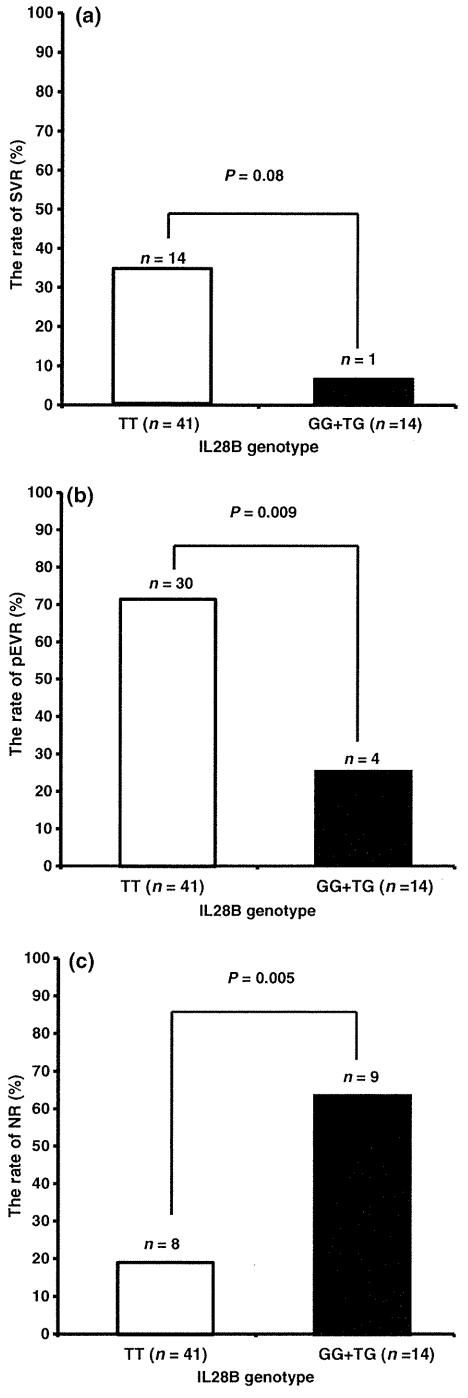

Fig. 2 Relationship between IL-28B and viral response in patients infected with hepatitis C virus genotype 1. (a) Sustained viral response rate, (b) Partial early viral response rate, (c) null response rate.

analysis identified three parameters that influenced NR: IL-28B genotype (genotype; GG+TG, $P = 0.005$), AFP (>30 ng/dL; $P = 0.054$) and gender (male; $P = 0.022$) (Table 5). Multivariate analysis identified two parameters that independently influenced the NR: IL-28B genotype (genotype GG+TG; odds ratio 7.8, 95%CI 1.81–34.4, $P = 0.006$) and AFP (>30; odds ratio 5.6, 95%CI1.40–22.8, $P = 0.015$) (Table 6). Mutations of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region were not significantly different between the NR and SVR+TR groups among patients infected with HCV genotype 1b.

Survival rates

The overall survival rate was significant different between patients of the SVR and non-SVR groups ($P = 0.034$). The survival rate of the SVR groups was 100% at 1 year, 100% at 3 years and 100% at 5 years. In contrast, the rates of the non-SVR group were 100%, 96% and 74%, respectively (Fig. 3).

Comparison of the first and second recurrence rates of hepatocellular carcinoma

Finally, we compared the overall cumulative rates of the first and second recurrence of HCC between the SVR and non-SVR groups (Fig. 4). The 1-, 3- and 5-year rates of the first recurrence of HCC in the SVR and non-SVR group were not different (0% *vs* 6.7%, 38.1% *vs* 37% and 48% *vs* 68%, respectively, Fig. 4A, $P = 0.41$). The 1-, 3- and 5-year rates of the second recurrence in the SVR and non-SVR groups were 0% *vs* 0%, 41% *vs* 64% and 48% *vs* 78%, respectively (Fig. 4(B), $P = 0.054$). These results demonstrated that patients of the SVR group tended to have a better chance of escaping a second HCC recurrence compared with those of the non-SVR group.

DISCUSSION

Several recent studies have reported that IFN therapy can prevent HCC recurrence and improve survival, especially in patients with SVR, even when administered after curative treatment for HCV-related HCC [10–21,31,39]. While there are a few reports of the use of PEGIFN/RBV after curative treatment for HCV-related HCC [30,31], none have discussed the SVR rate and the factors that determine the viral response to PEGIFN/RBV in such patients. In the present study, we reported the viral response and determinants (specially SNPs) of viral response with PEGIFN/RBV after treatment of HCC.

In our study, the SVR rate was 33.3% (26/78) for all patients, while that for patients with genotype 1 was 25.8% (15/58) and genotype 2 was 55.0% (11/20). These SVR rates are lower than that of patients with chronic hepatitis. The lower rate in the present study was probably because of the low number of patients who completed the therapy. The reason for the latter was the relatively high rate of HCC recurrence [53% (17/32)].

One of the major reasons of the low SVR rate was probably of discontinuation of therapy because of HCC recurrence.

Table 2 Univariate analysis of factors associated with SVR in 55 patients with genotype 1 continued PEGIFN/RBV >12 weeks

	SVR (n = 15)	TR+NR (n = 40)	P
Gender (male/female)	1/14	25/15	0.043
Age (years)*	65 (54–74)	65 (53–83)	0.94
Body mass index (kg/m^2)*	21.2 (18.4–28.5)	23.0 (18.7–40.1)	0.174
White blood Cell ($\times 10^3/\mu$L)*	5050 (4390–6130)	4280 (2470–6660)	0.8
Haemoglobin (g/dL)*	13.7 (11.2–14.8)	13.4 (9.3–18.1)	0.96
Platelet count ($\times 10^4$/mm^3)*	12.5 (3.9–19.6)	10.0 (4.7–20.8)	0.138
T-bilirubin (mg/dL)*	0.7 (0.4–1.8)	0.7 (0.2–1.7)	0.58
Alanine aminotransferase (IU/L)*	33 (12–189)	45 (17–166)	0.25
Prothrombin time activity (%)*	88 (80–106)	86 (64–121)	0.49
Albumin (g/dL)*	4.1 (3.7–4.9)	4.0 (2.7–4.9)	0.52
Fibrosis stage (F1-3/F4/ND)	2/2/11	10/15/15	1.0
γ-glutamyl transpeptidase (IU/L)	43 (12–87)	46 (15–294)	1.2
HbA1c (%)	5.1 (4.2–10.2)	5.4 (3.9–10.8)	0.41
Indocyanine green retention rate (%)	17.7 (7.5–37.8)	17.4 (3.5–45.4)	0.92
HCV viral load (Log IU/mL)	5.59 (4.3–7.1)	6.23 (1.2–7.2)	0.08
HCV Core70(mutant/wild)	8/7	23/17	1.0
HCV Core91 (mutant/wild)	5/10	21/19	0.23
HCV ISDR (0–1/>2)	9/6	26/14	0.75
α-Fetoprotein (ng/mL)*	6.9 (5–286.8)	19.7 (5–63240)	0.11
IL28B genotype (TT/GG+TG)	14/1	27/13	0.08
pEVR (yes/no)	14/1	20/20	0.004
Dose of PEGIFN at administration (μg/kg)*	80 (40–100)	80 (50–120)	0.74
Dose of RBV at administration(mg)*	600 (200–800)	600 (200–1000)	0.26
Therapy were completed (yes/no)	12/3	20/20	0.06

ND, not done; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; NR, null response; PEGIFN/RBV, pegylated interferon-alpha plus ribavirin combination therapy; pEVR, partial early viral response; SVR, sustained viral response; TR, Transient viral response. *Data are median and (range).

Alternatively, the low rate could be because of a high proportion of patients with advanced liver fibrosis. In fact, 19 (11.5%) patients were classified as F4 stage, and the median of platelet count was 11.1 × 10^4/mm^3. These reasons may explain the low IFN therapy continuation rate (55.2%) and the low SVR rate.

We analysed the factors that affect SVR in 55 patients infected with HCV genotype 1 who were able to continue therapy for more than 12 weeks. Multivariate analysis identified a single parameter that independently influenced the SVR: pEVR. Among the 55 patients, 34 (61.8%) achieved pEVR. Among the pEVR group, 14 (41.1%) patients achieved SVR. Recent studies reported the importance of the response guide-based therapy in the treatment of chronic hepatitis; i.e. 70–80% of patients of the cEVR group achieved SVR [40–43].

On the other hand, gender (male) and IL-28B genotype (TT) were identified as significant and independent predictors of pEVR. These factors are probably also significant and independent predictors of SVR in patients with chronic hepatitis C.

Thus, male patients with IL-28B genotype TT were more likely to achieve pEVR even when PEGIFN/RBV treatment was introduced after curative treatment for HCV-related HCC.

Evidence suggests that the SVR rate could be improved by IFN therapy (long-term low-dose IFN of 72 weeks instead of 48 weeks). In fact, Pearlman *et al.* [43] reported that the SVR rate was superior in patients treated for 72 *vs* 48 weeks (38% *vs* 18%, respectively; P = 0.026) in the pEVR groups. Furthermore, the SVR rate could be improved by combination therapy for HCC and HCV. For example, to achieve SVR, it might be better to restart PEGIFN/RBV therapy immediately after curative treatment of HCC.

On the other hand, multivariate analysis identified IL-28B genotype (GG+TG) as an independent parameter that influenced the NR. In this group, it is better to select low-dose intermittent IFN therapy than PEGIFN/RBV based on the SVR. In fact, it is reported that low-dose intermittent IFN therapy after hepatectomy for HCC improved liver function of patients with HCV-related HCC, and the preservation of hepatic function increased the chance of successful treatment against recurrence [10]. In contrast, mutations of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region were not significant and independent predictors of pEVR and NR.

Table 3 Univariate analysis of factors associated with pEVR in 55 patients with genotype 1 continued PEGIFN/RBV >12 weeks

	pEVR (n = 34)	non-pEVR (n = 21)	P
Gender (male/female)	29/5	10/11	0.005
Age (years)*	67 (54–83)	63 (53–72)	0.977
Body mass index (kg/m^2)*	23.6 (18.7–40.1)	22.2 (18.4–30.0)	0.151
White blood Cell (×10^3/μL)*	5150 (4390–6660)	3610 (2470–4930)	0.8
Haemoglobin (g/dL)*	13.8 (10.2–18.1)	12.3 (9.3–17.4)	0.745
Platelet count (×10^4/mm^3)*	11.1 (3.9–20.8)	10.0 (4.7–18.2)	0.126
T-bilirubin (mg/dL)*	0.7 (0.2–1.8)	0.7 (0.5–1.7)	0.53
Alanine aminotransferase (IU/L)*	44 (17–189)	37 (12–134)	0.319
Prothrombin time activity (%)	88 (68–114)	85 (64–121)	0.41
Albumin (g/dL)*	4.0 (3.4–4.9)	4.0 (2.7–4.9)	0.405
Fibrosis stage (F1-3/F4/ND)	8/8/18	9/4/8	0.43
γ-glutamyl transpeptidase (IU/L)	52 (12–219)	26 (15–294)	0.172
HbA1c (%)	5.5 (4.2–8.8)	5.0 (3.9–10.8)	0.49
Indocyanine green retention rate (%)	17.4 (3.5–37.8)	18.7 (7.6–45.4)	0.92
HCV viral load (Log IU/mL)	6.04 (4.3–7.2)	6.23 (1.2–6.7)	0.068
HCV Core70(mutant/wild)	19/15	13/8	0.78
HCV Core91 (mutant/wild)	13/21	13/8	0.17
HCV ISDR (0–1/>2)	19/15	14/7	0.24
α-Fetoprotein (ng/mL)*	9.1 (5.0–909.2)	42.0 (5.0–63240)	0.116
IL28B genotype (TT/GG+TG)	30/4	11/10	0.009
Dose of PEGIFN at administration (μg/kg)*	80 (40–120)	80 (50–100)	0.689
Dose of RBV at administration (mg)*	600 (200–1000)	600 (200–800)	0.20
Therapy were completed (yes/no)	21/13	11/10	0.4

HCV, hepatitis C virus; PEGIFN/RBV, pegylated interferon-alpha plus ribavirin combination therapy; pEVR, partial early viral response. *Data are median and (range).

Table 4 Multivariate analysis of factors associated with pEVR

Factor	Category	Odds ratio (95%CI)	P
Gender	Female	1	0.001
	Male	8.72 (2.1–41.6)	
IL28B genotype	GG+TG	1	0.007
	TT	7.93 (1.7–36.0)	

pEVR, partial early viral response.

Achieving SVR by PEGIFN/RBV treatment, even when administered after curative treatment for HCV-related HCC, could prevent HCC recurrence and improve survival. Although achieving SVR had no impact on the occurrence of HCC at the initial site, patients of the SVR group tended to show a lower rate of second HCC recurrence in this and another study [31]. It was reported that IFN therapy had no impact on the occurrence of HCC shortly after IFN therapy was started. It was speculated that IFN therapy does not suppress latent HCC. In our study, although the first recurrence rate of HCC was similar between patients with and without SVR, the second HCC recurrence rate tended to be lower in patients with SVR than in those without SVR (P = 0.054). Therefore, efforts should be directed to achieve SVR by PEGIFN/RBV therapy after curative treatment of HCV-related HCC, whenever possible. Importantly, the SVR rate for PEGIFN/RBV combination therapy was better than that for IFN monotherapy. On the other hand, the high rate of incomplete PEGIFN/RBV therapy (44.8%) was one of the causes of the high HCC recurrence rate and the advanced liver fibrosis. Our study identified factors that affect the viral response to PEGIFN/RBV therapy, and the identification of these factors should help in the selection of patients who will best benefit from such therapy.

On the other hand, the SVR rate was 55.0% (11/20) in patients infected with HCV genotype 2. Although the sample size was small, 78.5% (11/14) patients who showed pEVR achieved SVR. Therefore, continuation of treatment is likely to result in achievement of SVR even when PEGIFN/RBV treatment is started after curative treatment for HCV-related HCC. Efforts should be made to achieve SVR by PEGIFN/RBV therapy in patients infected with HCV genotype 2 after curative treatment for HCV-related HCC. Recently, the relationship between IL-28B and the effect of PEGIFN/RBV