

## Predictive value of the *IL28B* polymorphism on the effect of interferon therapy in chronic hepatitis C patients with genotypes 2a and 2b

Tomokazu Kawaoka<sup>1,2,3</sup>, C. Nelson Hayes<sup>1,2,3</sup>, Waka Ohishi<sup>3,5</sup>, Hidenori Ochi<sup>1,2,3</sup>, Toshiro Maekawa<sup>1</sup>, Hiromi Abe<sup>1,2,3</sup>, Masataka Tsuge<sup>2,3</sup>, Fukiko Mitsui<sup>2,3</sup>, Nobuhiko Hiraga<sup>2,3</sup>, Michio Imamura<sup>2,3</sup>, Shoichi Takahashi<sup>2,3</sup>, Michaki Kubo<sup>4</sup>, Tatsuhiko Tsunoda<sup>6</sup>, Yusuke Nakamura<sup>7</sup>, Hiromitsu Kumada<sup>8</sup>, Kazuaki Chayama<sup>1,2,3,\*</sup>

<sup>1</sup>Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN (The Institute of Physical and Chemical Research), 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; <sup>2</sup>Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan; <sup>3</sup>Liver Research Project Center, Hiroshima University, Hiroshima, Japan; <sup>4</sup>Laboratory for Genotyping Development, the RIKEN Center for Genomic Medicine, Yokohama, Japan; <sup>5</sup>Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan; <sup>6</sup>Laboratory for Medical Informatics, The RIKEN Center for Genomic Medicine, Yokohama, Japan; <sup>7</sup>Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan; <sup>8</sup>Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan

**Background & Aims:** Common *IL28B* locus polymorphisms (SNPs rs8099917 and rs12979860) have been reported to affect peg-interferon plus ribavirin combination therapy (PEG-RBV) for hepatitis C virus (HCV) genotype 1b, but few reports have examined their effect on other two common genotypes, 2a and 2b.

**Methods:** We analyzed predictive factors for sustained virological response (SVR) in a retrospective study of 719 patients with either genotype 2a (530) or 2b (189). Of these patients, 160 were treated with PEG-RBV and 559 were treated with interferon monotherapy. We evaluated predictive factors including HCV RNA, histological findings, *IL28B* SNP genotypes (rs8099917, rs12979860, and rs12980275), and the effect of treatment regimen and prior treatment history.

**Results:** HCV RNA viral load, treatment regimen, and rs8099917 genotypes independently contributed to the effect of the therapy. For patients treated with PEG-RBV, rs8099917 and viral load were independent predictive factors for SVR in genotype 2b but not in genotype 2a. Conversely, in patients treated with interferon monotherapy, viral load and rs8099917 were independent

predictive factors for SVR in genotype 2a but not in genotype 2b. The favorable rs8099917 genotype is also associated with a steep decline in viral load by the second week of treatment.

**Conclusions:** Initial viral load and rs8099917 genotype are significant independent predictors of SVR in genotype 2 patients.

© 2010 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

### Introduction

Hepatitis C virus (HCV) infection is a major worldwide cause of chronic liver diseases, affecting an estimated 170 million people [1]. Chronic HCV infection may progress to hepatocellular carcinoma (HCC) or liver cirrhosis (LC) [2–6], and in Japan, 60–70% of patients with HCC or LC are HCV carriers [7]. There are two major genotypes (1 and 2) and three sub-genotypes (1b, 2a, and 2b) in Japan as well as in many other countries [8]. Although pathological features of these genotypes are similar [9,10], interferon therapy is more effective against genotype 2 than genotype 1 [11,12]. Compared to the less than 50% of genotype 1 patients who respond to therapy [13–19], more than 80% of genotype 2 patients who received 24-week peg-interferon and ribavirin (PEG-RBV) combination therapy achieved sustained virological response (SVR), defined as absence of HCV RNA six months after the cessation of therapy. Because of this otherwise high success rate, the small subset of genotype 2 patients who fail to respond to therapy should be examined more closely. Although treatment-resistant genotype 2 sub-populations have been reported [20–22], the mechanism underlying variable response to treatment is unclear. Multiple viral (e.g., HCV genotype, amino acid substitutions in the NS5A and core region [22–26]) and host factors (e.g., age [14], body mass index [27], and insulin resistance

**Keywords:** Interferon therapy; Single nucleotide polymorphism; Ribavirin; Hepatitis C.

Received 12 March 2010; received in revised form 1 July 2010; accepted 2 July 2010; available online 19 September 2010

\*Corresponding author at: 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 address: chayama@hiroshima-u.ac.jp (K. Chayama).

**Abbreviations:** HCV, hepatitis C virus; IFN, interferon; PEG-IFN, pegylated interferon; RBV, ribavirin; PEG-RBV, pegylated interferon plus ribavirin combination therapy; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NR, non-responder.



Table 1. Baseline characteristics of patients with HCV genotypes 2a and 2b.

	All (n = 719)	2a (n = 530)	2b (n = 189)
Sex (M/F)	403/316	301/229	102/87
Age	57 (49-64)	56 (48-64)	59 (50-66)
Body weight (kg)	59.8 (51-71.4)	60.15 (53.75-71.65)	57.4 (48.5-70)
BMI (kg/m <sup>2</sup> )	23.2 (20.3-25.7)	24.48 (21.43-26.4)	21.78 (19.89-24.79)
Fibrosis (F0-2/F3-4)	484/101	359/68	125/33
Treatment (IFN/PEG-RBV)	559/160	477/53	82/107
Treatment naïve (Y/N)	689/30	523/7	166/23
HCV RNA (log IU/ml)	5.3 (4.7-5.9)	5 (4.6-5.7)	5.9 (5.5-6.5)
rs8099917 (TT/GT/GG)	572/135/11	425/97/7	147/38/4
rs12979860 (CC/TC/TT)	565/137/11	422/98/7	143/39/4
rs12980275 (AA/GA/GG)	543/158/16	402/116/10	141/42/6
SVR/non-SVR	455/264	340/190	115/74

IFN, interferon monotherapy; PEG-RBV, peg-interferon plus ribavirin combination therapy; SVR, sustained viral responder.

[28]) have been reported to affect the outcome of interferon therapy in genotype 1-infected patients but such factors have not been closely examined in genotype 2 patients.

Single nucleotide polymorphisms (SNPs) and other genetic factors have been reported to be useful in predicting the outcome of interferon therapy. Polymorphisms in MxA [29,30], interferon alpha-receptor 1 [31], and osteopontin [32] have also been reported to be associated with interferon response. We also identified a MAPKAPK3 SNP [33] that is a predictive factor for interferon mono-therapy. Recently, several groups have reported an association between several SNPs in the *IL28* locus and the effect of PEG-RBV combination therapy for genotype 1b [34-38] but only a few studies have examined the role of these SNPs in the treatment of other genotypes. In this study, we analyzed predictive factors for SVR in genotype 2a and 2b patients treated with PEG-RBV. Because PEG-RBV was only approved for use in Japan in 2005, we also examined predictive factors in patients who were treated with interferon monotherapy, which is still used in the event of an adverse reaction to ribavirin.

## Patients and methods

### Patients and study design

We studied 719 Japanese patients with chronic hepatitis C (positive for HCV RNA for more than 6 months) who received interferon therapy with or without ribavirin between 2002 and 2008. Patients were treated at Toranomon Hospital in Tokyo, Hiroshima University Hospital, and hospitals belonging to the Hiroshima Liver Study Group (<http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html>). All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease, and had not received immunosuppressive therapy before enrollment in the study. All patients gave written informed consent to participate in the study in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and according to the process approved by the ethical committees of Hiroshima University and the SNP Research Center at the Institute of Physical and Chemical Research (RIKEN) in Yokohama.

PEG-RBV patients received weekly injections of peg-interferon-alpha-2b at 1.5 g/kg body weight for 24 weeks. Ribavirin was administered orally, and the dosage was determined based on the patient's body weight (600 mg for <60 kg, 800 mg for 60-80 kg, 1000 mg for >80 kg). Patients receiving interferon mono-

therapy were treated daily with 6 million units of IFN intramuscularly for 8 weeks, followed by the same dose three times a week for 16 weeks, for a total of 528 million units. Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA-negative six months after cessation of therapy. Fibrosis stage and activity were diagnosed by pathologists at each hospital according to the criteria of Desmet et al. [39]. Patients were classified as interferon treatment naïve or experienced based on prior interferon treatment but only parameters related to the most recent therapy were used in the analysis.

### SNP Genotyping and quality control

We genotyped each patient for three *IL28B* SNPs previously reported to be associated with therapy outcome: rs8099917, rs12979860, and rs12980275. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or the Invader assay, as described previously [40,41]. We were unable to determine genotypes for one of the 796 patients for rs8099917, six of the patients for rs12979860, and two for rs12980275.

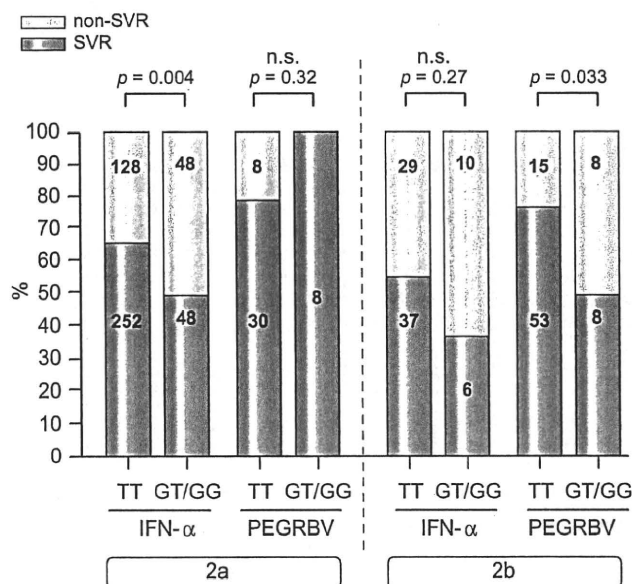


Fig. 1. Effect of interferon therapy on patients with genotype 2a and 2b infection. Sustained viral responders (SVR) and non-responders (non-SVR) were analyzed by *IL28B* SNP rs8099917 genotype, viral genotype, and treatment type. All patients were interferon-naïve.

# Research Article

**Table 2. Predictors for SVR in treatment-naïve patients treated with peg-interferon plus ribavirin combination therapy.**

Genotype	Variable	Simple		Multiple			
		n	p	n	OR	(95% CI)	p
2a + 2b	Age	130	0.42				
	Sex	130	0.62				
	Genotype	130	0.21				
	Viral load	127	0.002 **	127	0.19	(0.06-0.55)	0.002 **
	Fibrosis	110	0.25				
	rs8099917	130	0.23				
	rs12980275	129	0.79				
2a	Age	46	0.77				
	Sex	46	0.62				
	Viral load	44	0.16				
	Fibrosis	39	0.75				
	rs8099917	46	0.8				
	rs12980275	45	0.77				
2b	Age	84	0.14				
	Sex	84	0.58				
	Viral load	83	0.01 *	83	0.13	(0.03-0.62)	0.01 *
	Fibrosis	71	0.08				
	rs8099917	84	0.03 *	83	0.23	(0.06-0.80)	0.02 *
	rs12980275	84	0.21				

\*p <0.05; \*\*p <0.01; \*\*\*p <0.001.

### HCV RNA levels

HCV RNA levels, corresponding to initial viral load, were measured using one of several RT-PCR-based methods (the original Amplicor method, the high range method, or the TaqMan RT-PCR test). The measurement ranges of these assays were 0.5–850 KIU/ml, 5–5000 KIU/ml, and 1.2–7.8 log IU, respectively. Saturated samples were diluted with PBS and reanalyzed. All values were reported as log IU/ml.

### Statistical analysis

Genotype-based associations were tested using the Cochran–Armitage trend test. Combined analysis was performed using the Mantel–Haenszel method. Simple and multiple regression analyses were used to examine the association between viral and clinical factors using  $p < 0.05$  as the criterion for inclusion in the multivariate model. HCV RNA was converted into a binary variable based on the median. Multivariate logistic regression analysis was performed using the Design package in R (<http://www.r-project.org>) with fast backward elimination and validation based on AIC score for model construction.

### Results

Clinical characteristics are summarized by genotype in Table 1. The SVR rate was slightly but not significantly higher among patients with genotype 2a (340 out of 530; 64%) compared to genotype 2b patients (115 out of 189; 61%) ( $p = 0.43$ ). Patients who were treated with PEG-RBV had a slightly but not significantly higher rate of SVR (111 out of 160; 69%) than patients treated with interferon monotherapy (344 out of 559, 61%) ( $p = 0.08$ ). Because the number of patients treated with interferon monotherapy (559) greatly exceeds the number of patients treated with

PEG-RBV (160), patients were analyzed separately by treatment type. Because 30 out of the 719 patients (4%) had received prior interferon treatment, only treatment-naïve patients were included in the analyses mentioned below, followed by a separate analysis of the effect of prior interferon treatment on SVR rate.

### IL28B polymorphisms

Minor allele frequencies for rs8099917, rs12979860, and rs12980275 were 0.109, 0.112, and 0.132, respectively. The frequency of the rs8099917 risk allele was lower in SVR patients than non-SVR patients (0.089 vs. 0.14;  $p = 1.03 \times 10^{-5}$ ). The risk allele frequency among all patients was slightly higher than in the HapMap-JPT population (0.109 vs. 0.093;  $p = 0.01$ ) but lower than in the HapMap-CEU population (0.109 vs. 0.183;  $p = 1.6 \times 10^{-5}$ ). We compared rs8099917 allele and genotype frequencies with 900 healthy Japanese subjects but found no significant differences. 67% of patients (372 out of 552) with the favorable rs8099917 TT genotype achieved SVR, compared to 51% (70 out of 136) of patients with GT or GG genotypes. Fig. 1 shows the joint effects of treatment type, viral genotype, and rs8099917 genotype. In every case results for rs8099917 and rs12979860 are the same, but both factors cannot be included in a multivariate model simultaneously due to multicollinearity, so results for rs8099917 are presented due to the higher genotyping success rate.

### Predictive factors for SVR in patients treated with PEG-RBV

Among treatment-naïve patients treated with PEG-RBV, 78% (83 out of 106) of patients with rs8099917 TT achieved SVR compared

Predictive factors for SVR in patients treated with interferon monotherapy

Among patients treated with interferon monotherapy, 65% of patients with rs8099917 TT achieved SVR, compared to only 48% of patients with GT or GG genotypes ( $p = 0.002$ ). Viral load and the rs8099917 and rs12980275 genotypes were significant univariate predictors of SVR, and under multivariate analysis viral load and rs8099917 remained as independent predictors (Table 3). When genotypes 2a and 2b were analyzed separately, viral load ( $p = 0.001$ ) and rs8099917 genotype ( $p = 0.014$ ) were independent predictive factors for SVR in patients with genotype 2a but no significant univariate or multivariate terms were found for genotype 2b.

Effect of prior interferon treatment

Thirty out of the 719 patients (4%) had previously received treatment with interferon. Among these patients, only 40% achieved SVR, compared to the 64% SVR rate among treatment-naïve patients. Initial viral load was the only independent predictor of SVR in these patients, whereas in treatment-naïve patients, viral load, rs8099917 genotype, and treatment type (PEG-RBV vs interferon monotherapy) were independent predictors of SVR (Table 4).

Development of resistance to interferon therapy

Over the course of therapy five patients developed resistance to PEG-RBV treatment. In each case the patient showed an initial drop in viremia followed by viral breakthrough. Three out of the five patients were heterozygous (T/G) for the rs8099917 genotype and two out of the five were homozygous for the favorable allele (T/T).

Discussion

As the effect of *IL28B* polymorphism has not been reported separately for genotype 2 and its subtypes so far, we investigated whether the polymorphism influences treatment outcome in patients with HCV genotype 2a and 2b infections. In addition to previously reported effects for genotypes 1 and 4, our results demonstrate that polymorphisms in the *IL28B* locus are also predictive for SVR in genotype 2 (Table 2). We also showed that the favorable *IL28B* SNP genotype is associated with a rapid decrease in HCV RNA levels, which is itself a predictive factor for SVR [42]. Several studies have reported that polymorphisms at the *IL28B* locus affect the outcome of peg-interferon and ribavirin combination therapy in patients with HCV genotype 1b [34–36,38]. In particular, associations with therapy outcome have been reported for two SNPs in strong linkage disequilibrium, rs8099917 (T/G), and rs12979860 (C/T). Only a few studies have examined the effect of the SNP on the treatment outcome for other genotypes. Rallón et al. reported that the rs12979860 genotype is associated with treatment outcome for genotypes 1 and 4 but not genotype 3 in patients with HIV/HCV co-infection [43]. Similarly Rauch et al. reported an association between rs8099917 polymorphism and NVR for genotypes 1 and 4 (difficult-to-treat) but not for genotypes 2 and 3 (easier-to-treat) but the effect due to genotype 2 alone is unclear [38]. In a recent study, Mangia et al. also exam-

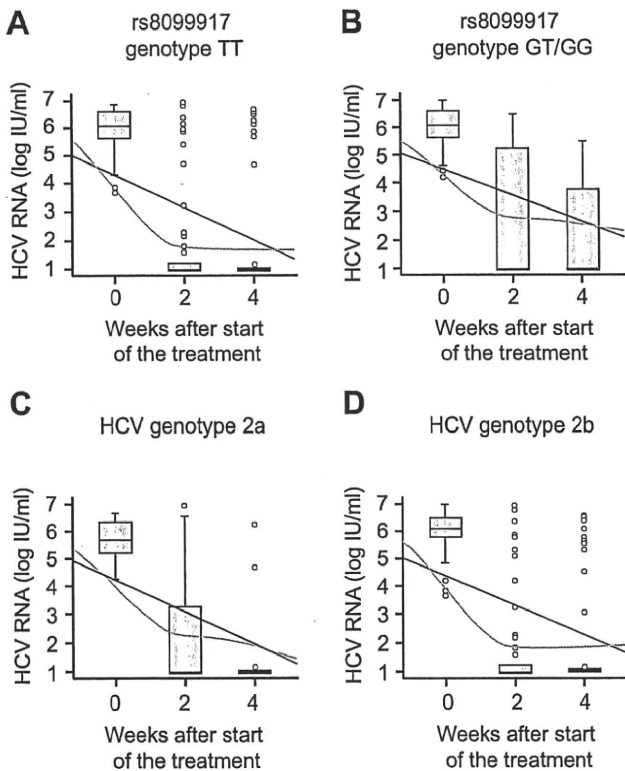


Fig. 2. Effect of rs8099917 genotype and HCV genotype on change in HCV RNA levels. HCV RNA levels at 0, 2, and 4 weeks after the start of peg-interferon plus ribavirin combination therapy in treatment-naïve patients. (A and B) Change in viral load for patients with the protective TT genotype for rs8099917 (A) compared to patients with the GT or GG genotypes (B). (C and D) Change in viral load for patients with HCV genotype 2a (A) versus genotype 2b (B).

to 67% (16 out of 24) of patients with non-TT genotypes ( $p = 0.29$ ). In univariate and multivariate analyses, only viral load was an independent predictive factor for SVR ( $p = 0.002$ ; Table 2), but when we examined genotypes 2a and 2b separately, rs8099917 genotype ( $p = 0.02$ ) and viral load ( $p = 0.01$ ) were both significant independent predictors of SVR for patients with genotype 2b, whereas no significant univariate or multivariate predictors were found for patients with genotype 2a. Notably, however, all 8 patients with genotype 2a with rs8099917 GT/GG achieved SVR (Fig. 1). The same pattern held for patients with rs12979860 TC/TT (9 SVR, 0 non-SVR) and rs12980275 GA/GG (11 SVR, 0 non-SVR) genotypes. Moreover, none of these patients was homozygous for the risk allele at each SNP.

Change in HCV RNA levels for patients treated with PEG-RBV

HCV RNA levels at the start of PEG-RBV therapy and after 2 and 4 weeks of treatment are plotted by rs8099917 genotype and viral genotype in Fig. 2. Under multivariate analysis, rs8099917 genotype was an independent predictive factor for change in HCV RNA level by week 2 ( $p = 0.036$ ) but viral genotype was not significant ( $p = 0.15$ ). For changes in HCV RNA levels by week 4, neither the rs8099917 genotype nor the viral genotype was significant ( $p = 0.17$  and  $p = 0.22$ , respectively).

# Research Article

**Table 3. Predictors for SVR in treatment-naïve patients treated with IFN monotherapy.**

Genotype	Variable	Simple		Multiple			
		n	p	n	OR	(95% CI)	p
2a + 2b	Age	559	0.35				
	Sex	559	0.17				
	Genotype	559	0.068				
	Viral load	507	0.0002 ***	506	0.59	(0.45-0.77)	0.0001 ***
	Fibrosis	450	0.61				
	rs8099917	558	0.001 **	506	0.52	(0.33-0.82)	0.005 **
2a	rs12980275	558	0.009 **				
	Age	477	0.19				
	Sex	477	0.2				
	Viral load	425	0.001 **	424	0.6	(0.44-0.81)	0.001 ***
	Fibrosis	382	0.37				
	rs8099917	476	0.003 **	424	0.53	(0.32-0.88)	0.014 *
2b	rs12980275	476	0.01 **				
	Age	82	0.67				
	Sex	82	0.56				
	Viral load	82	0.47				
	Fibrosis	68	0.53				
	rs8099917	82	0.19				
	rs12980275	82	0.44				

\*p <0.05; \*\*p <0.01; \*\*\*p <0.001.

ined genotypes 2 and 3 and found a significant association between rs12979860 genotype and rapid virological response (RVR) at week 4 for genotype 2 [44]. While rs12979860 was not directly associated with SVR in their study, rs12979860 genotype was significantly associated with SVR among those patients who failed to achieve RVR. In this study, we found a significant association between rs8099917 genotype and RVR in multivariate analysis for genotype 2b ( $p = 0.028$ , data not shown) but not for genotype 2a. When RVR was included as a factor in multivariate logistic regression analysis for genotype 2b, RVR and rs8099917 genotype were both retained in the final model but only RVR was significant (RVR:  $p = 4.9e-05$ ; rs8099917:  $p = 0.0850$ ; data not shown). When only non-RVR patients were included, no factors were significant; however, there were only six patients who achieved SVR without RVR and only one patient who achieved RVR but then failed to achieve SVR.

Although SVR rate was generally higher for genotype 2a, as reported previously [20,21], we found few differences between genotypes 2a and 2b. However, when analyzed separately, the results suggest an interesting interaction between the *IL28B* genotype, the viral genotype, and treatment type. In particular, we found that rs8099917 was a predictive factor for genotype 2a treated with IFN but not PEG-RBV, and conversely for genotype 2b treated with PEG-RBV but not IFN. This result is likely due to the relatively small sample sizes, but nonetheless all 8 (100%) of the genotype 2a PEG-RBV patients lacking the favorable rs8099917 genotype achieved SVR, compared to less than 50% for IFN therapy or either type of treatment with genotype 2b. In fact, each patient was heterozygous for each of the three *IL28B* SNPs examined. A further complication is that each of the five patients who developed resistance to interferon therapy was infected with genotype 2a,

and two of these patients had the favorable rs8099917 TT genotype while the others were heterozygous (GT). More detailed analysis will be required to interpret these results.

Because PEG-RBV therapy was not covered by insurance in Japan until 2005, we also present data comparing the effects of *IL28B* polymorphisms on treatment with the older IFN monotherapy versus the more recent PEG-RBV combination therapy. Although the small sample sizes within each patient group likely underestimate the effect of SNP genotype, we found that rs8099917 influences response to IFN monotherapy in patients with genotype 2a and also influences the response to PEG-RBV therapy in patients with genotype 2b. Although PEG-RBV is currently the standard treatment for chronic hepatitis C infection, interferon monotherapy may still be used in the case of intolerance to ribavirin; therefore, it is important to understand the direct effects of interferon with and without ribavirin. Moreover, even with the advent of protease inhibitors and other antiviral drugs undergoing clinical trials, they are likely to be co-administered with interferon to prevent the otherwise rapid emergence of resistant quasispecies [45].

In summary, we showed that the *IL28B* SNP genotype is an important predictive factor for SVR and early viral dynamics in patients with HCV genotypes 2a and 2b.

### Conflict of interest

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

Table 4. Comparison of predictive factors for SVR based on prior treatment with interferon.

Variable	Simple		Multiple				
	n	p	n	OR	(95% CI)	p	
All	Age	719					
	Sex	719					
	Genotype	719					
	Viral load	663	6.00E-02 ***	662	0.63	(0.51-0.79)	4.30E-05 ***
	Fibrosis	585	0.83				
	rs8099917	718	0.002 **	662	0.57	(0.38-0.85)	0.0055 **
	rs12980275	717	0.03 *				
	Treatment	719	0.054				
Naïve	Age	689					
	Sex	689					
	Genotype	689					
	Viral load	634	0.0011 **	633	0.53	(0.41-0.69)	2.00E-06 ***
	Fibrosis	560	0.95				
	rs8099917	688	0.00059 ***	633	0.5	(0.33-0.77)	0.0015 **
	rs12980275	687	0.013 *				
	Treatment	689	0.0013 **	633	3.01	(1.82-4.99)	1.80E-05 ***
Experienced	Age	30					
	Sex	30					
	Genotype	30					
	Viral load	29	0.032 *	29	0.21	(0.05-0.87)	0.032 *
	Fibrosis	25	0.53				
	rs8099917	30	0.12				
	rs12980275	30	0.1				
	Treatment	30	N/A				

\*p <0.05; \*\*p <0.01; \*\*\*p <0.001.

Acknowledgments

This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and the Ministry of Education, Culture, Sports, Science, and Technology, of the Government of Japan. Part of this work was carried out at the Analysis Center of Life Science, Hiroshima University. The authors thank Yasufumi Hayashida, Rie Akiyama, Yoshie Yoshida, Kazuyo Hattori, Mariko Shiota, Hiromi Ishino, and Takako Yokogi for excellent technical assistance, and Yuko Nagai, Junko Sakamiya and Aya Furukawa for clerical assistance.

References

[1] Alter MJ. Epidemiology of hepatitis C in the West. *Semin Liver Dis* 1995;15:5-14.  
 [2] Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997;349:825-832.  
 [3] Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990;12:671-675.  
 [4] Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993;328:1797-1801.

[5] Takano S, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995;21:650-655.  
 [6] Shiratori Y, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, et al. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology* 1995;22:1027-1033.  
 [7] Tomimatsu M, Ishiguro N, Taniai M, Okuda H, Saito A, Obata H, et al. Hepatitis C virus antibody in patients with primary liver cancer (hepatocellular carcinoma, cholangiocarcinoma, and combined hepatocellular-cholangiocarcinoma) in Japan. *Cancer* 1993;72:683-688.  
 [8] Takada N, Takase S, Takada A, Date T. Differences in the hepatitis C virus genotypes in different countries. *J Hepatol* 1993;17:277-283.  
 [9] Zeuzem S, Franke A, Lee JH, Herrmann G, Ruster B, Roth WK. Phylogenetic analysis of hepatitis C virus isolates and their correlation to viremia, liver function tests, and histology. *Hepatology* 1996;24:1003-1009.  
 [10] Adinolfi LE, Utili R, Andreato A, Tripodi MF, Rosario P, Mormone G, et al. Relationship between genotypes of hepatitis C virus and histopathological manifestations in chronic hepatitis C patients. *Eur J Gastroenterol Hepatol* 2000;12:299-304.  
 [11] Martinot-Peignoux M, Marcellin P, Pouteau M, Castelneau C, Boyer N, Poliquin M, et al. Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. *Hepatology* 1995;22:1050-1056.  
 [12] Tsubota A, Chayama K, Arase Y, Koida I, Saitoh S, Ikeda K, et al. Factors useful in predicting the response to interferon therapy in chronic hepatitis C. *J Gastroenterol Hepatol* 1993;8:535-539.  
 [13] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncalves Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975-982.

## Research Article

- [14] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958–965.
- [15] Mangia A, Minerva N, Bacca D, Cozzolongo R, Agostinacchio E, Sogari F, et al. Determinants of relapse after a short (12 weeks) course of antiviral therapy and re-treatment efficacy of a prolonged course in patients with chronic hepatitis C virus genotype 2 or 3 infection. *Hepatology* 2009;49:358–363.
- [16] von Wagner M, Huber M, Berg T, Hinrichsen H, Rasenack J, Heintges T, et al. Peginterferon-alpha-2a (40 kDa) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. *Gastroenterology* 2005;129:522–527.
- [17] Mangia A, Santoro R, Minerva N, Ricci GL, Carretta V, Persico M, et al. Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2005;352:2609–2617.
- [18] Fujiwara K, Yokosuka O, Komine F, Moriyama M, Kato N, Yoshida H, et al. Twenty-four weeks of interferon alpha-2b in combination with ribavirin for Japanese hepatitis C patients: sufficient treatment period for patients with genotype 2 but not for patients with genotype 1. *Liver Int* 2006;26:520–528.
- [19] Kawaoka T, Kawakami Y, Tsuji K, Ito H, Kitamoto M, Aimitsu S, et al. Dose comparison study of pegylated interferon-alpha-2b plus ribavirin in naive Japanese patients with hepatitis C virus genotype 2: a randomized clinical trial. *J Gastroenterol Hepatol* 2009;24:366–371.
- [20] Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, et al. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. *J Hepatol* 2002;37:831–836.
- [21] Akuta N, Suzuki F, Tsubota A, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype 2a low viral load and response to interferon monotherapy. *J Med Virol* 2003;69:376–383.
- [22] Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. Association of Amino Acid Substitution Pattern in Core Protein of Hepatitis C Virus Genotype 2a High Viral Load and Virological Response to Interferon-Ribavirin Combination Therapy. *Intervirology* 2009;52:301–309.
- [23] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. *Hepatology* 2007;46:1357–1364.
- [24] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. *J Med Virol* 2007;79:1686–1695.
- [25] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007;46:403–410.
- [26] Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka T, Takaki S, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: Correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. *J Med Virol* 2009;81:640–649.
- [27] Bressler BL, Guindi M, Tomlinson G, Heathcote J. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. *Hepatology* 2003;38:639–644.
- [28] Romero-Gomez M, Del Mar Viloria M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, et al. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005;128:636–641.
- [29] Hijikata M, Ohta Y, Mishiro S. Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt -88) correlated with the response of hepatitis C patients to interferon. *Intervirology* 2000;43:124–127.
- [30] Knapp S, Yee LJ, Frodsham AJ, Hennig BJ, Hellier S, Zhang L, et al. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. *Genes Immun* 2003;4:411–419.
- [31] Matsuyama N, Mishiro S, Sugimoto M, Furuichi Y, Hashimoto M, Hijikata M, et al. The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. *Hepatol Res* 2003;25:221–225.
- [32] Naito M, Matsui A, Inao M, Nagoshi S, Nagano M, Ito N, et al. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. *J Gastroenterol* 2005;40:381–388.
- [33] Tsukada H, Ochi H, Maekawa T, Abe H, Fujimoto Y, Tsuge M, et al. A polymorphism in MAPKAPK3 affects response to interferon therapy for chronic hepatitis C. *Gastroenterology* 2009;136:1796–1805, e1796.
- [34] Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401.
- [35] Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;41:1105–1109.
- [36] Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;41:1100–1104.
- [37] Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;461:798–801.
- [38] Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010;138:1338–1345, e1331–1337.
- [39] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis – diagnosis, grading and staging. *Hepatology* 1994;19:1513–1520.
- [40] Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. *J Hum Genet* 2001;46:471–477.
- [41] Suzuki A, Yamada R, Chang X, Tokuhira S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidyl-larginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003;34:395–402.
- [42] Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, Shianna KV, et al. IL28B polymorphism improves viral kinetics and is the strongest pre-treatment predictor of SVR in HCV-1 patients. *Gastroenterology* 2010;139:120–129.
- [43] Rallon NI, Naggie S, Benito JM, Medrano J, Restrepo C, Goldstein D, et al. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in HIV/hepatitis C virus-coinfected patients. *AIDS* 2010;24:F23–29.
- [44] Mangia A, Thompson AJ, Santoro R, Piazzolla V, Tillmann HL, Patel K, et al. IL28B polymorphism determines treatment response of patients with hepatitis C genotypes 2 or 3 who do not achieve a rapid virologic response. *Gastroenterology* 2010;139:821–827.
- [45] Lange CM, Sarrazin C, Zeuzem S. Review article: HCV – STAT-C era of therapy. *Aliment Pharmacol Ther* 2010;31:31.

ORIGINAL ARTICLE

## Prolongation of interferon therapy for recurrent hepatitis C after living donor liver transplantation: Analysis of predictive factors of sustained virological response, including amino acid sequence of the core and NS5A regions of hepatitis C virus

TOMOKAZU KAWAOKA<sup>1</sup>, NOBUHIKO HIRAGA<sup>1</sup>, SHOICHI TAKAHASHI<sup>1</sup>, SHINTARO TAKAKI<sup>1</sup>, FUKIKO MITSUT<sup>1</sup>, MASATAKA TSUGE<sup>1</sup>, YUKO NAGAOKI<sup>1</sup>, YUKI KIMURA<sup>1</sup>, YOSHIMASA HASHIMOTO<sup>1</sup>, YOSHIO KATAMURA<sup>1</sup>, AKIRA HIRAMATSU<sup>1</sup>, KOJI WAKI<sup>1</sup>, MICHIO IMAMURA<sup>1</sup>, YOSHIKU KAWAKAMI<sup>1</sup>, HIROSHI AIKATA<sup>1</sup>, HIROTAKA TASHIRO<sup>2</sup>, HIDEKI OHDAN<sup>2</sup> & KAZUAKI CHAYAMA<sup>1</sup>

<sup>1</sup>Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan, and <sup>2</sup>Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan

### Abstract

**Objective.** The aim of the present retrospective study was to evaluate the therapeutic efficacy and predictive factors of prolongation of treatment with peginterferon (PEGIFN) combined with ribavirin (RBV) for recurrent hepatitis C after living donor liver transplantation (LDLT). **Methods.** Fifty-three patients underwent LDLT due to HCV-related end-stage liver disease. Sixteen patients were removed from the study as a result of early death ( $n = 14$ ), no recurrence of HCV ( $n = 1$ ) and refusal of antiviral therapy ( $n = 1$ ). Therapy is ongoing in another 10 patients. The remaining 27 patients were available to establish the efficacy of IFN therapy. HCV genotype was 1b in 24 patients. All patients with genotype 1b were treated with IFN therapy for at least 48 weeks after HCV RNA levels had become undetectable. Amino acid substitutions in the HCV core region and NS5A region were analyzed by direct sequencing before LDLT. **Results.** The rate of sustained virological response (SVR) was 37.0% (10/27). SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in patients with genotype 2. Most patients with genotype 1b whose HCV RNA reached undetectable levels achieved SVR (87.5%; 7/8). However, mutation of the HCV core region and number of ISDR mutations were not associated with SVR rate in LDLT in our study. **Conclusions.** Prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels might prevent virological relapse of HCV.

**Key Words:** Core and NS5A regions, HCV, IFN, LDLT

### Introduction

Hepatitis C virus (HCV)-related end-stage liver disease is currently the leading indication for liver transplantation (LT). Unfortunately, prevention of HCV infection after transplantation is difficult and, unlike

the situation with the prevention of hepatitis B virus after transplantation [1], HCV re-infection after LT is almost universal, with histological evidence of chronic hepatitis in approximately 50% of patients within 1 year and cirrhosis in about 30% after 5 years. This in turn yields an excess risk of death or

Correspondence: Shoichi Takahashi, 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. Tel: +81 82 257 5192. Fax: +81 82 257 5194. E-mail: shoichit@hiroshima-u.ac.jp

(Received 26 April 2010; accepted 26 June 2010)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2010 Informa Healthcare  
DOI: 10.3109/00365521.2010.505657



retransplantation for liver failure 10–15 years after transplantation [2]. Given this risk, it seems reasonable to offer antiviral therapy to liver transplant recipients. Many reports have noted rates of sustained viral response (SVR) ranging from only 10% to 30% in liver transplant recipients with recurrent HCV treated for 48 weeks [3–10], indicating the need for new treatment regimens with higher SVR rates. Recent reports have indicated that the extension of treatment with peginterferon and ribavirin (PEGIFN/RBV) from 48 to 72 weeks significantly increases the rate of SVR in immunocompetent patients, particularly slow virological responders [11,12], and one report noted that extended treatment for recurrent hepatitis C infection after liver transplantation (LT) was effective [13,14].

There are many predictive factors of successful treatment with the combination of peginterferon and ribavirin in immunocompetent patients, including the viral factors, such as HCV genotype, pretreatment viral load, amino acid (aa) 70 and/or 91 in the HCV core protein, amino acid substitutions in the HCV NS5A region [15–17]. In their multivariate analyses of predictors of SVR, Akuta and colleagues identified substitutions of aa 70 and 91 in the HCV core region (double-wild-type; odds ratio 5.988) as predictive [18], whereas Enomoto identified substitutions of amino acids of the HCV NS5A region (mutant type; odds ratio 5.3) as predictive [15].

With regard to length of treatment, one study reported that an early viral response at 3 months was useful in predicting a lack of response to antiviral therapy in liver transplant recipients with recurrent hepatitis C [19,20]. To our knowledge, however, no study has analyzed viral factors in extended treatment for recurrent hepatitis C infection after liver transplantation.

The aim of the present study was to evaluate the therapeutic efficacy of peginterferon in combination with ribavirin (PEGIFN/RBV) on long-term treatment for recurrent hepatitis C after LDLT, and predictive factors of virological response to this treatment, particularly viral factors. This study is first report of predictive factors associated with virological response in recurrent hepatitis C patients after LDLT, including amino acid substitutions in the core region and NS5A region.

## Material and methods

### Patients

A total of 53 patients who underwent LDLT due to HCV-related end-stage liver disease from 2000 to January 2009 were enrolled for this retrospective

study. Among them, 14 patients died before the start of therapy, 1 refused treatment with antiviral therapy, and 1 did not become positive for HCV RNA after LDLT. Eventually, leaving 37 patients treated with PEGIFN/RBV in our institution. Of these, 10 patients are currently continuing antiviral therapy.

We introduced all patients to IFN therapy in principle. The efficacy of IFN therapy could thus be established in 27 patients (Figure 1).

### Antiviral treatment protocol

Patients received 1.5 µg/kg body weight (BW) PEGIFN (Peg-Intron; Schering-Plough, Segrate, Italy) subcutaneously (s.c.) once weekly and 200 mg RBV (Rebetol; Schering-Plough). PEGIFN/RBV was continued for more than 1 year after serum HCV RNA becomes negative. At the end of active treatment, the patients were followed for further 24 weeks without treatment.

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.

### Safety assessments

Safety was assessed by clinical and laboratory testing, and by evaluating all adverse events reported at each visit. In accordance with the protocol, growth factors were recommended to encourage optimum patient compliance in relation to predictable hematological side effects such as anemia.

Erythropoietin (EPO; Epogin, Chugai) from 6000 IU/week was used to treat anemia (Hb levels <10 g/dl). RBV was administered from 200 mg. When Hb increased by more than 10 g/dl, 200 mg per day of RBV was added. The daily dose of RBV was reduced by 200 mg when Hb fell below 10 g/dl, an acute decrease was followed by stabilization of Hb concentration at more than 3 g/dl from baseline, or the appearance of clinical symptoms of anemia (e.g. palpitation, dyspnea on effort, 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 if patients complained of anemia-related symptoms of fatigue or pallor. However, RBV was discontinued when Hb fell below 8.5 g/dl or when patients manifested more severe anemia, including orthostatic hypotension. PEGIFN was stopped if significant side effects occurred or if cytopenia persisted (neutrophil count <750/mm<sup>3</sup>, platelet count <20,000/mm<sup>3</sup>).

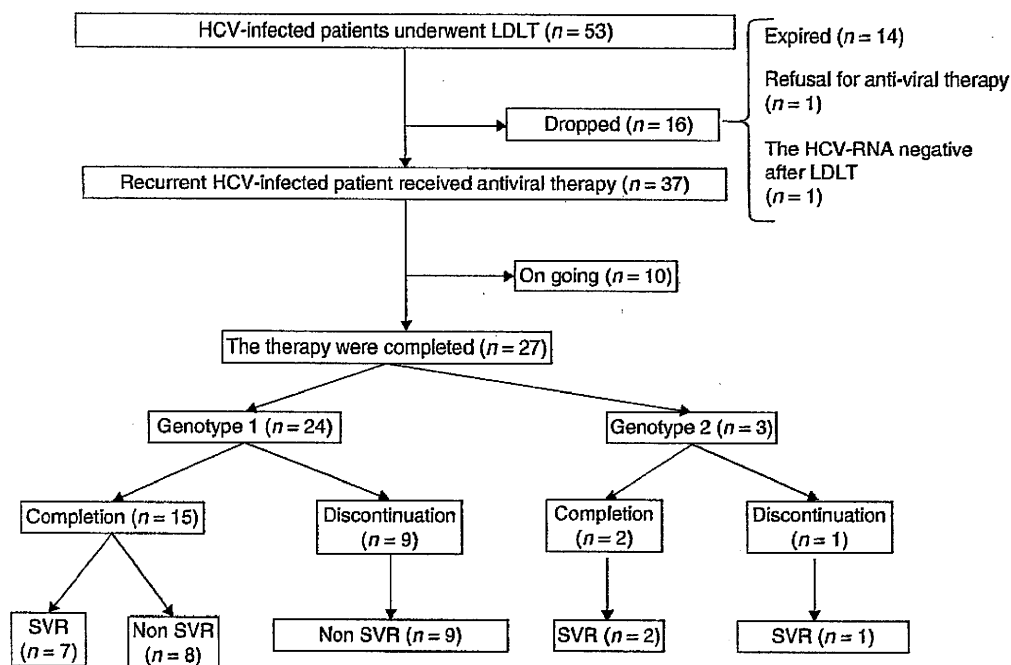


Figure 1. Flow diagram showing the course of HCV-infected patients after living donor liver transplantation. Twenty-seven patients treated with PEGIFN/RBV combination therapy were divided into two groups, namely sustained virological response (SVR) and non-SVR. *n*, number of patients. HCV genotype is shown.

#### Assessment of efficacy

HCV RNA levels were measured using one of several RT-PCR-based methods (original Amplicor method, high range method, or TaqMan RT-PCR test) at weeks 2 and 4, every 4 weeks of treatment thereafter, and at 24 weeks after the cessation of therapy.

The response was considered to be a SVR after another 6 months of negative serologic results without antiviral treatment. Patients with positive qualitative HCV RNA PCR tests during all examinations were categorized as having a non-virological response. Virological response (VR) was defined as becoming PCR-negative at least once during treatment; early virological response (EVR) as HCV-RNA-positive at 4 weeks after the start of treatment and HCV-RNA-negative at 12 weeks; and late virological response (LVR) as HCV-RNA-negative at more than 13 weeks after the start of treatment.

#### Analysis of nucleotide sequence of the core and NS5A region

Fifteen patients with genotype 1b completed our protocol. Seven patients achieved SVR whereas eight did not. Using serum obtained before LDLT, we analyzed amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region (HCV CR) and

mutation at the interferon sensitivity-determining region (ISDR) in the nonstructural 5A (NS5A) region of HCV by the direct sequencing method. The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR]) [15] sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the primers below. Nucleotide sequences of the core region: first-round PCR was performed with primers CC11 (forward, 5'-GCC ATA GTG GTC TGC GGA AC-3') and e14 (reverse, 5'-GGA GCA GTC CTT CGT GAC ATG-3'), and second-round PCR with primers CC9 (forward, 5'-GCT AGC CGA GTA GTG TT-3') and e14 (reverse), as described by Akuta et al. [16,18,21]. After denaturation at 95°C for 5 min, 35 cycles of amplification were set as follows: denaturation for 30 s at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions as those used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. Nucleotide sequences of ISDR in NS5A: PCR was performed

with IM11 (forward, 5'-TTC CAC TAC GTG ACG GGC AT-3') and 5OA2KI (reverse, 5'-CCC GTC CAT GTG TAG GAC AT-3'). After denaturation at 98°C for 30 s, 35 cycles of amplification were set as follows: denaturation for 10 s at 98°C, annealing of primers for 30 s at 66°C, and extension for 15 s at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated on a 2% agarose gel and purified by GENECLAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [22].

#### Statistical analysis

Variables between the SVR and non-SVR groups were compared using non-parametric tests (Mann-Whitney *U* test, two-tailed test and Fisher's exact probability test). Analyses for efficacy and safety were conducted on an intention-to-treat (ITT) basis, performed on patients who received at least one dose of the study medication.

Predictors of SVR were determined using univariate analyses. All *p* values <0.05 by two-tailed tests were considered significant. Potential predictive factors associated with SVR included sex, age, body mass index (BMI), viremia level, number of mutations in the ISDR, HCV core region (double mutant/non-double mutant), time from transplantation to therapy, duration of treatment, adherence to PEGIFN treatment, and adherence to RBV and EVR treatment. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

## Results

#### Patients characteristics

Table I shows the baseline characteristics of the 27 patients with recurrent hepatitis C after LT who were treated with PEGIFN/RBV combination therapy. The median age of patients was 56 years, and 17 were male. Median body mass index was 24.3. Most patients were infected with HCV genotype 1 (*n* = 24) and genotype 2 (*n* = 3). Median time for the initiation of antiviral therapy after transplantation was 4 months, and median pretreatment serum HCV RNA levels were 6.6 log IU/ml. Immunosuppressive therapy included tacrolimus in 22 of 27 patients, and cyclosporine in 5 of 27.

Table I. Characteristics of 27 patients with recurrent hepatitis C after living donor liver transplantation.

Age (years)*	56 (29–69)
Gender (male/female)	17/10
Body mass index*	24.3 (14.8–42.2)
Genotype (1/2)	24/3
Viral load at therapy (log IU/ml)*	6.6 (4.9–7.8)
Time from transplantation to therapy (months)*	4 (1–41)
Immunosuppression (tacrolimus/cyclosporine)	22/5

\*Values are median (range).

#### Efficacy and safety assessment

Among 27 patients who were treated with antiviral therapy, 17 were able to complete our protocol (15 patients with genotype 1, 2 patients with genotype 2), whereas 10 patients had to discontinue the protocol (9 patients with genotype 1, 1 patient with genotype 2). SVR rate with PEGIFN/RBV was 37.0% (10/27). By genotype, SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in those with genotype 2 (Figure 1). Most patients with genotype 1b whose HCV RNA reached undetectable level achieved SVR, at 87.5% (7/8), with only one patient not achieving SVR (Table II) (Figure 2).

Ten patients discontinued treatment, due to liver failure owing to the recurrence of HCV in 5 patients, general fatigue in 2, ALT flare due to acute rejection in 1 patient, anemia in 1, and depression in 1 (Figure 1).

#### Efficacy of long-term interferon therapy for genotype 1b patients

Table II shows details of patients who were treated with PEGIFN/RBV until HCV RNA had reached undetectable levels and were then further treated for at least more than 1 year.

Seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Seven patients were male.

Eight patients had reached undetectable levels of HCV RNA and 7 patients had never reached undetectable levels of HCV RNA. Although 5 of the 8 patients were classified as LVR, 4 patients of these 5 achieved SVR. One male patient aged 69 years (patient no. 5) who had double mutation of aa 70 and aa 91 in the core region and zero substitutions in ISDR achieved SVR after prolongation of therapy (Figure 3). By contrast, another male aged 51 years (patient no. 9) who had double wild aa 70 and aa 91 in the core region and five substitutions in the ISDR did not achieve SVR after prolongation of therapy (Figure 4).

Table II. Details of 15 patients (genotype 1).

Patient no.	Age (years)	Gender	HCV RNA (log IU/ml)	HCV core region (aa 70/aa 91)	Number of mutations in the ISDR	Time from transplantation to therapy (months)	Time to reach undetectable levels of HCV RNA (weeks)	Treatment duration of VR (weeks)	Treatment duration (weeks)	Adherence to PEGIFN (%)	Adherence to RBV (%)	SVR
1	63	Male	6.1	m/m	4	41	3	103	106	35	29	Yes
2	60	Male	5.8	w/w	1	13	10	48	58	100	62	Yes
3	66	Male	6.6	m/m	3	12	12	60	72	80	11	Yes
4	54	Male	6.5	w/w	3	4	16	56	72	77	54	Yes
5	69	Male	6.3	m/m	0	12	21	57	78	42	14	Yes
6	44	Male	6.6	w/m	0	1	28	76	104	88	47	Yes
7	53	Male	6.1	m/w	1	2	54	125	179	57	25	Yes
8	56	Male	6.6	w/w	0	2	27	52	79	66	7	No
9	51	Male	5.9	w/w	5	6	NR	NR	173	80	25	No
10	47	Female	6.6	m/m	1	3	NR	NR	124	63	15	No
11	59	Female	6.6	m/w	1	7	NR	NR	86	100	65	No
12	64	Female	5	m/m	0	3	NR	NR	81	72	25	No
13	58	Female	6.6	m/m	1	3	NR	NR	79	83	63	No
14	65	Female	5.9	w/m	0	3	NR	NR	79	45	19	No
15	56	Male	7.2	m/w	0	3	NR	NR	58	51	26	No

Abbreviations: m = mutant; w = wild; NR = non-virological responder; VR = virological response; SVR = sustained virological response.

Predictive factors of SVR in genotype 1b patients

Among 15 patients who completed our protocol with genotype 1b, Potential predictive factors associated with SVR were analyzed. Variables were follow up, the age, gender, body mass index, duration for the initiation of antiviral therapy after transplantation, pretreatment serum HCV RNA levels, immunosuppressive therapy, the number of mutations in the ISDR, HCV core region (double mutant/non-double mutant) adherence of PEGIFN and adherence of RBV.

There was no significance difference between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study (Table III). EVR rates in the SVR group tend to be higher than that of the non-SVR group, albeit that the difference was not significant ( $p = 0.07$ ) (Table III). Mutation of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region did not significantly differ between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study.

Although it has been reported that mutation of aa 70 and aa 91 in the core region of the HCV protein is predictive of a non-virological response [17,18], all three patients who had double mutation of aa 70 and aa 91 in the core region achieved SVR in this study.

Moreover, although it has also been reported that fewer mutations in the ISDR region of the HCV protein is predictive of a non-virological response [15], all four patients with 0 or 1 mutation in the ISDR achieved SVR.

Discussion

The optimal duration of therapy for liver transplant recipients with recurrent HCV is unclear. The treatment period for immunocompetent patients in the majority of published studies is 48 weeks. Among immunocompetent patients, the probability of relapse was greater in those responding later [23,24]. Using a mathematical model, Drusano and Preston reported that genotype 1-infected patients require the continuous absence of detectable HCV RNA in serum for 36 weeks to attain 90% probabilities of an SVR (i.e. relapse rate 10%) [25]. It is recently recommended that 72-week IFN treatment, compared to 48-week standard IFN treatment, was effective for the untransplanted patients with chronic hepatitis C whose HCV RNA does not reach undetectable level within 12 weeks [11,12]. It is also well known that patients with recurrent chronic hepatitis after LDLT are unlikely to achieve SVR, compared to immunocompetent

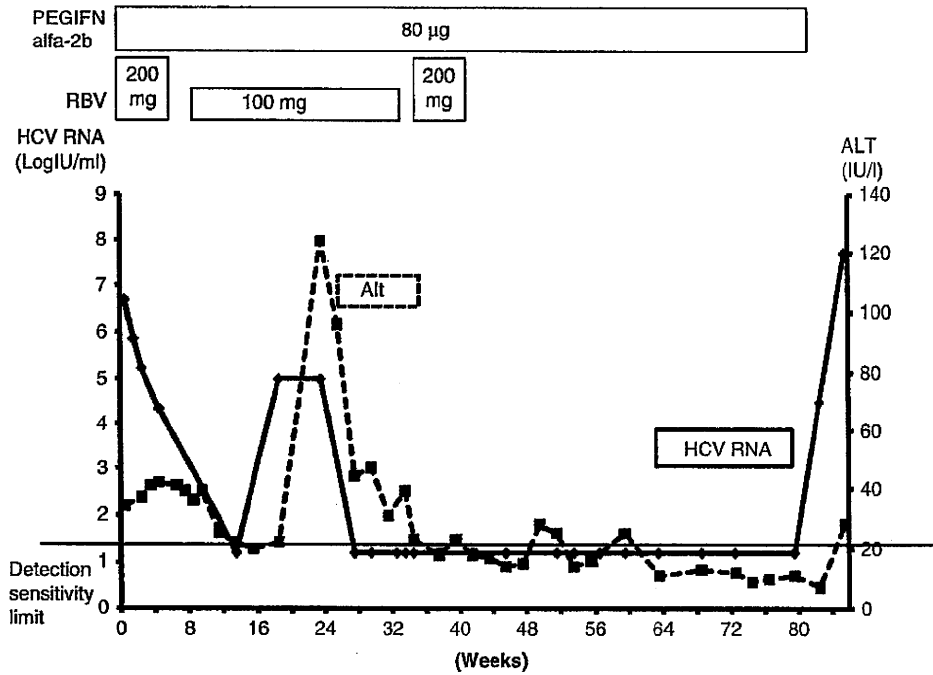


Figure 2. Clinical course in a male patient aged 56 years with genotype 1b, HCV Core 70mutant 91wild, and 0 ISDR mutations. Serum HCV RNA became negative at 27 weeks, after which treatment duration was 52 weeks. However, HCV RNA became positive at 1 week after the cessation of treatment.

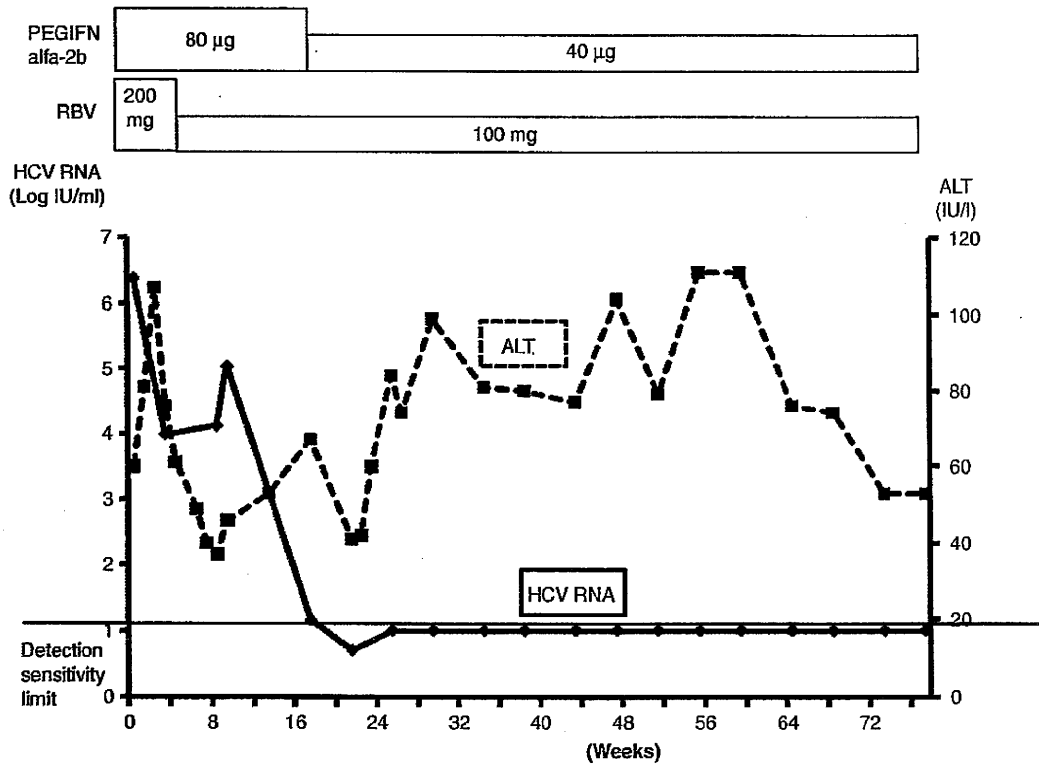


Figure 3. Clinical course in a male patient aged 69 years with genotype 1b, HCV Core 70mutant 91mutant, and 0 ISDR mutations. Virological response occurred at 21 weeks and therapy continued to 78 weeks. Final status was SVR.

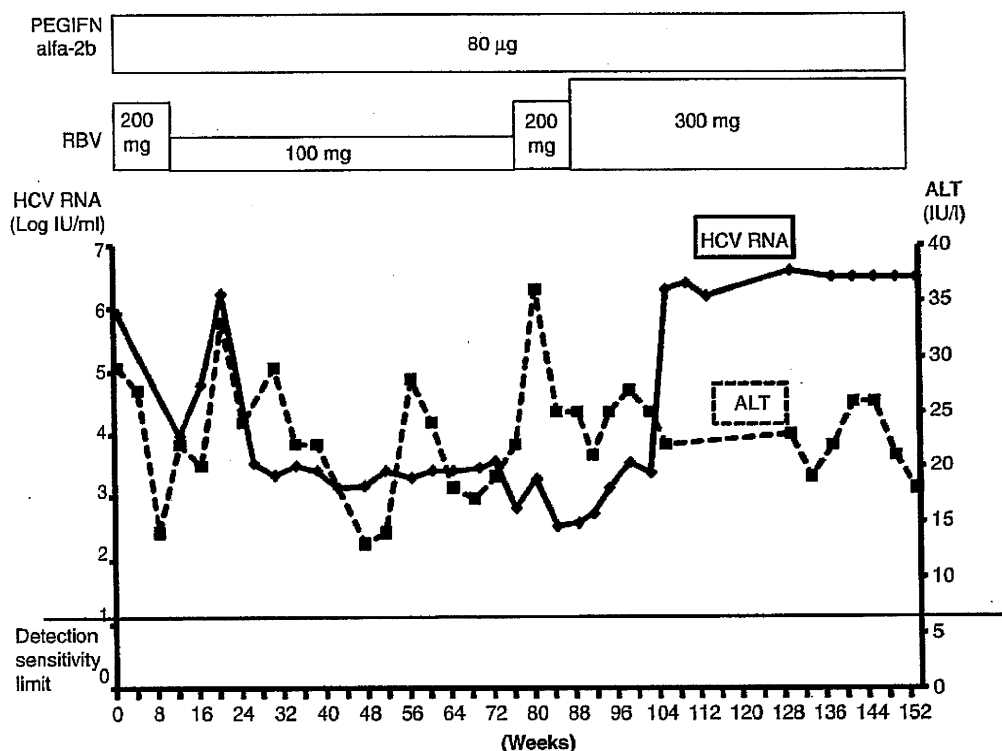


Figure 4. Clinical course in a male patient aged 51 years with genotype 1b, HCV Core 70wild 91wild, and 5 ISDR mutations. He did not develop VR during the dosing period.

untransplanted patients. Consequently, prolonged IFN treatment would be useful and improve the SVR rate for transplanted patients.

We treated recipients with PEGIFN/RBV until HCV RNA had reached undetectable levels and then to continue treatment for at least 1 year. 62.9% of patients (17/27) could complete therapy without severe adverse affects (Figure 1) and relapse rate under this study was 12.5% (Table II), whereas Tamura and Ueda reported a relapse rate of 14% and

3% under the same treatment. As a result, SVR rate was 34% and 50%, respectively [13,14]. With the aid of these results, it might indicate that prolonged PEGIFN/RBV therapy would be useful in eradicating HCV in LDLT patients.

In our study, seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Three of seven patients were EVR and four were LVR.

By contrast, in eight patients who were non-SVR, only one patient had reached undetectable levels of HCV

Table III. Predictive factors associated with SVR in genotype 1b patients.

	SVR (n = 7)	Non-SVR (n = 8)	p-Value
Age (years)*	60 (44-69)	57 (47-65)	0.64
Gender (male/female)	7/0	3/5	0.07
Body mass index*	24.1 (21.4-26.5)	24.2 (18.9-42.2)	0.67
Viral load at therapy (log IU/ml)*	6.3 (5.8-6.6)	6.6 (5.9-7.2)	0.48
Time from transplantation to therapy (months)*	12 (1-41)	3 (3-7)	0.21
Number of mutations in the ISDR (0-1/2-5)	4/3	7/1	0.28
HCV core region (double mutant/non-double mutant)	3/4	3/5	0.6
Duration of treatment (week)*	72 (48-179)	75 (61-133)	0.7
Immunosuppression (tacrolimus/cyclosporine)	6/2	7/1	1
Adherence of PEGIFN (%)*	80 (35.5-100)	71.5 (45.4-100)	0.39
Adherence of RBV (%)*	47.4 (11.2-62.5)	25.5 (15.3-65.9)	0.74
Early virological response (yes/no)	3/4	0/8	0.07

\*Values are median (range).

RNA and other seven patients had never reached undetectable levels of HCV RNA. That is, if patients had reached undetectable levels of HCV RNA, they could eradicate HCV RNA in the liver tissue by prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels. This regimen is similar to that of a recent recommendation that PEGIFN/RBV therapy for 72 weeks is necessary for patients with chronic hepatitis C whose HCV RNA does not reach undetectable levels within 12 weeks.

Recent findings among immunocompetent patients of pretreatment factors that could predict treatment efficacy of 72-week PEGIFN/RBV identified substitution of either or both aa 70 or 91 in the HCV core region, and the number of substitutions in amino acids 2209–2248, the ISDR of NS5A in HCV genotype 1b [26]. By contrast, however, our present results showed that substitution of aa 70 and/or 91 in the HCV core region or the number of ISDR were not predictive of SVR (Table III). All three patients who had double mutation of aa 70 and aa 91 in core region of HCV protein achieved SVR in this study, as did all four patients whose number of mutations in the ISDR was 0 or 1 (Table II).

Recently Fukuhara et al. reported that mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates in 50 patients [27]. Although the number of our patients included was less than Fukuhara's, we think that our result is still worth reporting because, in the case of acute hepatitis C, 24-week IFN treatment is enough to eradicate HCV in most cases, suggesting that HCV core mutant and the substitutions of amino acids of the HCV NS5A region are not likely to affect the SVR rate for acute hepatitis C. Since the recurrence of hepatitis C for transplanted patients is another acute hepatitis C, those substitutions might not affect the SVR rate of IFN treatment. Further studies would reveal whether the mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates.

Only one patient had HCV relapse after 79 weeks treatment, a male aged 56 years with genotype 1b, HCV Core 70mutant 91wild and number of ISDR mutation 0 (Figure 2). He had a VR at 27 weeks, which lasted for 52 weeks, and continued therapy to 79 weeks. However, he subsequently experienced relapse of HCV. One of many possible reasons was likely low adherence to RBV (7%).

Several reasons may account for the lack of association of HCV core region mutation and number of ISDR mutations with SVR rate. One reason is that it is acute hepatitis after LDLT, which is usually treated as soon as possible: even in those infected with genotype 1, HCV could be eradicated with regular IFN for 24 weeks after acute infection [28–31], meaning

that mutation of the core region and NS5A could not be determinants of PEGIFN therapy in LDLT cases. A second reason might be poor adherence to PEGIFN and RBV treatment in patients with LDLT. Among patients who experience severe leucocytopenia, thrombocytopenia and anemia after LDLT, dose reductions in PEGIFN or RBV are therefore inevitable. Therefore, it is reasonable to prolong the duration of PEGIFN/RBV therapy. Taken together, recurrent hepatitis C after LDLT is different from hepatitis C in immunocompetent patients. This might be the reason why any predictive factor but EVR was an only predictive factor of SVR in this study.

There were several limitations in present study. One was that our study is retrospective.

Since it was scheduled that the end point of treatment should be 1 year after serum HCV RNA became negative, it compelled to design the retrospective study as a pilot study. Further prospective study will prove our protocol strongly and help achieving high SVR and low relapse rate. Another limitation was the low number of patients included. Although other institutes also demonstrated good results with similar interferon protocol, as mentioned above [13,14], another study with more number of patients will prove the consistence of our study.

In conclusion, for recurrent hepatitis C after LDLT, our findings indicate that PEGIFN therapy for at least 1 year after HCV RNA reaches undetectable levels might prevent HCV viral relapse. Combination of the new selective inhibitors of HCV, named STAT-C (specifically targeted antiviral therapy for HCV), is expected to further improvements in SVR rates.

#### Acknowledgements

The authors thank Rie Akiyama and Hiromi Abe for their excellent technical assistance.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

#### References

- [1] Samuel D, Muller R, Alexander G, Fassati L, Ducot B, Benhamou JP, et al. Liver transplantation in European patients with the hepatitis B surface antigen. *N Engl J Med* 1993;329:1842–7.
- [2] Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; 122:889–96.
- [3] Ueda Y, Takada Y, Haga H, Nabeshima M, Marusawa H, Ito T, et al. Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C

- after living-donor liver transplantation. *Transplantation* 2008;85:855–62.
- [4] Sugawara Y, Makuuchi M, Matsui Y, Kishi Y, Akamatsu N, Kaneko J, et al. Preemptive therapy for hepatitis C virus after living-donor liver transplantation. *Transplantation* 2004;78:1308–11.
- [5] Sugawara Y, Makuuchi M. Living donor liver transplantation to patients with hepatitis C virus cirrhosis. *World J Gastroenterol* 2006;12:4461–5.
- [6] Roche B, Sebah M, Canfora ML, Antonini T, Roque-Afonso AM, Delvart V, et al. Hepatitis C virus therapy in liver transplant recipients: response predictors, effect on fibrosis progression, and importance of the initial stage of fibrosis. *Liver Transpl* 2008;14:1766–77.
- [7] Lodato F, Berardi S, Gramenzi A, Mazzella G, Lenzi M, Morelli MC, et al. Clinical trial: peg-interferon alfa-2b and ribavirin for the treatment of genotype-1 hepatitis C recurrence after liver transplantation. *Aliment Pharmacol Ther* 2008;28:450–7.
- [8] Dinges S, Morard I, Heim M, Dufour JF, Mullhaupt B, Giostra E, et al. Pegylated interferon-alpha2a/ribavirin treatment of recurrent hepatitis C after liver transplantation. *Transpl Infect Dis* 2009;11:33–9.
- [9] Saab S, Oh MK, Ibrahim AB, Durazo F, Han S, Yersiz H, et al. Anemia in liver transplant recipients undergoing antiviral treatment for recurrent hepatitis C. *Liver Transpl* 2007;13:1032–8.
- [10] Sugawara Y, Makuuchi M. Living donor liver transplantation for patients with hepatitis C virus cirrhosis: Tokyo experience. *Clin Gastroenterol Hepatol* 2005;3(10 Suppl 2):S122–4.
- [11] Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006;130:1086–97.
- [12] Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. *Hepatology* 2007;46:1688–94.
- [13] Tamura S, Sugawara Y, Yamashiki N, Kaneko J, Kokudo N, Makuuchi M. Pre-emptive antiviral therapy in living donor liver transplantation for hepatitis C: observation based on a single-center experience. *Transpl Int* 2009;23:580–8.
- [14] Ueda Y, Takada Y, Marusawa H, Egawa H, Uemoto S, Chiba T. Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. *Transplantation* 2010 (In press).
- [15] Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the non-structural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996;334:77–81.
- [16] Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. *J Med Virol* 2006;78:83–90.
- [17] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007;46:403–10.
- [18] Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005;48:372–80.
- [19] Berenguer M, Palau A, Fernandez A, Benlloch S, Aguilera V, Prieto M, et al. Efficacy, predictors of response, and potential risks associated with antiviral therapy in liver transplant recipients with recurrent hepatitis C. *Liver Transpl* 2006;12:1067–76.
- [20] Hanounch IA, Miller C, Aucejo F, Lopez R, Quinn MK, Zein NN. Recurrent hepatitis C after liver transplantation: on-treatment prediction of response to peginterferon/ribavirin therapy. *Liver Transpl* 2008;14:53–8.
- [21] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. *J Med Virol* 2007;79:1686–95.
- [22] Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* 1990;87:9524–8.
- [23] Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncales FL Jr, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KD)/ribavirin. *J Hepatol* 2005;43:425–33.
- [24] Zeuzem S, Buti M, Ferenci P, Sperl J, Horsmans Y, Cianciara J, et al. Efficacy of 24 weeks treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C infected with genotype 1 and low pretreatment viremia. *J Hepatol* 2006;44:97–103.
- [25] Drusano GL, Preston SL. A 48-week duration of therapy with pegylated interferon alpha 2b plus ribavirin may be too short to maximize long-term response among patients infected with genotype-1 hepatitis C virus. *J Infect Dis* 2004;189:964–70.
- [26] Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. *J Med Virol* 2009;81:452–8.
- [27] Fukuhara T, Taketomi A, Okano S, Ikegami T, Soejima Y, Shirabe K, et al. Mutations in hepatitis C virus genotype 1b and the sensitivity of interferon-ribavirin therapy after liver transplantation. *J Hepatol* 2010;52:672–80.
- [28] Kamal SM, Fouly AE, Kamel RR, Hockenjos B, Al Tawil A, Khalifa KE, et al. Peginterferon alfa-2b therapy in acute hepatitis C: impact of onset of therapy on sustained virologic response. *Gastroenterology* 2006;130:632–8.
- [29] Ogata K, Ide T, Kumashiro R, Kumada H, Yotsuyanagi H, Okita K, et al. Timing of interferon therapy and sources of infection in patients with acute hepatitis C. *Hepatol Res* 2006;34:35–40.
- [30] Delwaide J, Bourgeois N, Gerard C, De Maeght S, Mokaddem F, Wain E, et al. Treatment of acute hepatitis C with interferon alpha-2b: early initiation of treatment is the most effective predictive factor of sustained viral response. *Aliment Pharmacol Ther* 2004;20:15–22.
- [31] Jaeckel E, Cornberg M, Wedemeyer H, Santantonio T, Mayer J, Zankel M, et al. Treatment of acute hepatitis C with interferon alfa-2b. *N Engl J Med* 2001;345:1452–7.



## Original article

# Amino acid substitutions in core and NS5A regions of the HCV genome can predict virological decrease with pegylated interferon plus ribavirin therapy

Shosuke Kitamura<sup>1,2</sup>, Masataka Tsuge<sup>1,2,3</sup>, Tsuyoshi Hatakeyama<sup>1,2</sup>, Hiromi Abe<sup>1</sup>, Michio Imamura<sup>1,2</sup>, Nami Mori<sup>2,4</sup>, Hiromi Saneto<sup>1,2</sup>, Tomokazu Kawaoka<sup>1,2</sup>, Fukiko Mitsui<sup>1,2</sup>, Nobuhiko Hiraga<sup>1,2</sup>, Shintaro Takaki<sup>1,2</sup>, Yoshiiku Kawakami<sup>1,2</sup>, Hiroshi Aikata<sup>1,2</sup>, Shoichi Takahashi<sup>1,2</sup>, Waka Ohishi<sup>1,5</sup>, Hidenori Ochi<sup>1,2</sup>, C Nelson Hayes<sup>1,2</sup>, Kazuaki Chayama<sup>1,2\*</sup>

<sup>1</sup>Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

<sup>2</sup>Liver Research Project Center, Hiroshima University, Hiroshima, Japan

<sup>3</sup>Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan

<sup>4</sup>Chuden Hospital, Hiroshima, Japan

<sup>5</sup>Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan

\*Corresponding author e-mail: chayama@hiroshima-u.ac.jp

**Background:** The current standard therapy for chronic hepatitis C is pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. Recently, it has been reported that amino acid (aa) substitutions in the core region, as well as the IFN-sensitivity-determining region (ISDR), were predictive of non-virological response (NVR), sustained virological response (SVR) and early virological response. Despite the importance of these two predictive factors for combination therapy, their interaction is poorly understood.

**Methods:** A total of 117 patients who were treated with PEG-IFN- $\alpha$ 2b plus RBV combination therapy were selected for participation in this study. We determined the aa sequences in the core region and ISDR by direct sequencing and analysed them along with clinical data to identify predictive factors for therapeutic response.

**Results:** The aa sequences in the core region and  $\gamma$ -glutamyl transpeptidase (GTP) levels were associated with SVR and NVR, but aa sequences in the ISDR were not. However, substitutions at both aa 70 and aa 91 in the core region without substitutions in the ISDR and higher levels of  $\gamma$ -GTP were independent predictive factors for NVR. Wild-type aa 70 and aa 91 in the core region, higher platelet counts and lower levels of  $\gamma$ -GTP were independent predictive factors for SVR.

**Conclusions:** These results indicate that analyses of aa substitutions in both the core region and the ISDR are useful for predicting the effectiveness of combination therapy, and could help to avoid therapy exposure for patients who have a low probability of SVR.

## Introduction

HCV is one of the most serious global health problems, affecting >170 million people worldwide [1–3]. Chronic HCV infection leads to development of chronic hepatitis, cirrhosis and hepatocellular carcinoma [4–8]. In an attempt to eradicate the virus and prevent the development of advanced liver diseases and hepatocellular carcinoma, interferon (IFN) is administered to patients with chronic HCV infection, with success in a subset of patients in whom marked biochemical and histological improvements

can be obtained [9,10]. However, patients with high virus titres who are infected with genotype 1b, which is the major genotype, affecting approximately 70% of Japanese HCV patients, show poor response to IFN monotherapy. Less than 20% of patients treated with IFN monotherapy show sustained virological response (SVR) [11–14]. With the advent of pegylated interferon (PEG-IFN) and a newer antiviral agent, ribavirin (RBV), the eradication rate of the virus has improved overall. However, the eradication rate of

**Table 1.** Clinical characteristics of 117 patients before pegylated interferon plus ribavirin combination therapy

Characteristic	Value
Male/female gender, <i>n</i>	60/57
Age, years	62 (15–82)
BMI, kg/m <sup>2</sup>	22.7 (17.4–31.8)
WBC count, cells/ $\mu$ l	5,100 (2,030–9,610)
Neutrophil count, cells/ $\mu$ l	2,831 (830–7,145)
Haemoglobin, g/dl	13.9 (9.6–17.3)
Platelet count, $\times 10^4$ cells/mm <sup>3</sup>	14.0 (5.0–75.9)
AST, IU/l	46 (10–209)
ALT, IU/l	48 (5–214)
$\gamma$ -GTP, IU/l	39 (10–371)
Total cholesterol, mg/dl	174 (107–248)
HCV RNA, log <sub>10</sub> IU/ml	6.34 (5.2–7.1)
SVR/non-SVR, <i>n</i>	46/71

Data are median (range) unless indicated otherwise. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; SVR, sustained virological response; WBC, white blood cell;  $\gamma$ -GTP,  $\gamma$ -glutamyl transpeptidase.

genotype 1b with high viral load still remains only 40–50% [15–17].

Many host and viral factors that are predictive of the response to PEG-IFN plus RBV combination therapy have been reported to date. The SVR rate of the combination therapy has been reported to be significantly lower in older patients and in female patients [18–22]. The SVR rate was only 30% in female genotype 1b patients older than 65 years who have high viral loads [23]. Recently, sequencing analysis of HCV genomes has been actively performed to predict the effects of PEG-IFN plus RBV combination therapy. According to sequence analysis of HCV hypervariable region 1, quasispecies in this region at baseline were related to early virological response (EVR) and virological rebounds following combination therapy [24]. It has also been reported that the number of amino acid (aa) substitutions in the IFN-sensitivity-determining region (ISDR; nucleotides 2209–2248 or aa positions 237–276 of the NS5A region) correlates with SVR rate for combination therapy in HCV genotype 1b patients [25]. Recently, Akuta *et al.* [18,19,26,27] reported that the presence of aa 70 and/or aa 91 substitutions in the HCV core region is an independent and significant predictor of virological response (VR; for example, EVR, SVR and non-virological response [NVR]) to combination therapy. Okanoue *et al.* [28] also reported that the wild-type HCV core aa 70 and  $\geq 2$  aa substitutions in the ISDR are useful predictive factors for SVR. These reports, however, did not analyse the interaction of aa substitutions in the core region and the ISDR.

We recently analysed aa substitutions in the HCV core region and ISDR from patients treated with conventional IFN- $\alpha$ 2b and RBV combination therapy and showed that substitutions in the core region and ISDR

are useful predictive factors of response to therapy [29]. To date, no report has precisely analysed the relationship between both of these viral and clinical factors in patients who received PEG-IFN plus RBV combination therapy. In the current study, we analysed the relationship between clinical background and aa substitutions in the core region and ISDR to clarify the predictive factors of VR to combination therapy.

## Methods

### Patients

A total of 353 adult Japanese patients infected with HCV genotype 1b provided written informed consent to participate in the present study at Hiroshima University Hospital (Hiroshima, Japan). Among these patients, 117 who were treated with the PEG-IFN- $\alpha$ 2b plus RBV combination therapy from August 2004 to June 2008, were selected as study participants based on the following criteria: serum HCV RNA titre  $>5.0$  log<sub>10</sub> IU/ml using HCV RNA quantitative analysis with AmpliCor™ PCR analysis (Roche Diagnostics, Indianapolis, IN, USA; HCV RNA  $>5.0$  log<sub>10</sub> IU/ml was included in the category of high viral load based on guidelines by the Ministry of Health, Labor and Welfare [MHLW] of Japan [30]); no coinfection with other viruses, such as HIV or HBV; no other liver diseases, such as alcoholic liver disease, autoimmune hepatitis or decompensated cirrhosis (alcoholic liver disease was defined as patients with a lifetime total ethanol intake  $>100$  kg [31]); and no coexisting conditions, such as poorly controlled diabetes mellitus, decompensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, haemophilia, autoimmune diseases or post-transplantation. All selected patients were followed until 6 months after the completion of combination therapy. Overall, 56 of 117 patients had received prior antiviral treatments, such as IFN monotherapy or IFN plus RBV combination therapy, but they had never previously received PEG-IFN- $\alpha$ 2b monotherapy or PEG-IFN- $\alpha$ 2b plus RBV combination therapy. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee. Baseline clinical characteristics of the 117 patients are shown in Table 1.

Patients were injected weekly with 1.5  $\mu$ g/kg of PEG-IFN- $\alpha$ 2b (PegIntron®; Schering-Plough, Kenilworth, NJ, USA) subcutaneously and were administered oral RBV (Rebetol®; Schering-Plough) daily for 48 weeks. The initial dose of RBV was adjusted according to body weight following the standard Japanese regimen (600 mg/day for  $\leq 60$  kg, 800 mg/day for 60–80 kg and 1,000 mg/day for  $>80$  kg) [30]. Adverse events were monitored clinically by careful

interview and haematological examination throughout the study. Blood samples were taken before the beginning of therapy and every 4 weeks thereafter. Biochemical and haematological tests were performed at the Hiroshima University Hospital laboratory. The remaining sera were stored at  $-80^{\circ}\text{C}$  for further virological analysis. HCV genotypes were determined by phylogenetic analysis after reverse transcription (RT)-PCR and direct sequencing.

#### Assessment of efficacy

Serum HCV RNA was detected by RT-nested PCR assay (limit of detection 50 IU/ml; Cobas Amplicor HCV Test version 2.0; Roche Diagnostics) every 4 weeks during treatment and 24 weeks after cessation of therapy. Positive samples were analysed further by quantitative assay (limit of detection 500 IU/ml; Cobas Amplicor HCV Monitor version 2.0; Roche Diagnostics).

The following are definitions of the VR to PEG-IFN- $\alpha$ 2b plus RBV therapy. Rapid virological response (RVR) and EVR were defined as undetectable HCV RNA by qualitative PCR test at 4 and 12 weeks after the start of the treatment, respectively. SVR was defined as continuously undetectable HCV RNA by qualitative PCR assay and normalized alanine aminotransferase (ALT) levels for 24 weeks after the end of treatment. VR was defined as negative HCV RNA by qualitative PCR assay at least once during the treatment. NVR was defined as HCV RNA that was never detected as negative by qualitative PCR assay in all examinations.

#### Determination of aa sequences in the HCV core region and ISDR

HCV RNA was extracted from 100  $\mu\text{l}$  of stored serum samples by SepaGene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan) and dissolved in 20  $\mu\text{l}$  of  $\text{H}_2\text{O}$  and converted to complementary DNA by RT with random primers and Moloney murine leukaemia virus RT (Takara Shuzo, Tokyo, Japan). The complementary DNA was then amplified by nested-PCR to determine the nucleotide sequences in the HCV core region. The PCR protocol involved initial denaturation at  $95^{\circ}\text{C}$  for 5 min, 35 cycles of denaturation for 30 s at  $94^{\circ}\text{C}$ , annealing of primers for 1 min at  $57^{\circ}\text{C}$  and extension for 1 min at  $72^{\circ}\text{C}$ , followed by final extension at  $72^{\circ}\text{C}$  for 7 min. The primers for the first-round PCR were cc11 (forward 5'-GCC ATA GTG GTC TGC GGA AC-3') and e14 (reverse 5'-GGA GCA GTC CTT CGT GAC ATG-3'), and the primers for the second-round PCR were cc9 (forward, 5'-GCT AGC CGA GTA GTG TT-3') and e14 (reverse), as previously described [18,27,32,33]. To determine the nucleotide sequences of the HCV ISDR, we also performed RT nested-PCR with the same protocol as in the HCV core region. The primers used were IM11 (forward 5'-TTC CAC TAC

GTG ACG GGC AT-3') and 5A02KI (reverse 5'-CCC GTC CAT GTG TAG GAC AT-3') for the first-round PCR and 5A05KI (forward 5'-GGG TCA CAG CTC CCA TGT GAG CC-3') and IM10 (reverse 5'-GAG GGT TGT AAT CCG GGC GTG C-3') for the second-round PCR. After amplification, the final PCR products were separated in 2% agarose gel and purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Sequence analysis was performed using the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were compared with the nucleotide sequences of genotype 1b HCV-J (GeneBank accession number D90208) [34].

To analyse the relationship between the aa substitutions in the core regions, we divided the study participants into four groups on the basis of the presence of mutant (M) and wild-type (W) substitutions as follows: coreMM included patients with both aa 70 and aa 91 substitutions in the core region; coreMW included patients with only an aa 70 substitution in the core region; coreWM included patients with an aa 91 substitution in the core region; and coreWW included patients with neither aa 70 nor aa 91 substitutions in the core region. To analyse the effect of aa substitutions in the ISDR, we divided the patients into two groups as follows: ISDR $\geq$ 1 included patients with  $\geq$ 1 aa substitutions in the ISDR and ISDR0 included patients with no aa substitutions in the ISDR.

#### Statistical analyses

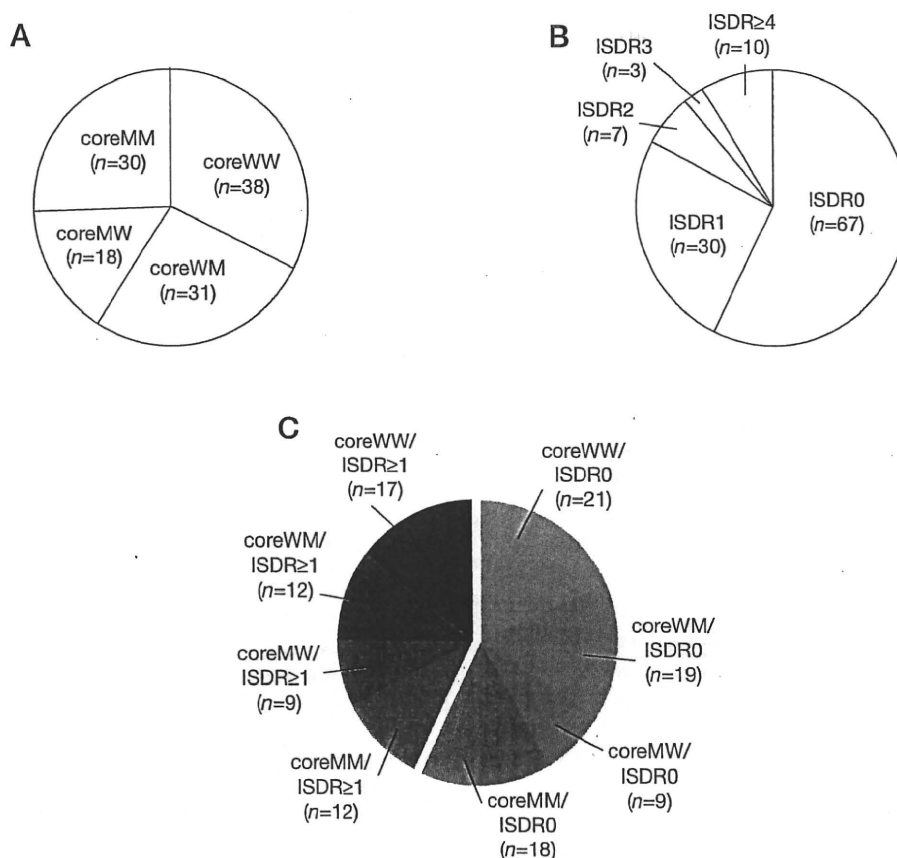
The baseline characteristics of the patients in the two groups were compared and the differences were assessed by  $\chi^2$  test with Yate's correction, Fisher's exact probability test and the Mann-Whitney U test. All *P*-values  $<0.05$  by two-tailed test were considered statistically significant. To determine the predictors of VR, univariate and multiple logistic regression analysis was performed. Variables with statistical significance ( $P<0.05$ ) or marginal significance ( $P<0.10$ ) in univariate analysis were retained for multiple logistic regression to identify significant independent predictive factors. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Core region and ISDR aa sequences, and their relationship with clinical parameters

To analyse the relationship between clinical background and aa sequences of the core region and ISDR, we compiled the clinical backgrounds of 117 patients who were treated with PEG-IFN- $\alpha$ 2b plus RBV combination therapy for 48 weeks (Table 1). The amount of PEG-IFN- $\alpha$ 2b injection was reduced in 48 of 117

Figure 1. Frequency of HCV amino acid substitutions in the core region and ISDR



(A) A total of 117 study patients were partitioned into four groups based on HCV core amino acid (aa) 70 and aa 91 substitution patterns. (B) Patients were grouped based on the number of HCV interferon-sensitivity-determining region (ISDR) substitutions. (C) Patients were divided into eight groups by the presence of HCV core substitutions and the number of ISDR substitutions. coreMM, substitutions in both core aa 70 and aa 91; coreMW, substitutions in core aa 70 only; coreWM, substitutions in core aa 91 only; coreWW, no substitutions in either core aa 70 or aa 91; ISDR0, absence of substitutions in the ISDR; ISDR $\geq$ 1, substitutions in  $\geq$ 1 region of the ISDR.

patients because of moderate or severe thrombocytopenia and/or neutropenia during the course of combination therapy. The dose of RBV was also reduced in 55 of 117 patients because of progression of anaemia, but none of the study patients received erythropoietin or blood transfusions during treatment. In total, 19 patients failed to complete the 48-week treatment because of very poor reduction of HCV RNA, and these patients were categorized as NVR; however, none of the study patients discontinued treatment because of adverse effects. We analysed the nucleotide and aa sequences of the core region and ISDR. In these 117 patients, core aa 70 (R70Q/Y/H) and core aa 91 (L91M) substitutions were observed in 48 (41.0%) and 61 (52.1%) patients, respectively. A marginal association was observed between the sequences of core aa 70 and core aa 91 ( $P=0.0920$ ; Figure 1A). For the aa sequence

of the ISDR, the wild-type was the most frequently detected sequence, and the frequency decreased as the number of aa substitutions increased (Figure 1B). We then evaluated the relationship between the aa substitutions of the core region and the ISDR (Figure 1C). The Breslow–Day test showed no heterogeneity in core mutation patterns between patients with or without aa substitutions in the ISDR ( $P=0.84$ ). We then compared the clinical parameters and core region aa substitution patterns. There was no relationship between age or gender and aa patterns of the core region (SK *et al.*, data not shown).

Clinical and virological predictive factors for VR and NVR to the combination therapy  
Statistical analysis was used to compare VR and NVR patients in order to identify predictive factors