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# Mutations in Two PKR-Binding Domains in Chronic Hepatitis C of Genotype 3a and Correlation With Viral Loads and Interferon Responsiveness

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Interferon (IFN) induces the double-stranded RNA-dependent protein kinase (PKR) to inhibit viral replication. Two motifs of the PKR-binding domain exist in the E2 and the NS5A regions of the hepatitis C virus (HCV). These regions are called the PKR-eukaryotic transcription factor (eIF2- $\alpha$ ) phosphorylation homology domain (PePHD), and the IFN sensitivity-determining region (ISDR). Both regions are inhibited by PKR. Thus, several studies have reported the relationship between these regions and IFN responsiveness and the HCV viral load. However, the data obtained from these studies remain controversial. The aim of this study was to investigate the genomic heterogeneity of the PePHD and the ISDR in patients with genotype 3a and how this impacts HCV replication and the response to IFN therapy. Twenty-one male patients infected with HCV genotype 3a were studied. The PePHD was well conserved, and mutations were found in only one amino acid position in two patients. Patients with three or more mutations in the ISDR had lower viral loads than those with fewer than two mutations ( $192.2 \pm 176.7$  vs.  $1279.4 \pm 997.6$  KIU/ml,  $P=0.0277$ ). Ten (71.4%) of 14 patients achieved a sustained virological response to IFN therapy. No specific amino acid substitutions in the PePHD and the ISDR were associated with IFN responsiveness; however, the number of mutations in the ISDR was significantly associated with the HCV viral load. The findings from this study suggest that the ISDR plays an important role in regulating viral replication in patients infected with HCV genotype 3a. *J. Med. Virol.* 83:1727–1732, 2011. © 2011 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis C virus (HCV); genotype 3a; double-stranded RNA-dependent protein kinase (PKR); E2-PePHD; NS5A-ISDR

## INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma (HCC) [Seeff, 2002]. HCV is classified into six genotypes that have different clinical courses, including the incidence of HCC and interferon (IFN) responsiveness. A part of the NS5A region of HCV genotype 1b is closely associated with the response to IFN, and is named the IFN sensitivity-determining region (ISDR) [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004]. IFN acts to inhibit viral replication by inducing the double-stranded RNA-dependent protein kinase (PKR). The ISDR is at the front of the PKR-binding domain and is thought to be inhibited by PKR, according to an in vitro study [Gale et al., 1998]. Thus, ISDR heterogeneity is likely to be an important factor determining the response to IFN treatment. However, despite recent reports investigating ISDR analysis as a predictor of the response to IFN therapy [Zeuzem et al., 1997; Squadrito et al., 2002], the clinical applicability of this approach remains to be established. In addition, it is thought that the mode of HCV infection and the racial background influence the predictions of IFN outcome by the ISDR [Layden-Almer et al., 2005; Jenke et al., 2009]. Although PKR-independent effects on NS5A have been reported, some reports have not confirmed the interaction between PKR and NS5A [Podevin et al., 2001; Tan and Katze, 2001; Liu et al., 2006]. Thus, the role of the ISDR in the interaction of NS5A with PKR remains controversial.

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Another PKR-binding domain, the protein kinase-RNA activated (PKR)-eukaryotic transcription factor (eIF2-alpha) phosphorylation homology domain (PePHD), exists in the E2 region of HCV. Several studies have reported a relationship between these two regions and IFN responsiveness [Saiz et al., 1998; Berg et al., 2000; Sarrazin et al., 2000; Durante et al., 2003; Hung et al., 2003; Ukai et al., 2006]. However, this is controversial for genotype 1b, and there are no reports analyzing the NS5A-ISDR and the E2-PePHD in genotype 3a in Japanese patients because of the rarity of genotype 3a in the Japanese population [Hayashi et al., 2003]. The aim of the present study was first to determine whether the genomic heterogeneity of the E2-PePHD and the NS5A-ISDR in Japanese patients with genotype 3a impacts HCV replication. The second aim was to evaluate the efficacy of IFN therapy in Japanese patients with HCV genotype 3a.

## MATERIALS AND METHODS

### Patients

A total of 21 hemophilic patients infected with HCV (mean age  $45.5 \pm 14.6$  years; range, 25–80 years) were studied. None of the patients had a history of intravenous drug use, chronic alcohol abuse, autoimmune disease, or metabolic disease. Six patients were co-infected with human immunodeficiency virus (HIV) and were well controlled by highly active antiretroviral therapy (HAART). The clinical characteristics of each patient are summarized in Table I.

### Schedule of IFN Therapy

Six patients received six mega units (MU) of IFN alpha 2b (INTRON A; Schering-Plough, Osaka, Japan) injected subcutaneously daily for the first 2 weeks and then three times a week for 22 weeks, in conjunction with oral ribavirin (Rebetol; Schering-Plough) at a dose of 800 mg (for those with a weight <80 kg) daily. Seven patients received Peginterferon alfa-2b (PEG-Intron, Schering-Plough) at a dose of 1.5 µg per kilogram of body weight injected subcutaneously once a week for 48 weeks. Ribavirin (Rebetol; Schering-Plough) was given orally for 48 weeks. One patient had severe anemia, which is a contraindication for ribavirin, and received monotherapy with IFN beta (Feron; Toray,

Tokyo, Japan) 600 MIU three times a week for 24 weeks. After the end of the treatment, the patients were followed for a further 24 weeks. HCV-RNA in serum samples was examined at 2, 4, 8, and 12 weeks, at the end of IFN therapy, and again 6 months after the end of the treatment. Patients who were persistently negative for serum HCV-RNA with normal serum ALT levels at 24 weeks after withdrawal of IFN treatment were considered to have a sustained virological response, and all others had non-sustained virological response. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Serum was stored at  $-80^{\circ}\text{C}$  until required.

### Virological Tests

All of the patients were negative for hepatitis B surface antigen and positive for serum HCV antibody, by commercial ELISA (Dinabot, Tokyo, Japan). In all patients HCV RNA quantitative viremia load was determined by a commercial assay [Kawai et al., 2002]. Anti-HIV antibody was detected by a commercial particle agglutination assay (Fuji Rebio, Tokyo, Japan). HCV genotyping was performed by direct sequencing of the 5'-UTR and E1 regions according to a method described previously [Otagiri et al., 2002; Hayashi et al., 2003]. The genotypes were classified according to the Simmonds' nomenclature [Simmonds et al., 2005]. Direct sequencing of the E2-PePHD and the NS5A-ISDR was performed. Briefly, RNA was extracted from 140 µl of sera using the QIAamp Viral RNA kit (QIAGEN, Valencia, CA) and redissolved in 50 µl diethylpyrocarbonate-treated water. Reverse transcription using oligo and random hexamer primers was carried out on 10 ng of RNA with the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA). Amplification of the E2-PePHD and the NS5A-ISDR was performed by nested PCR, as previously reported [Sarrazin et al., 2000; Castelain et al., 2002]. The E2-PePHD primers were: sense 5'-AGAAYSCCCACACACTA-CACGCGGC-3' and antisense 5'-GGCAGTTTCTCYTCCTCAGCACT-3'. For NS5A-ISDR: sense 5'-GCCAACACAACTA-CACGCGGC-3' and antisense 5'-CTTTTCTCCGAG-GAGG-3'. Amplification conditions consisted of 10 min at  $94^{\circ}\text{C}$ , followed by 40 cycles of  $94^{\circ}\text{C}$  for 10 sec,  $55^{\circ}\text{C}$  for 30 sec, and  $72^{\circ}\text{C}$  for 30 sec in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). The second PCR was performed with the following sets of primers: for E2-PePHD, sense 5'-GTGGTACCATGAGCGGCGATTGGCTGCGTATCATC-3' and antisense 5'-GCTCTAG/ATCATCAGCAGCAGACCA-CGCTCTGCTC-3'; and for NS5A-ISDR, sense 5'-TTCTTCACTGAAGTGGATGG-3', and antisense 5'-GTTGGTGGTACATAATCCGG-3'. The PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. The PCR products were also subjected to cycle sequencing with an ABI PRISM Cycle Sequence Kit (Perkin Elmer, Branchburg, NJ) that uses second

TABLE I. Clinical Characteristics

	N = 21
Age (years)	$45.5 \pm 14.6$
Sex (male/female)	21/0
AST (IU/L)	$59.6 \pm 26.6$
ALT (IU/L)	$83.6 \pm 44.1$
Platelet count ( $10^4/\mu\text{l}$ )	$14.7 \pm 7.2$
HCV RNA level (KIU/ml)	640 (71–3,900)
HIV co-infection (yes/no)	15/6

Data are expressed as mean  $\pm$  standard deviation. HCV RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

NZL1	PSLKATCCTRRPDPAEILVDNLLWRQEMGNSNITRVESET	HCV(KIU/ml)	HIV
H4	-----	590	n
H6	-----	1000	p
H7	-----	640	n
H10	-----	560	n
H11	-----	500	p
H12	-----	640	n
H13	-----	320	n
H14	-----	1300	n
H15	-----	2600	n
H16	-----	760	n
H20	-----	2500	n
H21	-----	3900	n
H2	A-----	1400	n
H3	-----D-	1200	n
H8	-----D-	540	p
H1	-----G-	2000	p
H9	-----H-G-----E-	110	p
H17	-----S-L-----S-	71	n
H19	A-----G-----G-----	500	p
H5	A-----E--LY-----G-----K	100	n
H18	Y-----H-KLY-----D-----	180	n

Fig. 1. Alignment of the amino acid sequence of the ISDR. HCV viral loads and co-infection with HIV are also indicated. In the sequence alignment, dashes indicate amino acids identical to the consensus sequence NZL1. ISDR, interferon sensitivity-determining region; HCV, hepatitis C virus; HIV, human immunodeficiency virus; P, positive; N, negative.

round primers as the sequencing primer, and analyzed on a ABI 310 DNA Sequencer (PE Applied Biosystems). The sequence data were aligned and analyzed with GENETYX software (version 7; Software Development, Tokyo, Japan).

Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). The paired *t*-test, the chi-square test, and Fisher's exact test were used to analyze differences in variables. A value of *P* < 0.05 was considered statistically significant. The statistical software used was Statview 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Sequencing revealed that the E2 region including the PePHD was well conserved. In two cases, a mutation (S665R and H668Q) was found in the E2-PePHD. The relationship between the NS5A-ISDR substitutions, the HCV viral loads, and co-infection with HIV are shown in Figure 1. The number of mutations in the ISDR of the NZL1 strain was determined, and ISDR sequences with more than three mutations were defined as the mutant type. Five patients were classified with the mutant type

and 16 patients as infected with the wild type. Six patients were co-infected with HIV. The HCV viral loads and the frequency of substitutions in the NS5A-ISDR were not significantly different between patients with HIV and those without HIV. Clinical characteristics relating to the ISDR are presented in Table II. Patients with three or more mutations in the NS5A-ISDR (mutant type) had lower viral loads than those with fewer than two mutations (wild type; 192.2 ± 176.7 vs. 1279.4 ± 997.6 KIU/ml, *P* = 0.0277). No other factors were statistically significant between the mutant and the wild types. A virological response, defined as negativity for HCV after 2, 4, 8, and 12 weeks of IFN therapy, occurred in 8, 2, 1, and 3 patients, respectively. All patients were negative for HCV-RNA at the end of the treatment. Sustained virological response was defined as no detection of HCV-RNA at 24 weeks after the end of the treatment. Ten patients remained negative for HCV-RNA, but four patients relapsed with HCV viremia after the treatment ceased. Thus, 10 (71.4%) of 14 patients achieved sustained virological response. Statistical analyses for associations between the response to IFN therapy and clinical characteristics, including substitutions in the E2-PePHD and the NS5A-ISDR, are shown in Table III. Detection of HCV RNA and an early virological response at the point of HCV negativity after 12 weeks of IFN therapy was predictive of SVR. However, other factors, including amino acid substitutions in the E2-PePHD and the NS5A-ISDR, were not associated with IFN responsiveness. There were no significant differences in HCV RNA levels between patients that were HCV negative after 12 weeks of IFN therapy with an early virological response and those without an early virological response (996.5 ± 1189.1 vs. 1666.7 ± 1040.8 KIU/ml, *P* = 0.3947). HCV RNA levels were not associated with early virological response.

DISCUSSION

HCV has been classified into six genotypes that have different geographic distributions. In Japan, genotype 1b is predominant, and genotypes 2a and 2b account for the majority of cases in Japanese patients with chronic hepatitis C. Therefore, chronic hepatitis with HCV genotypes 1b, 2a, and 2b have been well investigated

TABLE II. Clinical Characteristics According to ISDR

Factors	Wild type (n = 16)	Mutant type (n = 5)	P-value
Age (years)	45.2 ± 13.9	46.6 ± 18.1	0.8554
ALT (IU/L)	79.1 ± 40.0	98.0 ± 58.0	0.4157
AST (IU/L)	52.1 ± 18.2	61.8 ± 35.3	0.5034
PLT (×10 <sup>9</sup> /mm <sup>3</sup> )	14.1 ± 8.0	16.6 ± 10.4	0.5065
HCV RNA level (KIU/ml)	1279.4 ± 997.6	192.2 ± 176.7	0.0277
HIV: P/N	4/12	2/3	0.5975
PePHD: wild/mutant	1/15	1/4	0.2857

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; HIV, human immunodeficiency virus; P, positive; N, negative; PePDH, protein kinase-RNA activated-eukaryotic transcription factor phosphorylation homology domain; ISDR, interferon sensitivity-determining region. Data are expressed as mean ± standard deviation.

TABLE III. Univariate Analysis: Factors Predictive of SVR

	SVR (n = 10)	Non-SVR (n = 4)	P-value
Age (years)	41.9 ± 13.3	52.5 ± 8.7	0.1705
ALT (IU/L)	82.4 ± 37.3	88.3 ± 57.1	0.8223
AST (IU/L)	52.1 ± 18.2	61.8 ± 35.3	0.5034
PLT (×10 <sup>4</sup> /mm <sup>3</sup> )	15.1 ± 5.2	19.3 ± 10.3	0.3188
HCV RNA level (KIU/ml)	706.1 ± 735.3	2225.0 ± 1403.2	0.0190
HIV: P/N	2/8	2/2	0.5205
PePHD: wild/mutant	10/0	3/1	0.2857
ISDR: wild/mutant	7/3	3/1	0.9999
EVR: yes/no	10/0	1/3	0.0110

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; HIV, human immunodeficiency virus; P, positive; N, negative; PePDH, protein kinase-RNA activated-eukaryotic transcription factor phosphorylation homology domain; ISDR, interferon sensitivity-determining region; EVR, early virologic response. Data are expressed as mean ± standard deviation.

in Japan. However, the other genotypes (3, 4, 5, and 6) are uncommon, and clinical data are scarce. Genotype 3a, a rare strain in Japan, is limited to patients with hemophilia because they receive clotting factors of foreign origin [Fujimura et al., 1996; Otagiri et al., 2002]. Therefore, little is known about the clinical characteristics including IFN responsiveness and the genomic heterogeneity of the E2-PePHD and the NS5A-ISDR in patients with chronic hepatitis C, genotype 3a in Japan. HCV genotype is one of the most important factors relating to the response to IFN therapy. For example, genotypes 1 and 4 show resistance to IFN therapy, whereas genotypes 2 and 3 show a good response. The present study revealed that 10 (71.4%) of 14 patients achieved sustained virological response. The high virological response rate in genotype 3a has been confirmed in the present Japanese patients as it has in Caucasian patients. HCV RNA levels and early virological response were also associated with sustained virological response, as reported previously. The role of mutations in the E2-PePHD region, which is a PKR-binding domain, among Japanese patients with chronic hepatitis C infected with genotype 3a was analyzed. The E2-PePHD was well conserved, as previously reported, and all strains but two were identical to the consensus sequence [Chayama et al., 2000; Sarrazin et al., 2000]. It was difficult to clarify the relationship between the HCV viral load and the response to IFN therapy because of the small sample number in the present study. An *in vitro* study has revealed the potential of the E2-PePHD [Taylor et al., 1999]; however, many more patients with this mutation would be needed to determine the function of the E2-PePHD. Mutations in the E2-PePHD are quite rare, so this region, as well as HCV genotype 1, is unsuitable for routine clinical assessment [Muñoz et al., 2008].

The usefulness of the NS5A-ISDR for the prediction of IFN therapy differs between regions, such as in Asia and Europe [Enomoto et al., 1996; Zeuzem et al., 1997; Nakano et al., 1999; Squadrito et al., 2002; Pascu et al., 2004; Muñoz et al., 2008; Yen et al., 2008; Okanoue et al., 2009; Hayashi et al., 2010]. HCV genotype 1b is distributed worldwide, yet subtypes differ in each region. It has been hypothesized previously that the geographic distribution of genotype 1b would be a factor

that influences the usefulness of the ISDR. Subtype J, found only in Japan, and subtype W, distributed worldwide, have been identified. Subtype J is closely associated with the ISDR and confers a good response to IFN therapy [Nakano et al., 1999, 2001]. Thus, the prevalence of specific subtypes is one reason behind the conflicting opinions towards the use of the ISDR. This extends to racial differences [Reddy et al., 1999; Shiffman et al., 2007]. Forming a control group when comparing racial effects on chronic hepatitis C proved difficult due to the heterogeneity of HCV genotypes and subtypes. Thus, a study to evaluate racial differences is not easy to perform because of varying HCV genotype distribution. Japanese hemophiliacs receive clotting factors that are imported from foreign countries and are frequently infected with genotype 3a. Thus, the present study on genotype 3a allowed the assessment of racial differences by avoiding the distribution bias of HCV genotype. In the present study, the ISDR was not associated with the response to IFN therapy, as in Western reports [Sarrazin et al., 2000]. The prevalence of HCV genotypes and subtypes impacts the association between the ISDR and the IFN response to a far greater degree than racial differences.

There are several limitations to the present study. Firstly, the small number of patients examined prevented the identification of a correlation between the ISDR and IFN responsiveness. Secondly, the low virological response rate and the small number of mutations in the ISDR do not favor the use of ISDR analysis in predicting IFN responsiveness, as previously reported for genotype 1 [Herion and Hoofnagle, 1997]. Furthermore, the high virological response rate and the low prevalence of patients with more than three mutations in the ISDR do not favor the use of ISDR analysis in predicting IFN responsiveness. Four patients had more than three mutations in the ISDR, which was defined as the mutant type, and only one patient did not achieve sustained virological response. This patient was co-infected with HIV. HIV co-infection is known to be a negative factor for sustained virological response [Hayashi et al., 2000; Shire et al., 2006, 2007]. Thus, patients with more than three mutations in the ISDR tend to have good IFN responsiveness, though no statistically significant differences have been noted.

This might explain why a strong correlation between the ISDR and IFN responsiveness was not observed. The number of amino acid substitutions in the ISDR inversely correlates with the HCV viral load in patients with genotype 1 [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004]. Similar observations were made in our study for genotype 3a suggesting an important role for the NS5A-ISDR in regulating viral replication in these patients. However, because of the small sample size in the present study, larger cohorts are needed to confirm these results.

In addition to racial factors, host factors also contribute to IFN treatment outcomes. Many investigators have reported that gender [Alvarez, 1996; Izopet et al., 1998], age [Hayashi et al., 1998], and past history of transfusion [Kleter et al., 1998] are associated with sustained virological response in IFN-treated patients with HCV, although this is somewhat controversial. Since all of the patients in the present study were males with a past transfusion history, and they were younger than the average HCV-infected patients, we were not able to investigate a possible effect of these factors in our study population.

In conclusion, in Japanese patients with HCV genotype 3a, the E2-PePHD and the NS5A-ISDR are not associated with IFN responsiveness. The NS5A-ISDR is significantly associated with the HCV viral load suggesting an important role for NS5A-ISDR in regulating viral replication in these patients.

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CLINICAL STUDIES

## Association of interleukin 28B and mutations in the core and NS5A region of hepatitis C virus with response to peg-interferon and ribavirin therapy

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### Keywords

core region – genotype 1b – hepatitis C virus – interleukin 28B – NS5A

### Abbreviations

aa, amino acid; ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; SVR, sustained virological response.

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### Abstract

**Background and aims:** Mutations in the core and NS5A region of hepatitis C virus (HCV) genotype 1b have been associated with response to interferon (IFN) therapy. Genome-wide association studies have revealed that the single-nucleotide polymorphism (SNP) of interleukin 28B (IL28B) contributes to IFN response. The aim of this study was to investigate whether the SNP of IL28B (rs8099917) and amino acid substitutions in the core and NS5A region affect the response to IFN therapy. **Methods:** A total of 299 patients (157 men, 142 women; mean age, 55.9 ± 10.3 years) infected with HCV genotype 1b were studied. The fibrosis stage was diagnosed as F0 (n = 23), F1 (n = 121), F2 (n = 62), F3 (n = 32) and F4 (n = 7) by liver biopsy. **Results:** Of the 299 patients, 138 achieved sustained virological response (SVR). On univariate analysis, predictors of SVR were age < 60 years, male gender, higher platelet count, lack of fibrosis, non-Q at core 70, mutant-type interferon sensitivity-determining region (ISDR) and IL28B genotype TT. The factors related to SVR on multivariate analysis were IL28B (P = 0.0001), fibrosis (P = 0.0111) and mutations in the core region 70 (P = 0.0267) and ISDR (P = 0.0408). The best SVR was achieved in patients with non-Q70, mutant-type ISDR and T allele (74.5%), and the worst was achieved in patients with Q70, wild-type ISDR and G allele (8.1%). **Conclusions:** The SNP of IL28B and mutations in the core region and NS5A are associated with IFN responsiveness. Both host and viral factors might be useful for predicting IFN response.

It has been estimated that 170 million worldwide are infected with hepatitis C virus (HCV), which causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma (1). Therefore, HCV infection is a major global health problem. Pegylated-interferon (IFN)- $\alpha$  and ribavirin combination therapy is standard treatment for patients with chronic hepatitis C, but it eradicates HCV for only 50% of patients with genotype 1 (2, 3). The difference in response was investigated, and several factors were identified, including age, liver fibrosis, HCV genotype, HCV RNA levels and race (4–7). Viral factors were frequently the focus for investigation of IFN responsiveness, and amino acid (aa) substitutions in the core and NS5A regions were reported as markers that could be used to predict the response to IFN therapy (8–14). However, these relationships were controversial (15, 16), and investigations were limited to viral factors alone to clarify IFN responsiveness. However, host genetic factors, as well as genetic heterogeneity in the HCV genome, contribute to IFN

treatment outcomes. Therefore, several genome-wide association studies were performed to understand the host factors that were associated with IFN responsiveness; these revealed that interleukin 28B (IL28B) polymorphisms are strongly associated with response to IFN therapy (17–20). The single-nucleotide polymorphisms (SNPs) of IL28B, rs12979860 and rs8099917 genotypes are significantly associated with the outcome of IFN therapy. Although Caucasians and Hispanics have weak linkage-disequilibrium between these two SNPs, Japanese patients have strong linkage-disequilibrium, with no discrepancy between rs12979860 and rs8099917. Thus, rs8099917, which is strongly associated in Japanese reports, was selected for the present study (21). SNP of IL28B and mutations in the core and NS5A regions had different effects on IFN responsiveness, and their combined use might improve the ability to predict the response to IFN. However, the relationships between IL28B and viral factors such as mutations in the core and NS5A regions are little known. The aim of this study



was to investigate whether the SNP of IL28B and aa substitutions in the core and NS5A regions in patients with HCV genotype 1b affect the response to pegylated-IFN- $\alpha$  2b and ribavirin combination therapy.

## Methods

A total of 432 patients with chronic hepatitis C genotype-1b and high viral load who were treated at Nagoya University Hospital, Fujita Health University Hospital and Ogaki Municipal Hospital were enrolled; 299 patients who completed IFN treatment for 48 weeks and had complete clinical data were selected for this study. Patients whose HCV RNA levels were < 100 KIU/ml were excluded. The patients' clinical characteristics are summarized in Table 1. The core region (aa 30–110) and interferon sensitivity-determining region (ISDR) (aa 2209–2248) were examined by direct sequencing. Identification of the SNP of IL28B (rs8099917) was performed by a real-time polymerase chain reaction (PCR) system. Liver biopsy was performed in 245 patients, and fibrosis stage was diagnosed according to the METAVIR criteria (22). Patients received subcutaneous injections of pegylated-IFN- $\alpha$  2b (1.5  $\mu$ g/kg) once each week plus oral ribavirin (600 mg for < 60 kg, 800 mg for 60–80 kg, 1000 mg for > 80 kg) daily for 48 weeks. Serum was stored at –80 °C for virological examination at pretreatment. Patients who were persistently negative for serum HCV RNA at 24 weeks after withdrawal of IFN treatment were considered to have a sustained virological response (SVR). The other patients were considered to have non-SVR. This study was approved by each hospital's ethics committee. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

## Virological analysis

The HCV-RNA quantitative viraemia load was determined by PCR. HCV was genotyped by direct sequencing of the 5'-untranslated region and/or E1 regions as described previously (23, 24). Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* (25). Direct sequencing of the HCV core and NS5A-ISDR region was performed as reported previously (9, 14). In brief, RNA was extracted from 140  $\mu$ l of serum with a commercial kit (QIAamp Viral RNA Kit, Qiagen, Valencia, CA, USA) and dissolved in 50  $\mu$ l of diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers using a commercial kit (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). The HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50  $\mu$ l PCR reaction contained 100 nM of each primer, 1 ng of template cDNA, 5  $\mu$ l of GeneAmp 10  $\times$  PCR buffer, 2  $\mu$ l of dNTPs and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for the core region were sense, 5'-GGGAGTCTCGTA

**Table 1.** Baseline characteristics of the patients

Clinical characteristics	N = 299
Age (years)	55.9 $\pm$ 10.3
Sex: male/female	157/142
AST (IU/L)	58.7 $\pm$ 48.9
ALT (IU/L)	69.8 $\pm$ 66.9
Platelet count (10 <sup>4</sup> / $\mu$ L)	16.6 $\pm$ 5.3
HCV RNA level (KIU/ml)	1760
The fibrosis stage	(100–7200)
F0, F1, F2, F3, F4	23, 121, 62, 32, 7
Body weight (kg)	57.9 $\pm$ 12.7

Data are expressed as mean  $\pm$  standard deviation.

HCV RNA level was shown by median (range).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus.

GACCGTGCACCATG-3' and antisense, 5'-GAGMGG KATRTACCCCATGAGRTCGGC-3', and primers for the NS5A-ISDR were sense 5'-TGGATGGAGTGGCGTTGCA CAGGTA-3' and antisense 5'-TCTTCTCCGTGGAGG TGGTATTG-3'. Amplification conditions consisted of 10 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template and with the following sets of primers: for the core region, sense primer 5'-AGACCGTGCACCATGAGCAC-3', and antisense 5'-TACGCCGGGGTCAKTRGGGGCCCCA-3'; and for the NS5A-ISDR, sense 5'-CAGGTACGCTCCGGCGTGCA-3' and antisense 5'-GGGGCCTTGGTAGGTGGCAA-3'. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

## Genomic analysis

Detection of the SNP of IL28B (rs8099917) was carried out by a real-time PCR system. In brief, genomic DNA was extracted from 150  $\mu$ l of whole blood using a commercial kit (QIAamp DNA Blood mini Kit, Qiagen) and was dissolved in 50  $\mu$ l of diethylpyrocarbonate-treated water. DNA (10 ng) was used for PCR with primers and probes from a commercial kit (Taqman SNP Genotyping Assays, Applied Biosystems). The SNP of IL28B (rs8099917) was amplified, and the results were analysed by real-time PCR in a thermal cycler (7300 Real-time PCR System, Applied Biosystems).

## Statistical analysis

Data are expressed as means  $\pm$  standard deviation. The paired *t*-test, the  $\chi^2$ -test and Fisher's exact test were used

to analyse differences in variables. A *P* value < 0.05 was considered significant. Multiple logistic regression models were used to identify factors predictive of SVR. The statistical software used was spss software (SPSS Inc., Chicago, IL, USA).

## Results

### Virological response

Of 299 patients, 35 (11.7%) showed a rapid virological response (RVR), with HCV negativity at 4 weeks, and 172 (57.5%) showed an early virological response (EVR), with HCV negativity at 12 weeks. Overall, 234 patients became HCV negative at the end of treatment (78.3%). However, 138 patients continued to be HCV negative after withdrawal of IFN treatment, and 138 of 299 (46.2%) patients were defined as achieving SVR. Of 35 patients with RVR, 33 (94.3%) achieved SVR. Of 172 patients with EVR, 126 (73.3%) achieved SVR. Of 127 patients without EVR, 115 became non-SVR (90.6%). Thus, RVR and EVR were associated with SVR (*P* < 0.001).

### Genetic heterogeneity in NS5A-interferon sensitivity-determining region and response to interferon therapy

The sequence of the HCVJ strain was defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in ISDR was used to analyse the ISDR system as in previous reports (12–14). Seventy-one patients with more than two mutations in the ISDR were defined as mutant type, and the other 228 patients were wild type. SVR was achieved in 41.2% (95/228) of the patients with wild-type ISDR and in 60.6% (43/71) of the patients with mutant-type ISDR (*P* = 0.0063). ISDR was associated with SVR.

### Amino acid substitutions in core regions of the hepatitis C virus genome and response to interferon therapy

Eighty-five patients with glutamine in core region 70 were defined as Q-type, and the other 214 patients were non-Q-type, as in previous reports (14). Overall, 118 of 214 patients with non-Q in the core region achieved SVR (55.1%). The SVR rate of patients with Q in core region 70 was 23.5% (20/85). Q70 in core region 70 was significantly associated with poor response to IFN therapy (*P* < 0.0001). The distribution of mutations in the HCV core region at aa 91 was leucine (L), 210 and methionine (M), 89. There were no significant differences between mutations in the HCV core region at aa 91 and SVR.

The prevalence of the single-nucleotide polymorphism of Interleukin28B (rs8099917) T (major allele) and G (minor allele) and response to interferon therapy

The frequencies of the IL28B genotypes were major homozygotes (TT), 219; heterozygotes (TG), 76; and

**Table 2.** Association between interleukin 28B genotypes and amino acid substitutions in hepatitis C virus core region and interferon sensitivity-determining region

	ISDR	
	Mutant	Wild
TG/GG	12	68
TT	59	160
<i>P</i> value = 0.0324		
HCV core region 70		
	Non-Q	Q
TG/GG	35	45
TT	179	40
<i>P</i> value < 0.0001		
HCV core region 91		
	L	M
TG/GG	46	34
TT	164	55
<i>P</i> value = 0.0044		

The number is patients' number.

HCV, hepatitis C virus; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; L, leucine; Q, glutamine; M, methionine.

minor homozygotes (GG), 4. The rates of SVR in the patients with TT, TG and GG were 57.9% (127/219), 14.5% (11/76) and 0% (0/4) respectively. The G allele of the IL28B genotype was significantly associated with poor response to IFN therapy (*P* < 0.0001).

The relationships between substitutions of aa in the HCV core region, NS5A-ISDR and the SNP of IL28B are shown in Table 2. NS5A-ISDR and both mutations in the HCV core regions were associated with IL28B genotypes. ISDR wild-type and Q70, which were resistant strains to IFN therapy, were more frequently found in patients with resistant TG/GG allele than in those with sensitive TT allele.

### Factors associated with sustained virological response

The results of univariate analysis for factors predictive of SVR are shown in Table 3. Patients with SVR were younger than those without SVR. Males were more frequent among SVR patients than non-SVR patients. SVR patients had higher platelet counts than non-SVR patients. SVR was achieved in 23.1% (9/39) of patients with advanced fibrosis and 50.5% (104/206) of patients without advanced fibrosis (*P* = 0.0016). SVR was achieved in 41.7% (95/228) of patients with wild-type ISDR and 60.6% (43/71) of patients with mutant-type (*P* = 0.0063). SVR occurred more frequently in patients without Q70 (55.1%; 118/214) than in those with Q70 (23.5%; 20/85; *P* = 0.0001). Achievement of SVR occurred more frequently in patients with TT allele (58%; 127/219) than in those with TG and GG alleles (13.8%; 11/80;

**Table 3.** Univariate analysis: factors predictive of sustained virological response

Factors	SVR (n = 138)	Non-SVR (n = 161)	P value
Age (years)	53.8 ± 11.5	57.9 ± 8.7	0.0005
Gender: male/female	82/56	75/86	0.0280
ALT (IU/L)	71.3 ± 76.7	68.4 ± 57.6	0.7110
AST (IU/L)	54.6 ± 46.7	62.1 ± 50.6	0.1983
PLT (× 10 <sup>4</sup> /mm <sup>3</sup> )	17.7 ± 5.5	15.6 ± 4.9	0.0008
Fibrosis: F0, 1, 2/3, 4	104/9	102/30	0.0016
HCV RNA level (KIU/ml)	2001.5 ± 1441.2	2168.3 ± 1432.4	0.3705
Core 70: non-Q/Q	118/20	96/65	0.0001
Core 91: L/M	104/106	34/55	0.0771
ISDR: wild/mutant	95/43	133/28	0.0063
IL28B: TT/TG+GG	127/11	92/69	0.0001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; L, leucine; M, methionine; PLT, platelet count; Q, glutamine.

**Table 4.** Factors associated with sustained virological response by multivariate analysis

Factor	Category	Risk ratio	95% CI	P value
IL28B genotype	TT	0.106	0.043–0.259	0.0001
Fibrosis	F3, F4	3.550	1.335–9.440	0.0111
Core70	Q	2.496	1.111–5.604	0.0267
ISDR	Wild	2.206	1.034–4.710	0.0408

Only factor that achieved statistical significance ( $P < 0.05$ ) on multivariate logistic regression analysis are shown.

IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region.

$P = 0.0001$ ). Age, sex, platelet count, liver fibrosis, core 70, ISDR and IL28B were associated with SVR. The same 11 factors used in univariate analysis were used in multivariate analysis. The factors related to SVR on multivariate analysis were IL28B genotype, liver fibrosis, core 70 and ISDR, as shown in Table 4. The other factors were not significant.

The virological response according to interleukin28B genotypes and amino acid substitutions in the 70 core region and interferon sensitivity-determining region

The SVR rates according to IL28B genotypes and aa substitutions in the 70 core region and ISDR are shown in Table 5. The best SVR rate was achieved in patients with non-Q70, mutant-type ISDR and T allele, and the worst response was achieved in patients with Q70, wild-type ISDR and G allele.

## Discussion

Viral factors associated with SVR have been the most frequently studied, and several regions, including 5'UTR, core, E2, NS5A and NS5B, have been suggested to play important roles in IFN responsiveness (8–16, 26–31). The aa substitutions in the HCV core and NS5A region would be two major viral factors that have strong associations with IFN response. The ISDR located in the

**Table 5.** The sustained virological response rate according to interleukin 28B and amino acid substitutions in 70 core region and interferon sensitivity-determining region

Core70/ISDR	IL28B; TT 58% (127/219)	IL28B; GT/GG 13.8% (11/80)
Q/wild 20% (13/65)	35.7% (10/28)	8.1% (3/37)
Q/mutant 35% (7/20)	50% (6/12)	12.5% (1/8)
non-Q/wild 50.3% (82/163)	57.6% (76/132)	19.4% (6/31)
non-Q/mutant 70.6% (36/51)	74.5% (35/47)	25% (1/4)

$P$ -value = 0.0001 by Cochran–Armitage test.

IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; Q, glutamine.

NS5A region was originally reported in 1996 by Enomoto et al. (8) and confirmed by several Asian studies (9, 12–14), but controversial results were reported by Western studies (15, 16). Meta-analysis showed the relationships between ISDR and SVR and suggested that unidentified factors have an effect on IFN responsiveness (32). The ISDR interacts with protein kinase R (PKR) and inactivates replication of HCV *in vitro* (33). Therefore, ISDR heterogeneity plays an important role that may affect response to IFN. However, some reports have not confirmed the interaction between PKR and NS5A (34, 35), and they suggested the PKR-independent effects of NS5A (36, 37). Thus, the effects of aa substitutions of the ISDR are unclear, and investigators searched for other viral factors. The aa substitutions at 70 and 91 in the HCV core region were reported as factors that could predict IFN responsiveness (10). Thus, several studies have reported that combining aa substitutions in the HCV core region and NS5A region could improve the predictive value of SVR in patients with genotype 1b (12–14). These results were useful to develop individualized treatment strategies for chronic hepatitis C patients.

For instance, in this study, only 20% of patients with Q70 and wild-type ISDR achieved SVR, compared with 70.6% of those with non-Q70 and mutant-type ISDR. However, the majority of patients were classified into those with non-Q70 and wild-type (50.3%), and another factor for improving SVR prediction was considered necessary. Three genome-wide association studies of SVR to pegylated-IFN- $\alpha$  and ribavirin combination therapy for chronic hepatitis C patients with genotype 1 from Japan, the USA and Australia identified SNPs of IL28B associated with IFN responsiveness (17–19). SVR was achieved in 13.8% of patients with IL28B minor allele (TG and GG) and in 58% with IL28B major allele (TT) in this study, and the SNP of IL28B was associated with the response to IFN in patients with HCV genotype 1b, as in previous reports. The effects of both host and viral factors on IFN responsiveness would affect the IFN treatment outcome. Thus, the SNP of IL28B was considered in the analysis of aa substitutions in the HCV core and the NS5A region for improving the prediction of SVR. The strain with the worst SVR outcome was Q70 and wild-type ISDR, with an SVR of 20%. When IL28B was considered in the analysis of patients with Q70 and wild-type ISDR, 8.1% of patients with TG/GG for IL28B achieved SVR compared with 35.7% of those with TT for IL28B. These results indicate the effects of both host and viral factors on IFN responsiveness. The best responders were 47 patients who simultaneously had non-Q70, mutant-type ISDR and TT allele; 35 (74.5%) achieved SVR. The clear suggestion of a correlation between the combination of the SNP of IL28B and aa substitutions in the core region 70 and ISDR with IFN responsiveness would not be supported in the non-Q/mutant/G allele and the Q/mutant/G allele groups because of the small number of patients. Both mutations in core region 70 and ISDR were strongly associated with IL28B genotype. Thus, the prevalence of patients with core 70 non-Q, ISDR mutant, and IL28B genotype G was rare, and it was difficult to find these combinations. Patients infected with IFN-resistant strains Q70 and wild-type ISDR could be clearly identified as non-responders to IFN therapy (8.1%) by the IL28B genotype; the positive predictive value for non-SVR was 91.9%. Meanwhile, patients infected with IFN-sensitive strains non-Q70 and mutant-type ISDR showed that the positive predictive value for SVR was 74.5%. Montes-Cano *et al.* (38) reported that the influence of IL28B would be stronger among patients infected with an IFN-resistant genotype (HCV genotype 1) than in those infected with an IFN-sensitive genotype (HCV genotype non-1). The SNP of IL28B would be strongly associated with the response to IFN, especially for poor responders.

Interleukin28B genotype was associated with spontaneous viral clearance, as well as IFN responsiveness (17, 20). A Spanish study found that the prevalence of HCV genotype depends on IL28B genotype and speculated that IL28B would be a candidate to explain HCV genotype differences in the IFN response (38). The

IFN-resistant strain (Q70) was detected more frequently in patients with the IL28B minor allele (TG and GG) (56.3%) than in those with the IL28B major allele (TT) (18.3%). The present study showed similar results: patients with IL28B G allele, which is associated with poor response to IFN, seemed to more frequently have the IFN-resistant strain (Q70). Further study is needed to clarify the effect of the IL28B gene on differences in IFN response between each HCV genotype and subgenotype. The IL28B polymorphism might regulate the expression of hepatic interferon-stimulated genes and cause the difference in IFN responsiveness (39). The association between IL28B genotypes and IL28B gene expression is controversial (18, 19, 39). The effects of the SNP of IL28B on gene expression and mechanisms against HCV infection are still under debate. Although the effect of the SNP of IL28B was unclear, as were the aa substitutions of the core region and ISDR, these factors could be used to predict SVR in patients infected with genotype 1b. The SNP of IL28B plays an important role in choosing optimal therapy and avoiding unnecessary treatment.

In conclusion, the SNP of IL28B and aa substitutions in the core region and ISDR were associated with response to IFN in patients with HCV genotype 1b. Combined use of both host and viral factors could improve prediction of the IFN response.

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# Antiviral Combination Therapy With Peginterferon and Ribavirin Does not Induce a Therapeutically Resistant Mutation in the HCV Core Region Regardless of Genetic Polymorphism Near the *IL28B* Gene

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An association has been reported between genetic polymorphism near *IL28B* gene and the prevalence of mutation of hepatitis C virus (HCV) core region residue 70, both of which have been associated with a lack of virologic response to antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin. This study investigated whether PEG-IFN/ribavirin combination therapy induces amino acid (AA) mutation at residue 70 of HCV and whether genetic polymorphism near *IL28B* gene affects it. AA substitutions at residue 70 of the HCV core region were measured and compared before and after combination therapy in 65 non-responders and 88 relapsers to the combination therapy. In three patients in whom both wild-type AA (arginine) and mutant-type AA (glutamine or histidine) were detected at residue 70 before treatment, only mutant-type AA was identified after treatment. In two patients who had wild-type AA solely before treatment, both wild-type and mutant-type AAs were identified at residue 70 after treatment. In five patients, in whom the AA had changed at residue 70 between before and after treatment, four patients carried the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene and one carried the TG/GG genotype. No difference was found in the prevalence of this change of AA at residue 70 between the TT and the TG/GG genotype. Antiviral combination therapy with PEG-IFN and ribavirin does not appear to induce mutation of HCV core region residue 70 regardless of genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. *J. Med. Virol.* 83:1559–1564, 2011. © 2011 Wiley-Liss, Inc.

**KEY WORDS:** chronic hepatitis C; peginterferon and ribavirin; amino acid substitution of HCV core region residue 70; genetic polymorphisms near the *IL28B* gene; mutation; non-sustained virologic responder

## INTRODUCTION

Hepatitis C virus (HCV) causes chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998; Kenny-Walsh, 1999]. The current standard therapy for patients with chronic HCV infection is the combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients with sustained virologic response, which indicates the eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieve a sustained virologic response.

Many studies have investigated the potential baseline host- or virus-related factors that are associated

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with the lack of virologic response to IFN-based antiviral therapy. As a host-related factor, recent studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 are strongly associated with a resistance to the combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Patients having the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene show a favorable response to the combination therapy with PEG-IFN and ribavirin, whereas patients having the GG genotype or those who are TG heterozygote show a resistance to the therapy. As for virus-related factors, amino acid (AA) mutations at residue 70 in the HCV core region have been reported to be associated strongly with a resistance to PEG-IFN/ribavirin combination therapy in patients infected with HCV genotype 1b [Akuta et al., 2005, 2007a; Donlin et al., 2007]. Patients with the mutant-type AA (glutamine or histidine) at residue 70 in the HCV core region show a resistance to the combination therapy in comparison to those with the wild-type AA (arginine) at this residue. These host- and virus-related factors are both associated with the outcome of the combination therapy with PEG-IFN and ribavirin independently in a previous report [Hayes et al., 2011].

A previous study reported that the percentage of patients with the mutant-type AA at residue 70 of the HCV core region increases with the progression of chronic hepatitis, suggesting that the mutation of AA at residue 70 (from arginine to glutamine or histidine) occurs in the natural course of chronic HCV infection [Kobayashi et al., 2010a]. Several recent studies have reported a higher prevalence of the mutant-type AA at residue 70 in patients who have the TG/GG genotype of genetic polymorphism of rs8099917 near the *IL28B* gene, which is associated with an unfavorable response to the combination therapy with PEG-IFN and ribavirin, than in patients who have the TT genotype [Abe et al., 2010; Kobayashi et al., 2010b]. These reports suggest that the mutation of AA residue 70 of the HCV core region may occur more frequently in patients with the TG/GG genotype. Especially, the induction of this mutation may occur easily in patients who underwent PEG-IFN/ribavirin combination therapy and failed to clear HCV (non-sustained virologic response), wherein HCV obtained a resistance to combination therapy.

Mutation at HCV core region residue 70 has reportedly been associated with a hepatocarcinogenesis and an insulin resistance [Akuta et al., 2007b, 2009; Nakamoto et al., 2010]. In addition, a recent study reported that patients who have both the TG/GG genotype of rs8099917 near the *IL28B* gene and the mutant-type AA at residue 70 of the HCV core region have shown further resistance even to the triple therapy with telaprevir, PEG-IFN, and ribavirin [Akuta et al., 2010]. It is, therefore, important to clarify whether PEG-IFN/ribavirin combination

therapy induces the mutation of the HCV core region residue 70 in patients who failed to eradicate HCV, and whether genetic polymorphism near the *IL28B* gene are correlated with this mutation. If so, some patients should not undergo the current standard combination therapy in order to prevent the acquisition of the resistance (i.e., mutation at residue 70).

The present study investigated the effects of the combination therapy with PEG-IFN and ribavirin and genetic polymorphisms near the *IL28B* gene on the mutation of HCV core region residue 70 in patients who failed to achieve a sustained virologic response.

## PATIENTS AND METHODS

### Patients and Treatment

Three hundred and forty six patients with chronic hepatitis C who had been infected with HCV genotype 1b (as assessed by amplification of core-gene sequences with polymerase chain reaction (PCR) using genotype-specific primers [Ohno et al., 1997]) and pretreatment HCV-RNA level of  $>100 \times 10^3$  IU/ml [as assessed by a quantitative PCR assay (Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA)] underwent antiviral combination therapy with PEG-IFN and ribavirin between January, 2007 and December, 2009 at the Ogaki Municipal Hospital or the Nagoya University Hospital. Of these patients, 19 patients dropped out and their outcome could not be defined. Among the remaining 327 patients, 274 patients who gave written informed consent for genetic analyses were enrolled to the study (Fig. 1). No patients were coinfecting with hepatitis B virus or human immunodeficiency virus.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The initial doses of PEG-IFN and ribavirin and the dose reductions were according to the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of the treatment. Some patients had an extended treatment duration of up to 72 weeks. In some patients, the treatment was discontinued before 48 weeks because they had a low likelihood of achieving a sustained virologic response, when serum HCV-RNA was positive 24 weeks after starting the therapy. The outcomes of the combination therapy were classified as a sustained virologic response when serum HCV-RNA became undetectable during the treatment and remained undetectable for 6 months after the treatment ended (i.e., eradication of HCV), a relapse when the serum HCV-RNA became undetectable during the treatment period but returned detectable after the treatment, and no-response when the serum HCV-RNA remained detectable during and after the treatment period.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Written



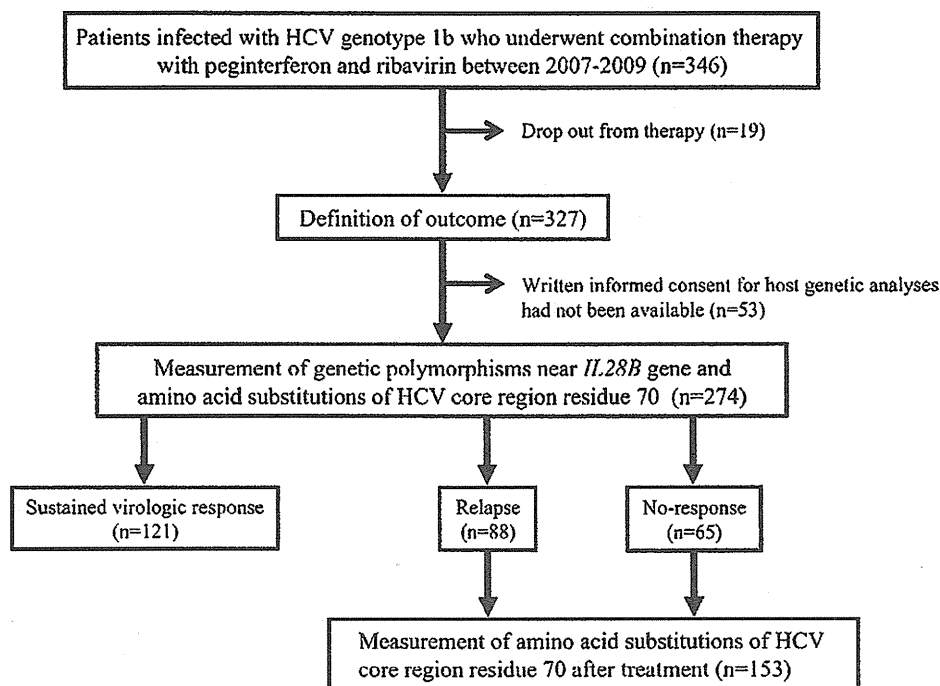


Fig. 1. Schematic representation of the study design.

informed consent was obtained from all patients prior to the study for the measurement of genetic polymorphism of rs8099917 near *IL28B* gene and AA substitution of HCV core region residue 70, and for the use of the laboratory data.

**Measurements of Genetic Polymorphism Near the *IL28B* Gene and Amino Acid Substitution of the HCV Core Region Residue 70**

Genotyping of polymorphisms of the rs8099917 locus near the *IL28B* gene was carried out in all 274 patients using a Taqman SNP assay (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C\_11710096\_10, Applied Biosystems).

The AA at residue 70 of the core region of HCV was measured before the treatment in all patients. In patients who failed to achieve a sustained virologic response, that is, patients who showed a relapse or no-response, the AA identity was measured at residue 70 after the treatment and compared pre- to post-treatment AA identity at this residue (Fig. 1). The AA at residue 70 after the treatment was measured in serum samples obtained at the end of treatment in patients who showed no-response. In patients with a relapse, it was measured in serum samples obtained upon the reappearance of HCV-RNA after the completion of the therapy. The AA identity was analyzed by direct nucleotide sequencing according to

a previous report [Akuta et al., 2007c]. The primer pairs used for PCR for direct sequencing the HCV core region were 5'-GCCATAGTGGTCTGCGGAAC-3' (outer, sense primer), 5'-GGAGCAGTCCTTCGTGACATG-3' (outer, antisense primer), 5'-GCTAGCCGAGTAGTGTT-3' (inner, sense primer), and 5'-GGAGCAGTCCTTCGTGACATG-3' (inner, antisense primer).

**Statistical Analysis**

The chi-square test was used to analyze the differences in percentages between groups.

**RESULTS**

**Patient Characteristics and the Outcome of the Combination Therapy**

The characteristics of study patients are shown in Table I. The study patients comprised 139 males (50.7%) and 135 females (49.3%), with a mean age of 58.0 ± 10.4 years. The grade of liver fibrosis according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994] was F0 in 31 patients (11.6%), F1 in 122 patients (45.9%), F2 in 75 patients (28.2%), and F3 in 38 patients (14.3%). Analysis of the genetic polymorphism of the rs8099917 near the *IL28B* gene indicated 202 patients (73.7%) had the TT genotype, three patients (1.1%) had the GG genotype, and the remaining 69 patients (25.2%) were TG heterozygous. Before the treatment, 204 patients (74.4%)

TABLE I. Baseline Characteristics of the Study Patients (n = 274)

Age (years)	55.9 ± 11.2
Sex (female/male)	135 (49.3)/139 (50.7)
Body weight (kg)	58.0 ± 10.4
Alanine aminotransferase (IU/L)	64.5 ± 56.3
Aspartate aminotransferase (IU/L)	53.7 ± 42.2
Gamma-glutamyl transpeptidase (IU)	49.7 ± 48.5
Alkaline phosphatase (IU/L)	267.9 ± 100.6
Albumin (g/dl)	4.07 ± 0.38
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4933 ± 1331
Hemoglobin (g/dl)	14.0 ± 1.4
Platelet count (×10 <sup>3</sup> /μl)	164 ± 50
Liver histology-activity (A0/A1/A2/A3) <sup>a</sup>	2 (0.7)/147 (55.3)/99 (37.2)/18 (6.8)
Liver histology-fibrosis (F0/F1/F2/F3) <sup>a</sup>	31 (11.6)/122 (45.9)/75 (28.2)/38 (14.3)
HCV-RNA concentration (log <sub>10</sub> IU/ml) <sup>b</sup>	6.34 ± 0.54
Genetic polymorphisms near the <i>IL28B</i> gene (TT/TG/GG) <sup>b</sup>	202 (73.7)/69 (25.2)/6 (2.2)
Amino acid at HCV core 70 (wild type/mutant type/both) <sup>c</sup>	204 (74.4)/64 (23.4)/6 (2.2)
Response (SVR/relapse/NR)	121 (44.2)/88 (32.1)/65 (23.7)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no-response.

Percentages are shown in parentheses.

<sup>a</sup>Liver biopsy was not performed in eight patients.

<sup>b</sup>rs8099917 genetic polymorphism

<sup>c</sup>Before the treatment.

carried HCV with the wild-type AA at residue 70 of the HCV core region, 64 patients (23.4%) carried the mutant-type AA at residue 70, and both the wild-type AA and the mutant-type AA were identified at residue 70 in the remaining six patients (3.5%).

As a final outcome, 121 patients (44.2%) achieved a sustained virologic response, 88 patients (32.1%) relapsed, and the remaining 65 patients (23.7%) showed no-response (Fig. 1). Treatment was discontinued before 48 weeks in 11 of 65 patients who showed no-response because HCV-RNA remained detectable in serum 24 weeks after starting the therapy. The identity of the AA 70 of the core region of HCV was determined after the treatment in serum obtained at the discontinuation of the therapy in these 11 patients. Table II shows the association between the genetic polymorphisms of the rs8099917 near the *IL28B* gene, the AA substitutions of the HCV core region residue 70, and the outcome of the combination therapy. The wild-type AA was more frequently identified at residue 70 in patients with the TT genotype in comparison to those with the TG/GG genotype (82.2% vs.

52.8%,  $P < 0.0001$ ). The rate of a sustained virologic response was significantly higher in patients with the TT genotype than those with the TG/GG genotype (107 of 202 patients, 53.0% vs. 14 of 72 patients, 19.4%,  $P < 0.0001$ ), as well as being higher in patients carrying HCV with the wild-type AA at residue 70 of the core region than those with the mutant-type AA at this residue (101 of 204 patients, 49.5% vs. 19 of 64 patients, 29.7%,  $P = 0.0083$ , one patient had both the wild-type and the mutant-type AAs).

#### Comparison of the Amino Acid at Residue 70 of the HCV Core Region Before and After the Combination Therapy in Patients Who Showed a Relapse or No-Response

Table III shows the comparison of the AA at residue 70 of the HCV core region before and after the combination therapy in patients who showed a relapse or no-response, according to the genetic polymorphisms of the rs8099917 near the *IL28B* gene. In three of five

TABLE II. Association Between the Genetic Polymorphisms Near the *IL28B* Gene, the Amino Acid at the HCV Core Region Residue 70, and the Final Outcome of Peginterferon/Ribavirin Combination Therapy

Genetic polymorphism of rs8099917 near <i>IL28B</i> gene	Amino acid at residue 70 of the HCV core region		
	Wild type (n = 204)	Mutant type (n = 64)	Wild type + mutant type (n = 6)
TT (n = 202)	166 (92/60/14)	31 (14/9/8)	5 (1/2/2)
TG/GG (n = 72)	38 (9/9/20)	33 (5/7/21)	1 (0/1/0)

Outcomes of the combination therapy with peginterferon and ribavirin are shown in parentheses as sustained virologic response/relapse/no-response.

TABLE III. Amino Acid Substitutions of HCV Core Region Residue 70 Before and After the Combination Therapy With Peginterferon and Ribavirin in No-Responders or Relapsers

Amino acid at HCV core region residue 70	After treatment		
	Wild type	Wild + Mutant	Mutant type
Before treatment			
(A) Genetic polymorphisms near the <i>IL28B</i> gene (rs8099917): TT (n = 91)			
No-responders (n = 24)			
Wild type (n = 14)	13	1	0
Wild + mutant (n = 2)	0	0	2
Mutant type (n = 8)	0	0	8
Relapsers (n = 71)			
Wild type (n = 60)	60	0	0
Wild + mutant (n = 2)	0	1	1
Mutant type (n = 9)	0	0	9
(B) Genetic polymorphisms near the <i>IL28B</i> gene (rs8099917): TG/GG (n = 57)			
No-responders (n = 41)			
Wild type (n = 20)	19	1	0
Wild + mutant (n = 0)	0	0	0
Mutant type (n = 21)	0	0	21
Relapsers (n = 17)			
Wild type (n = 9)	9	0	0
Wild + mutant (n = 1)	0	1	0
Mutant type (n = 7)	0	0	7

HCV, hepatitis C virus.

patients in whom both the wild-type and mutant-type AAs had been identified at residue 70 of the HCV core region before treatment, only the mutant-type AA was identified at this residue after the treatment. All three of these patients (two no-responders and one relapser) had the TT genotype of the rs8099917. Both the wild-type and mutant-type AAs were identified at residue 70 after the treatment in two no-responders in whom only the wild-type AA had been identified before the treatment. One of them had the TT genotype at the rs8099917 and the other patient was TG heterozygous. No change in the HCV core region residue 70 was found after the treatment in patients with the mutant-type AA at this residue before the treatment.

### DISCUSSION

The present study investigated whether the combination therapy with PEG-IFN and ribavirin causes the mutation of residue 70 of the HCV core region, and whether the genetic polymorphisms of the rs8099917 locus near the *IL28B* gene influence this mutation. It is thought to be important to verify this issue, because it may be advisable to avoid the treatment of patients who have the TG/GG genotypes by the combination therapy with PEG-IFN and ribavirin so as to avoid an acquisition of the further resistance to emerging new therapies against HCV, as well as to avoid a potential enhancement of hepatocarcinogenesis.

The mutation of the AA at residue 70 was not observed before and after the treatment in all patients who had failed to achieve a sustained virologic response. The mutant-type AA was identified solely at

residue 70 after the treatment in three patients who had both the wild-type and the mutant-type AAs at residue 70 before the treatment. This could be due to the selection of HCV strains with the mutant-type AA at residue 70 by the combination therapy with PEG-IFN and ribavirin, as reported previously [Kurbanov et al., 2010]. In two patients who carried only the wild-type AA before the treatment, the HCV with the mutant-type AA at residue 70 was also detected with the persistence of the wild-type AA at this residue after the treatment. The very minor HCV strain with the mutant-type AA at residue 70 which were not detected before the treatment may have been detected after the treatment due to the reduction of HCV with the wild-type AA at residue 70 by the combination therapy. Indeed, HCV with the mutant-type AA at core region residue 70 was not detectable in serum 6 months after the end of the combination therapy, suggesting that it returned to being a very minor population (data not shown). These two phenomena were observed in patients with both the TT genotype of the rs8099917, that is associated with a favorable response to the combination therapy and those with the TG/GG genotypes that is associated with an unfavorable response, without difference in the prevalence according to the genetic polymorphisms at the rs8099917 near the *IL28B* gene.

In conclusion, PEG-IFN/ribavirin combination therapy does not appear to induce the mutation of the AA at the HCV core region residue 70 regardless of the genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. The combination therapy can be attempted regardless of the genetic polymorphisms near the *IL28B* gene in

treatment-naïve patients without the anxiety for the acquisition of the further resistance to the antiviral therapy. However, future studies should be undertaken to confirm the absence of the mutation at residue 70 of the HCV core region induced by the combination therapy with PEG-IFN and ribavirin. In addition, the effect of the genetic polymorphisms near the *IL28B* gene on the mutation of the AA at the HCV core region residue 70 should be investigated in the long-term observation of the natural course of chronic hepatitis C.

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