were as follows: hepatitis B surface antigenemia; human immunodeficiency virus infection; chronic alcohol abuse; autoimmune liver disease; and history of phlebotomy. Degree of inflammatory activity and stage of fibrosis were assessed according to the histological scoring system of METAVIR[22] by pathologists who were blinded to clinical data. Hepatic iron storage was graded with Perls' Prussian blue stain on a scale of 0–4 as follows: grade 0, iron granules absent or barely discernible × 400; grade 1, barely discernible × 250 or easily discernible × 400; grade 2, discrete granules resolved × 100; grade 3, discrete granules resolved × 25; grade 4, massive visible × 10, or naked eye.[23] Patients received subcutaneous injections of PEG-IFN (1.5 μg/kg) once per week plus oral RBV (600 mg for < 60 kg, 800 mg for 60–80 kg, 1000 mg for > 80 kg) daily, in accordance with Japanese guidelines.[24] Sustained virological response (SVR) was defined as undetectable HCV RNA at 24 weeks after withdrawal of treatment. The other patients were considered to have non-SVR. This study was approved by the Nagoya University Hospital ethics committee, and was conducted in accordance with the principles of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients.

Genomic analysis. Genomic DNA was isolated from peripheral blood leukocytes by standard procedures. HFE mutations at position 63 (histidine to aspartic acid, H63D) and at position 65 (serine to cysteine, S65C) were determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism method with *Bcl*-I (for H63D) and *Hinf*-I (for S65C), as described previously.[11] Detection of the rs8099917 single-nucleotide polymorphism (SNP) in interleukin 28B (IL28B) was also performed by real-time PCR using custom-designed primers and probes (Taqman SNP Genotyping Assays; Applied Biosystems, Foster, CA, USA). IL28B SNP rs8099917 was amplified and the results were analyzed by real-time PCR in a thermal cycler (7300 Real-time PCR System; Applied Biosystems).

Statistical analysis. Quantitative variables were compared by the Mann–Whitney *U*-test, and are expressed as median values with interquartile range. The distribution of qualitative variables was compared by the χ^2-test or Fisher's exact test, as appropriate. Multiple logistic regression analysis was performed in order to determine the factors contributing to SVR. *P*-values less than 0.05 were considered to be statistically significant and $0.1 > P \geqq 0.05$ were referred to as marginally significant. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Influence of H63D mutation on clinical characteristics. Of the 251 patients with CHC, 14 carried the H63D mutation (5.6%); all were heterozygous. Patient characteristics according to the presence of H63D mutation are shown in Table 1. No significant differences in clinical, laboratory, or histological data were observed between H63D heterozygotes and wild-type patients, except that hemoglobin levels were higher in H63D heterozygous patients. There was no correlation between the presence of H63D mutation and genotype of IL28B. Histological evaluation of hepatic iron deposition using Perls' Prussian blue method in nine H63D heterozygous patients demonstrated low levels of iron staining in the liver: six had grade 0, two had grade 1, and one had grade 2 positive staining.

Influence of H63D mutation on response to PEG-IFN plus RBV therapy in patients with CHC. Of the 251 patients who received PEG-IFN plus RBV therapy, 116 (46.2%) achieved SVR. Univariate analysis identified seven factors that influenced SVR: younger age ($P = 0.003$); genotype 2 ($P < 0.001$); lower viral load (< 1000 KIU/mL) ($P < 0.001$); higher white blood cell count ($P = 0.039$); lower stage of fibrosis ($P = 0.002$); genotype TT of IL28B rs8099917 ($P < 0.001$); and presence of H63D heterozygosity ($P = 0.012$)

Table 1 Comparison of characteristics according to the presence of the H63D mutation

	H63D heterozygotes ($n = 14$)	Wild type ($n = 237$)	*P*-value
Sex (male/female)	11/3	132/105	0.093
Age (years)	53 (44–56.8)	56 (47–63)	0.148
Body mass index (kg/m²)	21.6 (19.6–24.4)	22.1 (20.3–24.4)	0.282
Alanine aminotransferase (IU/L)	35 (28.2–50.1)	38 (28–68)	0.520
Aspartate aminotransferase (IU/L)	53.5 (26.5–103.3)	46 (29–80)	0.748
Gamma-glutamyl transpeptidase (IU/L)	49 (25–62)	32.0 (21.0–62.3)	0.327
Total bilirubin (mg/dL)	1.00 (0.78–1.03)	0.8 (0.6–1.0)	0.247
Albumin (g/dL)	4.1 (4.0–4.3)	4.1 (3.8–4.3)	0.292
Serum ferritin (ng/mL)	110 (82–143)	89 (40–180)	0.580
White blood cell counts (/mm³)	4550 (3950–4950)	4600 (3900–5400)	0.652
Hemoglobin (g/dL)	14.7 (13.5–15.8)	13.7 (13.0–14.8)	0.040
Platelet counts (/mm³)	177 000 (135 000–158 000)	164 000 (132 000–203 000)	0.468
HCV genotype (1/2)	12/2	181/56	0.326
Viral load (KIU/mL) (< 1000/ 1000)	5/9	56/181	0.334
Stage of inflammatory activity (0/1/2/3)	0/6/5/0	4/96/82/4	0.592
Stage of fibrosis (0/1/2/3/4)	0/7/4/0/0	28/74/50/26/7	0.381
IL28B rs8099917 genotype (TT/TG or GG)	10/4	175/62	0.538

Data is expressed as median values and (interquartile range).

HCV, hepatitis C virus; IL28B, interleukin 28B.

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Table 2 Characteristics of SVR and non-SVR in patients with chronic hepatitis C

	SVR (*n* = 116)	non-SVR (*n* = 135)	*P*-value
Sex (male/female)	69/47	74/61	0.456
Age (years)	54 (43.3–60)	58 (49–64.5)	0.003
Body mass index (kg/m²)	22.0 (20.4–23.8)	22.3 (20.5–24.9)	0.140
Alanine aminotransferase (IU/L)	34 (26–63)	39.5 (29.3–69.8)	0.094
Aspartate aminotransferase (IU/L)	46 (26–94)	46 (29.3–77)	0.896
Gamma-glutamyl transpeptidase (IU/L)	31 (21–53)	34 (21.8–64.5)	0.248
Total bilirubin (mg/dL)	0.80 (0.60–1.00)	0.8 (0.6–1.0)	0.736
Albumin (g/dL)	4.1 (3.8–4.3)	4.0 (3.8–4.2)	0.109
Serum ferritin (ng/mL)	95.5 (40–178)	86.4 (44.3–161.5)	0.661
White blood cell counts (/mm³)	4800 (4000–5800)	4550 (3900–5200)	0.039
Hemoglobin (g/dL)	14.0 (13.0–15.0)	13.7 (13.0–14.6)	0.136
Platelet counts (/mm³)	167 000 (137 000–206 000)	160 000 (131 000–199 000)	0.499
HCV genotype (1/2)	76/40	117/18	< 0.001
Viral load (KIU/mL) (< 1000/≥ 1000)	42/74	18/117	< 0.001
Stage of inflammatory activity (0/1/2/3)	1/49/38/1	3/53/49/3	0.344
Stage of fibrosis (0/1/2/3/4)	13/45/21/6/1	15/36/33/20/6	0.002
H63D mutation (present/absent)	11/105	3/132	0.012
IL28B rs8099917 genotype (TT/TG or GG)	101/15	82/53	< 0.001

Data are presented as median values and (interquartile range).
HCV, hepatitis C virus; IL28B, interleukin 28B; SVR, sustained virological response.

Table 3 Factors associated with SVR in patients with chronic hepatitis C by multivariate analysis

Factor	Category	OR (95%CI)	*P*-value
IL28B rs8099917	Genotype TT	7.089 (2.961–16.976)	< 0.001
Age (years)	Younger age (each 1 year decrease)	1.062 (1.030–1.094)	< 0.001
HCV genotype	Genotype 2	2.978 (1.357–6.535)	0.001
Viral load (KIU/mL)	< 1000	4.631 (1.937–11.073)	0.007
H63D mutation	Present	5.281 (0.994–28.072)	0.051

Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on multivariate logistic regression analysis are shown.
CI, confidence interval; HCV, hepatitis C virus; IL28B, interleukin 28B; OR, odds ratio; SVR, sustained virological response.

(Table 2). On multivariate analysis, IL28B genotype (TT; $P < 0.001$), age (each 1-year decrease; $P < 0.001$), HCV genotype (2; $P = 0.001$), viral load (< 1000 KIU/mL; $P = 0.007$) and H63D mutation (heterozygosity; $P = 0.051$) were significant or marginal independent predictors of SVR (Table 3). In our study population, the SVR rate among patients with genotype TT in IL28B was 55.8% and that for genotypes TG and GG was 21.7%. In the analysis of IL28B SNP, H63D mutation was considered to improve prediction of SVR, as among 14 patients carrying the H63D mutation, the 10 patients with genotype TT in IL28B all achieved SVR. The characteristics of the patients are shown in Table 4.

Table 4 Characteristics of patients with both H63D mutation in HFE and genotype TT in IL28B (rs8099917)

Patient	Sex	Age (years)	HCV genotype	Viral load (KIU/mL)
1	Male	52	1	106
2	Male	57	2	342
3	Male	44	1	2080
4	Male	50	1	4950
5	Female	55	1	3220
6	Male	25	1	5100
7	Male	70	1	100
8	Female	28	2	2650
9	Male	66	1	2900
10	Female	57	1	250

HCV, hepatitis C virus; IL28B, interleukin 28B.

Discussion

The clinical penetrance of the HFE gene mutation is low. A large cohort study reported that, even in male carriers of the homozygous C282Y mutation, which is the most common genotype resulting in HH, only 28% of the subjects showed the clinical expression of iron overload, and in female patients, only 1% developed iron overload-related symptoms, possibly due to iron loss caused by menstruation.[25] With regard to the H63D mutation, its effect on body iron stores was much milder than the C282Y mutation and, particularly in heterozygotes, iron overload was scarcely observed.[20,26,27] However, in patients with chronic HCV infection, which is often associated with hepatic iron deposition, it was unclear whether HFE mutations were associated with iron

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overload.[11–18] These conflicting results may have been due to genetic and environmental factors that affect iron accumulation. The Hemochromatosis and Iron Overload Screening (HEIRS) Study group indicated the importance of considering the effects of ethnic differences on serum iron markers.[28] In this study, H63D heterozygotes with CHC did not show any differences in laboratory data, except for hemoglobin levels, as compared with wild-type patients (Table 1). In Korea, Won *et al.* showed no correlation between serum ferritin levels and the H63D heterozygous state; however, the number of subjects was small.[21] This study also confirmed the lack of correlation between the presence of H63D heterozygosity and serum ferritin levels in Asia. Histological examination of liver iron stores substantiated the notion that H63D heterozygosity had no influence on hepatic iron deposition. We did not analyze the C282Y mutation because of its very low prevalence in Asian countries, including Japan, as reported previously.[10,20,29–33] On the other hand, we assessed the frequency of the S65C mutation, as no reports on this mutation have been published in Japan. However, in line with other studies from Asia,[31–33] none of the present subjects carried the S65C mutation.

The effects of H63D heterozygosity on fibrosis progression in patients with chronic HCV infection also remain unclear.[11,12,14–18] Although we did not find a correlation between the H63D mutation and fibrosis stage, some reports have suggested that the H63D heterozygote state would accelerate progression of fibrosis.[16–18] In these studies, the H63D heterozygotes showed increased hepatic iron deposition. Iron-related oxidative stress can increase hepatic inflammation and promote fibrosis,[2] and the authors of these studies suggested that progression of hepatic fibrosis was due to hepatic iron overload. Our study showed no evidence of excess iron deposition, which explains why H63D heterozygotes showed no correlation with fibrosis stage.

This study demonstrated that H63D heterozygotes with CHC show a good response to PEG-IFN plus RBV therapy. The positive effect of H63D mutation on IFN responsiveness in patients with CHC was reported in 2004.[19] The Hepatitis C Anti-Viral Long-Term Treatment to Prevent Cirrhosis (HALT-C) trial, a large and well-designed study, showed the same results in advanced CHC patients who received PEG-IFN plus RBV therapy.[11] Hepatic iron overload was shown to be associated with lower response rates to interferon therapy[4–8] and recent meta-analysis confirmed the beneficial effects of phlebotomy on response to interferon therapy.[34] Whereas H63D mutation is a potential cause of hepatic iron overload, a better treatment response was observed in H63D mutation carriers. The present study does not confirm the effects of H63D mutation on iron overload, but supports the positive effect of this mutation on IFN responsiveness. The precise mechanism of this effect remains unclear. The immunologic functions of HFE mutations were thought to play an important role.[11,19] The HFE gene is closely linked to the human leukocyte antigen-A3 locus, and some immunological differences, such as decreased cell-surface expression of major histocompatibility complex class I[35] and elevated monocyte chemoattractant protein-1 levels,[36] have been reported in HFE mutation carriers, as compared with wild-type patients. Further studies on the association between HFE mutations and immunologic functions in CHC patients are necessary in order to clarify these issues.

Factors associated with SVR have been widely studied, and several factors, including HCV genotype, viral load, mutations in HCV core and NS5A region, sex, liver fibrosis, ethnicity and age, have been suggested to play important roles in IFN responsiveness. Recently, three genome-wide association studies validated the correlation between SVR and SNP near the IL28B gene.[37–39] A minor allele in this genetic variation strongly predicts the failure of PEG-IFN plus RBV therapy; the SVR rate for our patients with this minor IL28B allele was 21.7%, and the positive predictive value of the major IL28B allele for SVR was 55.8%. Thus, another factor was considered to be necessary for improving SVR prediction.

Our study suggests that the H63D mutation is correlated with the outcome of IFN treatment, and combining IL28B SNP and H63D mutation may improve the predictive value for SVR. All patients with both H63D mutation and the major allele (genotype TT) of IL28B (rs8099917) achieved SVR. Although the frequency of H63D mutations is low in the Japanese population, it is much higher in North American and European populations; this correlation may therefore be more useful as a predictive factor for SVR in Western regions.

In conclusion, 5.6% of patients with CHC in Japan carry the H63D mutation in the HFE gene, and the S65C mutation was not detected. The H63D mutation had no influence on hepatic iron overload, but the presence of this mutation was associated with a good response to PEG-IFN plus RBV therapy.

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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 ± 176.7 vs. 1279.4 ± 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); genotype3a; double-stranded RNA-dependent protein kinase (PKR); E2-PePHD; NS5A-ISDR

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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 (elF2-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 hemophiliac patients infected with HCV (mean age 45.5 ± 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°C until required.

TABLE I. Clinical Characteristics

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

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

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′-AGAAYSCCCCACYRCTGAGGACAT-3′ and antisense 5′-GGCAGTTTCTCYTCCTCAGCACT-3′. For NS5A-ISDR: sense 5′-GCCCAACACACACAACTA-CACGCGCGC-3′ and antisense 5′-CTTTTCCTCCGAG-GAGG-3′. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°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′-GTGGTA-CCATGAGCGGCGATTGGCTGCGTATCATC-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

```
NZL1   PSLKATCQTHRPHPDAELVDANLLWRQEMGSNITRVESET  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------H---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 ($\times 10^4$/mm^3)	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, interferes 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 ($\times 10^4$/mm^3)	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 region70 ($P=0.0267$) 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)-α and ribavirin combination therapy is standard treatment for patients with chronic hepatitis C, but it eradicates HCV for only 50% of patients with genotype1 (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

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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-α 2b and ribavirin combination therapy.

Methods

A total of 432 patients with chronic hepatitis C genotypelb 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-α 2b (1.5 μg/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 μl of serum with a commercial kit (QIAamp Viral RNA Kit, Qiagen, Valencia, CA, USA) and dissolved in 50 μl 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 μl PCR reaction contained 100 nM of each primer, 1 ng of template cDNA, 5 μl of GeneAmp 10 × PCR buffer, 2 μl of dNTPs and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for the core region were sense, 5′-GGGAGGTCTCGTA

Table 1. Baseline characteristics of the patients

Clinical characteristics	*N* = 299
Age (years)	55.9 ± 10.3
Sex: male/female	157/142
AST (IU/L)	58.7 ± 48.9
ALT (IU/L)	69.8 ± 66.9
Platelet count (10^4/μL)	16.6 ± 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 ± 12.7

Data are expressed as mean ± 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′-TGGATGGAGTGCGGTTGCA CAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGG 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′-TACGCCGGGGGTCAKTRGGGCCCCA-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 μl of whole blood using a commercial kit (QIAamp DNA Blood mini Kit, Qiagen) and was dissolved in 50 μl 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 ± standard deviation. The paired *t*-test, the χ^2-test and Fisher's exact test were used

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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 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.

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
P value = 0.0324		
	HCV core region 70	
	Non-Q	Q
TG/GG	35	45
TT	179	40
P value < 0.0001		
	HCV core region 91	
	L	M
TG/GG	46	34
TT	164	55
P 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.

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;

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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 ($\times 10^4$/mm^3)	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.

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.

$P=0.0001$). 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 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.

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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-α 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 region70 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.

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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 reponse.

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 coinfected 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

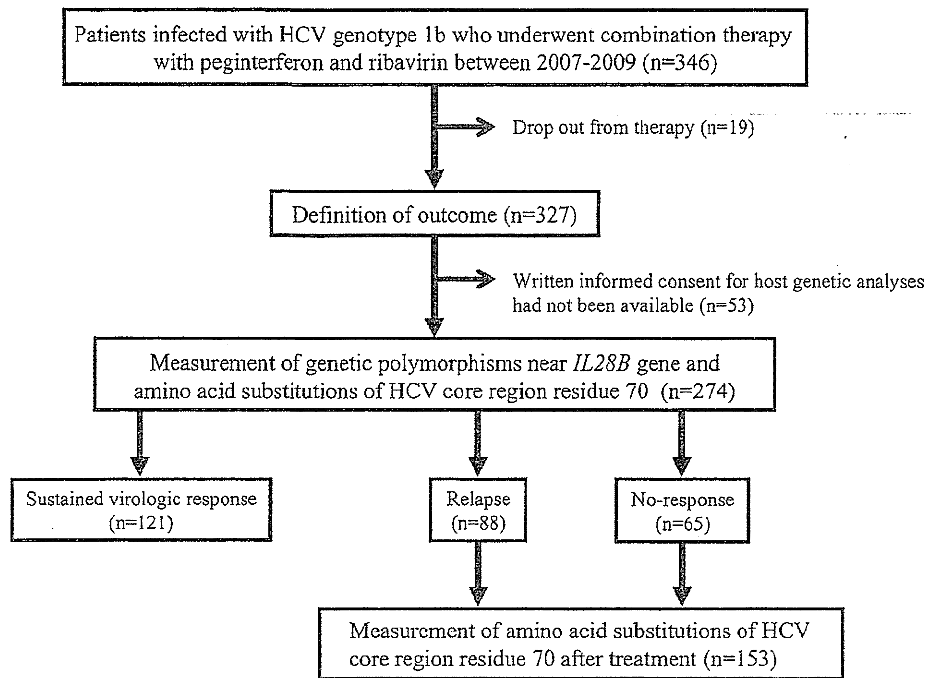

Fig. 1. Schematic representation of the study design.

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%)