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Original Article

High sensitivity assay using serum sample for *IL28B* genotyping to predict treatment response in chronic hepatitis C patients

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Aim: Recent human genome-wide association studies (GWAS) revealed a strong association between *IL28B* gene variation and the pegylated interferon-α with ribavirin (PEG-IFN-α/RBV) treatment response in chronic hepatitis C patients. Two single nucleotide polymorphisms (SNP), rs8103142 and rs11881222 located in the *IL28B* gene, were found in significant association with the viral clearance. The present study employed these SNPs to develop a new accessible screening method allowing identification of potential non-responders before starting the therapy.

Methods: Primer sets were designed to amplify rs8103142 and rs11881222 fragments from genomic DNA extracted from serum samples. This method was validated using microarray typing (GWAS) and applied for genotyping of 68 hepatitis C virus-infected patients with PEG-IFN-α/RBV treatment at baseline.

Results: In comparison with GWAS, the screening method showed 100% and 95.6% accuracy in typing of rs8103142 and rs11881222, respectively, indicating incomplete specificity but 100% of sensitivity in both. Genotyping by both SNP showed that 53 (77.9%), 14 (20.6%) and one (1.5%) of the patients were of major homozygous, heterozygous and minor homozygous type, respectively. The majority (85%) of homozygous patients exhibited response to therapy in contrast to heterozygous patients (29%). Among all genotyped only one case was found with the minor homozygous genotype which had late virological response to therapy before relapsing.

Conclusion: This study described a highly sensitive assay that can be useful in determining SNP genotypes as well as in predicting the response to IFN-based treatment.

Key words: hepatitis C virus, *IL28B*, pegylated interferon-α plus ribavirin, single nucleotide polymorphism typing.

INTRODUCTION

AN ESTIMATED 3% of the global population has been infected with hepatitis C virus (HCV). More than 170 million chronic carriers are at risk of liver cirrhosis and hepatocellular carcinoma.[1] Combination of pegylated interferon-α with ribavirin (PEG-IFN-α/RBV) is presently the most effective therapy for chronic HCV infection and is widely practiced as a standard treatment. However, not all patients are able to achieve viral clearance. A number of factors were associated with efficiency of the therapy; genotype of the infecting virus is one of them. HCV genotypes 1 and 4 respond poorly to PEG-IFN-α/RBV and often require therapy extended beyond 48 weeks to increase chances of sustained virological response (SVR).[2,3] More than half of the patients infected with genotype 1 could not elicit SVR and suffered from adverse side-effects such as influenza-like symptoms, depression, fever and anemia.[3,4]

Beside viral factors (genotype and viral load), host factors also influence the therapeutic outcome. Age, sex, body mass index and histological grade are considered to determine the individual's treatment regimen and

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outcome.[3,5,6] Recent studies have demonstrated a strong association between genetic variation of the *IL28B* gene on chromosome 19 with response to PEG-IFN-α/RBV therapy[7–9] and natural clearance of HCV.[10] *IL28B* is encoded as a cytokine known as interferon-λ3 (IFN-λ3) which is adjacent to *IL28A* (IFN-λ2) and near *IL29* (IFN-λ1) on chromosome 19. Genome-wide association studies (GWAS) exerted single nucleotide polymorphisms (SNP) in the *IL28B* region to be associated with response to IFN-based treatment among HCV-infected patients of Asian, African and European ancestry.[7–11] The two outstanding SNPs, rs12979860 and rs8099917 (located ~3 kb and 8 kb upstream of *IL28B*, respectively), have been found in strong association with the treatment response and several other SNPs within the *IL28B* gene were found in strong linkage disequilibrium with them (including rs8103142 and rs11881222).[7,9] The minor allele frequency of rs8099917 was significantly higher in the null virological response group (NVR) than the virological response (VR) group. By taking advantage of *IL28B* typing, it may be possible to predict NVR as well as SVR in order to tailor the most suitable treatment regimens.

The featured rs8103142 and rs11881222 SNP are located in the third exon and the third intron of the *IL28B* gene and have significant association with treatment response.[9,12] In this study, we took advantage of the two SNP to develop an accessible screening method. To allow determination of the polymorphism, the *IL28B* gene was performed using conventional polymerase chain reaction (PCR) on serum samples. The newly developed method was validated using microarray typing and enrolled clinical cohort of HCV-1b infected patients receiving PEG-IFN-α/RBV treatment.

METHODS

Patients

SERUM SAMPLES WERE collected from Japanese patients infected with HCV genotype 1b, after providing informed consent. All of the subjects had undergone a standard course of PEG-IFN-α/RBV therapy and had their virological response status established before this study.

Null virological response (NVR), transient virological response (TVR) and sustained virological response (SVR) were defined as previously described.[9] This study classified the response outcome into two categories: responders (including those with SVR and TVR) and non-responders (including patients with NVR). Sera were collected from 68 Japanese patients (age range 23–72 years) infected with HCV genotype 1b, who had received the IFN-based treatment. Additionally, serum samples of 23 patients who had genotyping profiles from a GWAS study[9] were included in this study to validate the PCR-based genotyping method outlined in this study.

DNA extraction and PCR amplification

Human genomic DNA was extracted from 100 μL of serum samples using a QIAamp blood kit according to the manufacturer's instruction (QIAGEN, Tokyo, Japan). To determine SNP genotype of rs8103142 and rs11881222 as well as rs8099917, specific sets of primers were designed within the *IL28B* gene and approximately 8-kb upstream of the non-coding region of the gene, respectively. Nested PCR amplified a short fragment containing rs8103142, rs11881222 or rs8099917 using specific primer pairs (Table 1). The PCR mixture comprised 1 μL DNA, 10 pmol of each primer, 160 μM of deoxyribonucleotide triphosphate, Mg^{2+} and 1.25 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The amplification conditions for the three SNP spanning regions was carried out under the following conditions: preincubation for 5 min at 94°C, followed by 40 cycles of three step holds (94°C for 30 s, 65°C for 30 s, and 72°C for 45 s) before final extension at 72°C for 7 min. The amplification products were subjected to electrophoresis in 2.5% agarose gel. Amplified fragments (~200 bp) were sequenced directly in both forward and reverse directions with Prism Big Dye (Fig. 2a) (Applied Biosystems) on an ABI 3100 DNA automated sequencer.

Nucleotide sequencing and data analysis

Genotyping was based on the chromatograms of nucleotide bases in positions 502 and 685 of rs8103142 and rs11881222, respectively (Figs 1,2b) (nucleotide numbering started at the ATG start codon of the *IL28B* gene). SNP typing of rs8099917 was analyzed in the same criteria. The superimposed chromatogram signals at those three SNP positions were interpreted as heterozygous genotype (Fig. 2b and Table 1). Specificity of this method was assessed by comparison with the SNP genotyping of rs8099917 carried out by GWAS.[9]

RESULTS

Validation of *IL28B* typing

ACCORDING TO THE GWAS, rs8099917, located upstream of the *IL28B* gene, is strongly associated with response to therapy of HCV-infected patients.[9] The present study employed PCR and sequencing-based

Table 1 Three single nucleotide polymorphisms (SNP) of *IL28B* used in this study. Allele genotype of each SNP was shown. Validation of SNP typing based on polymerase chain reaction and sequencing compared with genome-wide association studies showed the high specificity of this method

SNP	Location	Allele		Specificity	Primer name	Sequence	Direction	Size (bp)
		Major	Minor					
rs8103142	Exon 3	A	G	100% (23/23)	IL28B/ORF/392F	ttgtcagccatcctccaatcccatcagag	Forward	224
					IL28B/ORF/616R	gggccactacagagccaggtgagca	Reverse	
rs11881222	Intron 3	T	C	95.6% (22/23)	IL28B/ORF/579F	gcccaccctgccctcccagccctg	Forward	219
					IL28B/ORF/787R	ggtgaggggaggtgagggaacaggttg	Reverse	
rs8099917	Upstream	T	G	95.6% (22/23)	rs8099917/F	aagtaacactgttccttgtaaaagattcc	Forward	250
					rs8099917/R	cgctataattaaagatgtgggagatgcaa	Reverse	

Primer pairs and predicted product size of each amplified fragment were indicated.

assay to analyze rs8099917 in DNA extracted from serum samples. Of the 23 samples that have been genotyped in GWAS,[9] only one sample showed discrepancy in results obtained here by PCR and sequencing method (Table 1, Fig. 2c). Although rs8099917 has been associated with NVR and has high potential to use as the predictive factor for IFN-based treatment response, it is located far from the *IL28B* coding region. Two additional SNP located in the *IL28B* gene, rs8103142 and rs11881222, which were also significantly associated with the response to HCV therapy, may directly affect the anti-viral response of the IFN-λ3 function. Therefore, these SNP were proposed as a new useful tool to predict NVR or SVR.[9]

Due to the high homology of *IL28A* and *IL28B*, with 96% of amino acid identity,[12] two sets of primers were designed based on the variable regions (Fig. 1). The primers included unique nucleotide sequences of *IL28B* to specifically amplify fragments spanning rs8103142 and rs11881222. To validate the new method, serum of the 23 patients with known genotype identified by GWAS were used to test the specificity of the primer sets of this study. The single clear band of the respective target fragments amplified from serum DNA demonstrated that this method was highly sensitive and specific for the individual SNP (Fig. 2a). Genotyping by our new method showed results identical with the GWAS in 100% and 95.6% of rs8103142 and rs11881222, respectively. In total, of the 23 samples with GWAS data, 11 (47.8%), 10 (43.5%) and two (8.7%) samples were major homozygous, heterozygous and minor homozygous genotypes, respectively. Of the patients with major homozygous genotypes, 72.7% had achieved SVR to IFN-based therapy. However, most of heterozygous and minor homozygous patients, 70% and 100%, respectively, were non-responders. These results in correlation with GWAS, indicated that the method was highly specific and sensitive to amplify the respective SNP in the *IL28B* gene from serum samples.

IL28B typing in PEG-IFN-α/RBV treated patients

Hepatitis C virus genotype 1b infected patients treated with PEG-IFN-α/RBV were examined using this method. The *IL28B* genotype was determined based on both rs8103142 and rs11881222. With results identical for both SNP, most of the patients were of major homozygous genotype (77.9%) (Table 2). Patients with major homozygous allele exhibited higher prevalence

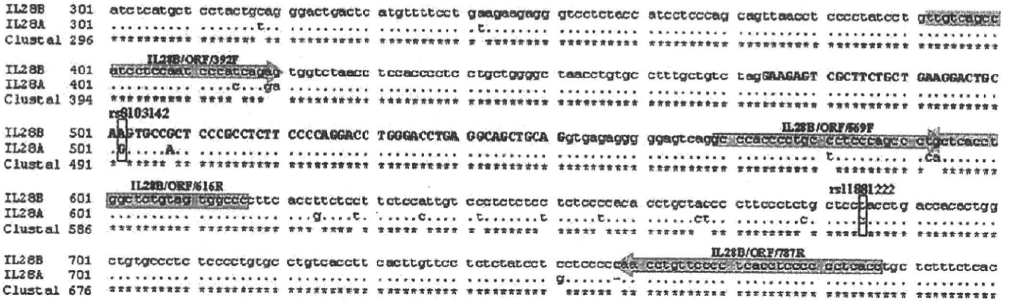

Figure 1 Nucleotide sequence alignment of *IL28B* and *IL28A* coding region. The gray arrows show the primer-specific site and direction. The numbering of the primers was according to the first AUG start codon. Upper case and lower case represent sequence of exon and intron, respectively. The boxes show the positions of rs8103142 and rs11881222.

of virological response (84.9%) compared to those with minor heterozygous or homozygous allele (5/15, 33.3%) (Table 2). Interestingly, none of the latter patients achieved SVR by PEG-IFN-α/RBV therapy for 48 weeks.

DISCUSSIONS

TREATMENT WITH PEG-IFN-α/RBV for 48 weeks is currently recommended for patients infected with HCV genotype 1, which is the most common genotype worldwide. However, less than 50% of HCV genotype 1 infected patients achieved SVR to IFN-based therapy.[13] It would be worthwhile to identify those patients who would not benefit from this regimen. In the present study, an effective tool to predict treatment response was developed and evaluated. Genetic variation in *IL28B*, rs8099917, was significantly associated with treatment response.[9] The rs8103142 and rs11881222 were found in linkage disequilibrium with the rs8099917. The rs8103142 is located in the exon 3 and an amino acid change of this location may have an effect on the function of the protein.[9] To achieve higher confidence of the genotyping, this method targets the two SNP (rs8103142 and rs11881222) located in relative proximity to each other within the *IL28B* gene.

Due to relatively low yield of intact human genomic DNA in serum samples, the method was designed for amplification of short DNA. Nested PCR was performed to amplify both alleles of chromosome 19 and increase the detection sensitivity (Fig. 2). Comparison of the results obtained by this screening method with GWAS data revealed its reliability and accuracy (100% accuracy with rs8103142 and 95.5% accuracy with rs11881222). It would be useful to detect the SNP using serum samples as the material is easier to collect, prepare and store in most clinical and research laboratories, however, it might be difficult to exclude possible contamination by nested PCR. Although a real-time detection PCR by TaqMan probe (rs8099917) might be more reliable (100% of the specificity), the sensitivity was lower than that of nested PCR (78.3% and 100%, respectively).

This method applied the conventional PCR with high sensitivity detection even using stored serum samples. The technique was easy and convenient to perform, as neither a high level of experience and training nor complicated analysis were required. Additionally, due to the fact that rs8103142 and rs11881222 are located close to each other, a single PCR (~395 bp using IL28B/ORF/392F and 787R) could be performed to detect genomic DNA from peripheral blood mononuclear cells (PBMC). Trial of the *IL28B* genotype with the single PCR using human DNA from PBMC was successful for 600 HCV-infected Japanese patients (data not shown), indicating that the results by the single PCR using PBMC might be more reliable than those by the nested PCR using serum samples. If patients need information on the *IL28B* SNP genotype, the results using PBMC should be presented.

Most of the major homozygous type patients (72.7%) achieved SVR while none of the heterozygous or minor homozygous patients had SVR to PEG-IFN-α/RBV for 48 weeks. Similar rates were observed when PEG-IFN-α/RBV-treated patients with no GWAS data were examined (Table 2). Although the minor allele was associated with NVR, one of the minor homozygous patients in this study exhibited late virological response and relapsed

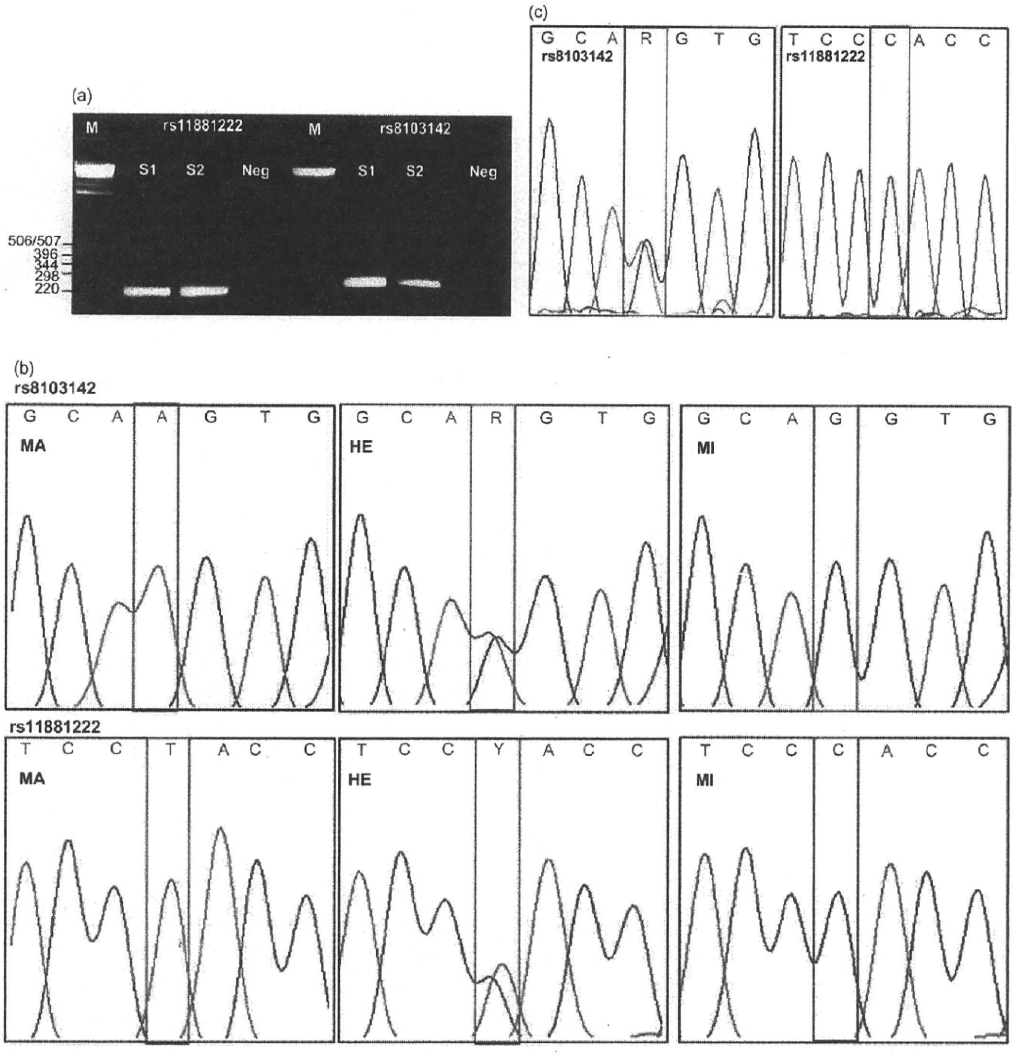

Figure 2 (a) High sensitivity and specificity of rs11881222 and rs8103142 typing. DNA marker (M) shows approximate product size of samples (S1 and S2) amplified from serum DNA. (b) Nucleotide sequence chromatogram of major homozygous (MA), heterozygous (HE) and minor homozygous (MI) of rs11881222 (lower panel) and rs8103142 (upper panel). (c) A discrepant data between rs8103142 (HE) and rs11881222 (MI).

after the end of 56 weeks of treatment. This may be due to other factors related to viral response.

Individuals with the minor allele genotype displayed significant association with low levels of *IL28A* and *IL28B* expression, which may be associated with the small proportion of SVR patients in heterozygous and minor homozygous groups.[8,9] IFN-λ, a new member of cytokines, has a distinct receptor complex and functions

Table 2 Correlation between clinical treatment outcome and SNP typing based on rs8103142 and rs11881222 in hepatitis C virus-infected patients

	MA (n = 53) (77.9%)	HE (n = 14) (20.6%)	MI (n = 1) (1.5%)	Total (n = 68)
Mean age, years (SD)	53.0 (10.8)	55.6 (10.9)	61 (0)	53.6 (10.6)
Sex (%)				
Female	23 (43.4)	6 (42.9)	0 (0)	29 (42.7)
Male	30 (56.6)	8 (57.1)	1 (100)	39 (57.4)
Response (%)	45 (84.9)	4 (28.6)	1 (100)	51 (75)
Non-response (%)	8 (15.1)	10 (71.4)	0 (0)	17 (25)

Single nucleotide polymorphism (SNP) typing based on the two SNP, clinical data as well as pegylated interferon-α plus ribavirin treatment response.
HE, heterozygous; MA, major homozygous; MI, minor homozygous genotype.

similar to type 1 IFN by inducing the JAK–STAT pathway.[12,14] The previous study reported that IFN-α induces IFN-λ expression,[15] suggesting that IFN-α and IFN-λ work synergistically. Consequently, lack of these cytokines or low levels of expression may lead to inadequate transduction signals and become resistant to therapy. Introduction of IFN-λ as an optional new antiviral agent may facilitate better response in the deficient group.

Among viral factors currently considered to be important in prognosis treatment outcome, viral load and genotype are important ones. Variations in the core and NS5A region of HCV genotype 1b and substitutions at amino acids 70 and 91 in the core region were associated with NVR,[16] in addition to host genetic factors may play a role in determining the susceptibility to HCV infection and viral clearance.[17] Most genetic variations presented in the innate immune system such as the interleukin family, tumor necrosis factor and the new member of IFN family (IFN-λ), *IL28A*, *IL28B* and *IL29*, were suggested to have an effect on spontaneous clearance and response to therapy.[12,18,19] Except for *IL28B*, none of them were proved to be significantly associated with response to antiviral therapy.[9,20,21] Hence, this study describes the new convenient approach to predict response to antiviral treatment using DNA from serum samples.

The high-sensitivity technique based on DNA genotyping in serum can be useful in determining SNP genotypes as well as in predicting the response to IFN-based treatment. Although it might be convenient for research purposes to determine *IL28B* SNP in serum, we have to be careful of showing the results in clinics. The method may be useful in IFN-based therapy not only for HCV patients, but potentially for patients infected with other viruses and receiving IFN-based therapy.

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MAJOR ARTICLE

Positive Selection of Core 70Q Variant Genotype 1b Hepatitis C Virus Strains Induced by Pegylated Interferon and Ribavirin

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Background. Approximately 20% of patients with hepatitis C virus (HCV) genotype 1b infection have nonresponse to the most current treatment, pegylated interferon with ribavirin. Mutations in the HCV core region were recently proposed to be associated with nonresponse. Our aim was to evaluate the viral factors associated with treatment failure.

Methods. HCV variants were determined directly and after cloning in 66 HCV-1b–infected Japanese patients and in 5 urokinase-type plasminogen activator transgenic severe combined immunodeficiency mice with human hepatocytes (chimeric mice), at baseline, during treatment, and after treatment.

Results. At baseline, glutamine at position 70 of the HCV core protein (70Q) was detected by direct sequencing in 20% of patients with virologic response and in 43.8% of patients with nonresponse. Among patients with nonresponse, who were examined during and after treatment, the prevalence of the 70Q substitution increased to 56.3%, which indicates that treatment-induced selection occurred in all patients with nonresponse who had 70Q quasispecies detectable by cloning. This observation was reinforced by the results from experimentally infected chimeric mice. Logistic regression analysis indicated that detection of 70Q quasispecies was associated with a statistically significantly increased risk of nonresponse (odds ratio, 15.1; $P = .004$) in the studied cohort.

Conclusion. Presence of the 70Q quasispecies at baseline was associated with an increased risk of treatment failure, as indicated by the positive selection of the 70Q clones induced by treatment with pegylated interferon with ribavirin. These results urge further investigation of the mechanisms of this association.

Hepatitis C is a global health problem that affects a significant portion of the world's population. There are ~170 million hepatitis C virus (HCV) carriers in the world, and 3–4 million new cases of infection are diagnosed each year [1]. The estimated burden of HCV infection exceeds 4 million persons in the United States, where hepatitis C represents the leading cause of cirrhosis and hepatocellular carcinoma, as well as the leading indication for liver transplantation [1]. The most effective of the currently available treatments for chronic hepatitis C, a combination of pegylated interferon alfa with ribavirin, does not provide sustained virologic response (SVR) in all patients who receive this treatment. Nonresponse and posttreatment relapse are the main forms of the treatment failure. Posttreatment relapse is often associated with a number of well-described adverse effects (such as hematologic abnormalities and neuropsychiatric symptoms) that require dose reduction and premature withdrawal from therapy [2]. It is thus important to predict individual response to the rather costly treatment with pegylated interferon–ribavirin, which is accompanied by many adverse effects. Factors that contribute to nonresponse have been extensively studied; of these, infection with HCV genotype 1 (vs 2 or 3), high

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Table 1. Primers Used for Polymerase Chain Reaction and Sequencing of Hepatitis C Virus Genotype 1b Genes

Primer	Sequence, 5′–3′	Gene	Nucleic acid position
HCV-264_F	AGGCCTTGTGGTACTGCCTGATAGG	Core	276–300
HCV-852_R	GAAGATAGAAAAGGAGCAACC	Core	872–852
HCV-6879_F	TCCATGCTCACCGAYCCCTCCC	NS5A	6861–6879
HCV-7203_R	AGTGGAGGGTTGTAATCCGGGCGTGCCCA	NS5A	7228–7203
HCV-7061_F	GGCAGGAGATGGGCGGTAACATC	Pre-V3	7042–7064
HCV-7563_R	GTAGACCAAGACCCGTCG	V3	7558–7544

NOTE. NS5A, nonstructural protein 5A; V3, variable region 3.

baseline viral load (vs low), viral kinetics characterized by late response to treatment (vs rapid), amino acid substitutions in nonstructural protein 5A (NS5A), and putative interferon sensitivity–determining region (ISDR; wild type or 1 substitution vs ≥2 substitutions) [3] have been found to be statistically significantly associated with nonresponse in a number of independent studies [4, 5]. A few recent studies have also indicated that amino acid substitutions in the HCV core protein (70Q and 91M vs 70R and 91L) are associated with treatment failure [6, 7].

The present study targeted HCV genomic regions that have been previously found to be associated with pegylated interferon–ribavirin treatment outcome. With the aim to investigate retrospectively whether treatment-related selection occurred in those genomic regions, we enrolled 66 patients with HCV genotype 1b infection whose treatment outcome had been assessed at the end of 6 months of posttreatment follow-up. Stored serum samples from all the patients were used for determining changes in HCV genetic heterogeneity at baseline, during treatment, at the end of treatment, and during posttreatment follow-up. Selection of the 70Q substitution was further investigated using an in vivo model of HCV infection in chimeric mice with livers repopulated by human hepatocytes.

MATERIALS AND METHODS

Patients. Sixty-six patients in Japan with chronic HCV-1b infection were enrolled in this study from a group of patients at 3 hospitals (Nagoya City University Hospital, Social Insurance Chukyo Hospital, and Nagoya City Johoku Hospital, Nagoya) who received therapy with pegylated interferon alfa-2b (1.5 μg/kg of body weight by subcutaneous injection once per week) plus ribavirin (600–1000 mg daily, according to body weight). Patients with other hepatitis virus infection or human immunodeficiency virus coinfection were not included in this study. The mean age of the population was 57 years, and 37 (57%) were men. The mean (± standard deviation [SD]) value for the alanine transaminase level was 66.3 (± 41.4) IU/L and for the platelet count was 17.6×10^4 ($\pm 17.3 \times 10^4$) platelets/μL. The mean HCV RNA level was 2041 (± 1557) kIU/mL, as measured by means of a Cobas Amplicor HCV Monitor test (version 2.0; Roche). The doses of pegylated interferon alfa-2b and ribavirin were individually reduced during the treatment whenever needed to lessen adverse effects, and these dose reductions were performed according to the labeling. Informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institutions' human research committees.

Definition of response. This is a retrospective study of a cohort of patients who underwent a standard protocol of 48 weeks of treatment and consecutive 24 weeks of follow-up. All the patients underwent HCV RNA testing at weeks 4, 8, 12, 16, 20, 24, and 48 of the combination therapy. Follow-up testing was performed at week 72. SVR, posttreatment relapse, and nonresponse were defined on the basis of test results (Cobas Amplicor HCV Monitor test, version 2.0; Roche). SVR was defined as a serum HCV RNA level that was undetectable 6 months after the end of therapy. Posttreatment relapse was defined as reappearance of HCV RNA in serum after treatment was discontinued in a patient whose HCV RNA level was undetectable during or at the completion of therapy. Nonresponse was defined as a decrease in HCV RNA level of <2 log copies/mL at week 12 and a detectable HCV RNA level at week 24 during combination therapy.

Determination of core and NS5A amino acid sequence. Total HCV RNA was extracted from serum by means of a SepaGene RV-R nucleic acid extracting kit (Sanko Junyaku) and reverse transcribed to complementary DNA by means of SuperScript II RNAse H⁻ reverse transcriptase (Invitrogen Tech-Line), with a random hexamer primer (Takara Shuzo). Target sequences were amplified by means of polymerase chain reaction with specific primers (Table 1). The nucleotide sequences of the amplicons were determined directly or after cloning in the thymine-adenine (TA) vector (Topo TA cloning kit; Invitrogen) in both forward and reverse directions, using the ABI Prism BigDye Terminator cycle-sequencing ready reaction kit on a fluorescent model 3100 DNA sequencer (Applied Biosystems). The amino acid sequences were deduced and aligned using Win software (version 7.0; Genetyx). Throughout this article, the amino acids

Table 2. Characteristics of Patients with Chronic Hepatitis C Virus (HCV) Genotype 1b Infection Who Received Pegylated Interferon Alfa-2b Plus Ribavirin

Characteristic	Nonresponse (n = 16)	Response (n = 50)	P
Age, years	60.4 (7.4)	52.5 (10.3)	.006
Male sex, no. (%) of patients	9 (56.3)	28 (56.0)	.9
ALT level, IU/L	57.1 (42.1)	69.4 (41.2)	.38
γ-GTP level, IU/L	60.4 (37.2)	58.0 (53.8)	.87
LDL cholesterol level, mg/dL	84.7 (26.4)	98.2 (26.4)	.19
Glucose level, mg/dL	102.4 (12.1)	96.5 (21.3)	.35
Platelet count, $10^4/\mu L$	15.2 (8.1)	15.6 (4.7)	.31
HCV RNA level, kIU/mL	2196.3 (1539.6)	1991.2 (1574.1)	.65

NOTE. Data are mean (standard deviation) values, unless otherwise indicated. ALT, alanine aminotransferase; γ-GTP, γ glutamyl transferase; LDL, low-density lipoprotein.

are numbered according to the full-length genome sequence of isolate H77 (GenBank and DDBJ accession number AF009606) [8].

HCV infection of chimeric urokinase-type plasminogen activator transgenic severe combined immunodeficiency mice. The chimeric mice were purchased from Phoenix Bio [9]. Human hepatocytes were imported from BD Biosciences. The human serum albumin level was measured in murine serum samples by means of commercial enzyme-linked immunosorbent assay kits (Eiken Chemical). The serum levels of the human albumins and the body weight were required to be identical among all of the mice to provide reliable comparison. All mice were infected successfully with HCV recovered from serum samples of patients. Four protocols for administration of the medication were consecutively applied to the same mice: monotherapy with interferon alfa-2a (Chugai) in doses of 3 μg/kg (standard treatment dose), monotherapy with interferon in doses of 30 μg/kg (10-fold treatment dose), monotherapy with ribavirin in doses of 10 mg/kg (standard clinical treatment dose) followed by 50 mg/kg (5-fold treatment dose), and finally combined therapy with interferon (standard treatment dose) and ribavirin (5-fold treatment dose) [10].

Statistical analyses. Statistical differences were evaluated by use of the Fisher exact test or the χ^2 test with the Yates correction. Contribution to the treatment outcome was evaluated for host and viral factors in multivariate and logistic regression models by use of Stata software (version 8.0; StataCorp) and SPSS software (version 16; SPSS).

RESULTS

Virologic response. Among the 66 patients, 16 (24.2%) did not respond to the treatment (nonresponse), 29 (43.9%) achieved SVR, and 21 (31.8%) had achieved virologic response but experienced relapse after completion of the therapy, during the posttreatment follow-up (posttreatment relapse). Patients in the nonresponse group were older than those in the SVR group (mean age ± SD, 60.4 ± 7.4 years vs 50.6 ± 9.8 years; P = .002). Except for age, no statistically significant differences were observed among the 3 groups for comparisons of age, sex, alanine transaminase level, γ glutamyl transferase level, low-density lipoprotein cholesterol level, triglyceride level, total cholesterol level, blood sugar level, platelet count, erythrocyte count, hemoglobin level, leukocyte count, or HCV load. To evaluate factors associated with the virologic nonresponse to therapy, patients who achieved SVR and those who were responsive to therapy but experienced relapse during the posttreatment follow-up were grouped together into a "response" category. Characteristics of the patients in the nonresponse (n = 16) and response (n = 60) groups are summarized in Table 2. Patients in the nonresponse group were statistically significantly older than those in the response group; however, there was no statistically significant difference observed between the groups in clinical laboratory characteristics.

Prevalence of amino acid substitutions in HCV polyprotein before, during, and after therapy. HCV polyprotein amino acid substitutions that have been reported to be associated with outcome of interferon or combination interferon-ribavirin therapies (HCV core amino acids 70 and 91 and NS5A amino acids 2209–2248) were examined in the cohort under study.

Core amino acids 70 and 91. Direct sequencing of HCV in pretreatment baseline samples revealed the presence of glutamine at position 70 of the core protein (70Q) in 5 (17.2%) of the patients with SVR, 5 (23.8%) of the patients with posttreatment relapse, and 7 (43.8%) of the patients with nonresponse. In 2 of the 7 patients with nonresponse and in 1 of the 5 patients with posttreatment relapse, an ambiguous amino acid (glutamine or arginine) at position 70 was identified by means of direct sequencing; these patients were considered to have the 70Q (mutant) clone. When samples obtained during treatment were examined, 70Q was detected in 9 (56.3%) patients in the nonresponse group, whereas all samples from the posttreatment relapse and SVR groups showed negative results

after a polymerase chain reaction was performed. The prevalences of the 70Q clone in posttreatment samples from patients with nonresponse and from patients with posttreatment relapse were 56.3% (9 patients) and 23.8% (5 patients), respectively, whereas all patients who achieved SVR had negative polymerase chain reaction results. The prevalence of HCV with the methionine substitution (91M) was relatively higher in the SVR group (44.8%) than that in the nonresponse group (25%) and that in the posttreatment relapse group (23.8%). The prevalence of HCV with 91M in the nonresponse and posttreatment relapse groups remained constant when posttreatment samples were sequenced.

The prevalence of the 70Q substitution tended to be higher in the nonresponse group than in the response group ($P = .1$; Yates corrected χ^2); however, the difference was more pronounced when the prevalence among patients in the response group was compared to the posttreatment prevalence among patients in the nonresponse group ($P = .01$) (Figure 1).

Amino acid patterns of ISDR. The amino acid sequences spanning the ISDR were compared with the reference HCV-J strain (DDBJ, EMBL, and GenBank accession number D90208), and the number of amino acid substitutions was calculated for each patient. The wild type (no substitutions) and intermediate type (1 substitution) were considered as markers predisposing to resistance (resistant type), whereas the mutant type (≥2 substitutions) was considered as a marker predisposing to virologic response to antiviral therapy [4]. At baseline, the prevalence of HCV with resistant type (<2 substitutions in the ISDR) was 55.2% in the SVR group, 93.8% in the nonresponse group, and 81.0% in the posttreatment relapse group. After treatment, the prevalence was constant in the posttreatment relapse group (81.3%), whereas HCV from 1 patient in the nonresponse group had shifted from 2 substitutions (at baseline) to 1 substitution (after therapy), which resulted in a relative increase of the resistant ISDR prevalence in the nonresponse group in this study (from 93.8% at baseline to 100% after therapy; $P = .99$). A comparison of the prevalence of HCV with the resistant-type ISDR alone or in combination with core 70Q or 91M is shown in Figure 1.

Changes in HCV quasispecies during and after treatment. Direct nucleotide sequence coding amino acid 70 in the HCV core region was carefully revised in all 66 pretreatment baseline samples to reveal the presence of minor electrophoregram "picks" indicating the presence of heterogeneous quasispecies. Samples from 2 patients in the nonresponse group (patients 4 and 10) showing obvious ambiguity (glutamine or arginine) and samples from 3 other nonresponse patients (patients 1, 2, and 5) showing minor "picks" were subjected to TA cloning and sequencing of samples taken at baseline, during treatment, at the end of treatment, and at the end of follow-up. A total of 360 clones (15–20 for each time point) were picked, sequenced, and analyzed. The result is summarized in Figure 2. In samples from 4 of the 5 patients (patients 1, 2, 4, and 10), 70Q was found in the majority of clones picked during and at the end of treatment. However, during the posttreatment follow-up, the prevalence of 70Q was found to be decreased in all of the 4 patients, in 2 of which (patients 2 and 4) the wild-type sequence (70R) became detectable in a majority of clones. These results suggest that in these 4 patients HCV quasispecies possessing the 70Q substitution in the core protein were positively selected during treatment with pegylated interferon–ribavirin. Interestingly, another patient in whom 70Q was detected in a minority of strains at baseline (patient 5) did not show substantial changes in the prevalence of 70Q strains during treatment. The sample obtained from this patient during treatment was injected into chimeric mice.

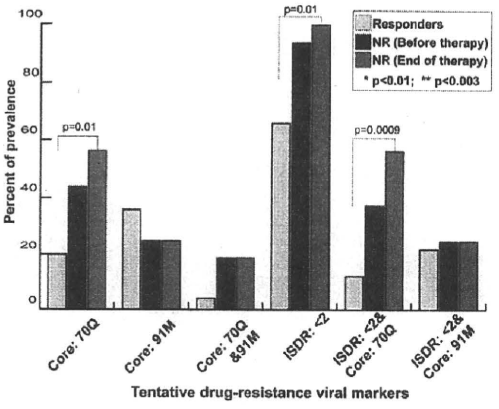

Figure 1. Independent or combined occurrence of the 70Q and 91M substitutions in hepatitis C virus (HCV) core protein and the resistant interferon sensitivity–determining region (ISDR; <2 substitutions), as detected by direct sequencing among patients with nonresponse (NR) to pegylated interferon plus ribavirin ($n = 16$) and patients with sustained ($n = 29$) or transient ($n = 21$) response to the therapy. The nonresponse group was assessed both at baseline and at the end of treatment; the response group was assessed at baseline. All patients had been infected with hepatitis C virus genotype 1b.

Minor clone changes in HCV-infected chimeric mice. To further investigate the effect of the presence of 70Q as a minor clone, a serum sample obtained from patient 5 (who experienced nonresponse) during treatment was injected into 2 chimeric mice with human hepatocytes for analysis. Because all patients with SVR in this study had achieved an undetectable viral load during and after treatment, the drug-selection effect was not available for estimation in this group. Therefore, 3 additional mice were infected with a baseline sample obtained from a patient who had achieved SVR. Serum samples were collected from each mouse and HCV RNA was quantified on

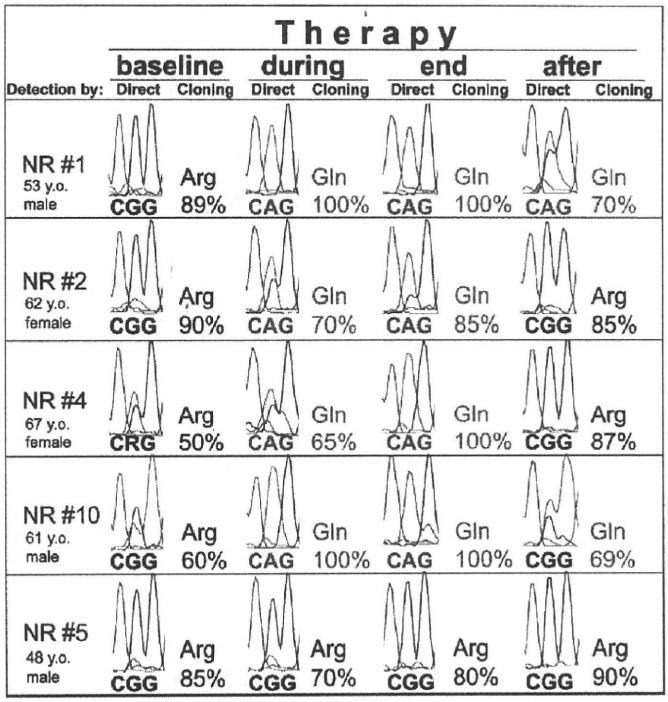

Figure 2. Changes in the proportion of clones with the 70Q substitution relative to wild type (70R) in patients with nonresponse (NR), as determined by both direct sequencing and cloning at different time points during treatment with pegylated interferon plus ribavirin. Each row summarizes the findings for a single patient identified by a unique ID, age, and sex. The direct sequencing results are demonstrated by 5′–3′ nucleotide sequence electrophoregrams. The cloning results are given as the translated amino acid (glutamine or arginine) from the dominant clone, and the proportion of the dominant clone is evaluated by the percentage of the total clones (15–20) sequenced at the respective time point.

a weekly basis. After inoculation, the HCV RNA titer increased rapidly in all mice, and by week 2 after inoculation the mean HCV RNA titers of the nonresponsive strains plateaued at ~7.6 log copies/mL. The HCV-1b RNA level in the mice inoculated with the sample from the SVR group increased to 8.5 log copies/mL until week 3 and then reached a plateau of ~8.0 log copies/mL (Figure 3). These 5 animals were subsequently treated with 4 different regimens, as outlined in Materials and Methods, and viral kinetics were described in detail in another report on the interferon sensitivity of the strains [10]. Briefly, no reduction of the HCV RNA titer was observed after administration of 3 injections of pegylated interferon alone at the clinical dosage (3 μg/kg) in the mice inoculated with a sample from the nonresponse group, whereas the mice inoculated with a sample from the SVR group achieved a 0.5 log copies/mL reduction in the HCV RNA titer. Administration of the 10-fold dose of interferon (30 μg/kg) induced reductions in the HCV RNA titer of 1.0 and 2.0 log copies/mL for the nonresponse and SVR groups, respectively ($P < .02$). No statistically significant difference was observed among the groups after administration of ribavirin in standard or 5-fold clinical doses. However, administration of the pegylated interferon in combination with ribavirin resulted in reductions of 0.9 and 0.2 log copies/mL in the SVR and nonresponse groups, respectively ($P < .02$). Mice serum samples collected at 4 time points, as indicated in Figure 3, were subjected to cloning. A total of 320 clones (15–20 for each time point) were analyzed. The results are summarized in Figure 3. The proportion of 70Q clones relative to 70R clones differed in the 2 mice infected with a nonresponse sample; after administration of 10-fold pegylated interferon (point 1), the proportion of 70Q clones was 15% in serum samples from mouse 5-1 and 90% in serum samples from mouse 5-2. Seven weeks later (point 2), the predominance of 70R clones was restored in both animals. After administration of pegylated interferon with ribavirin, 70Q was detected in the majority of clones retrieved from both mice (point 3), but 2

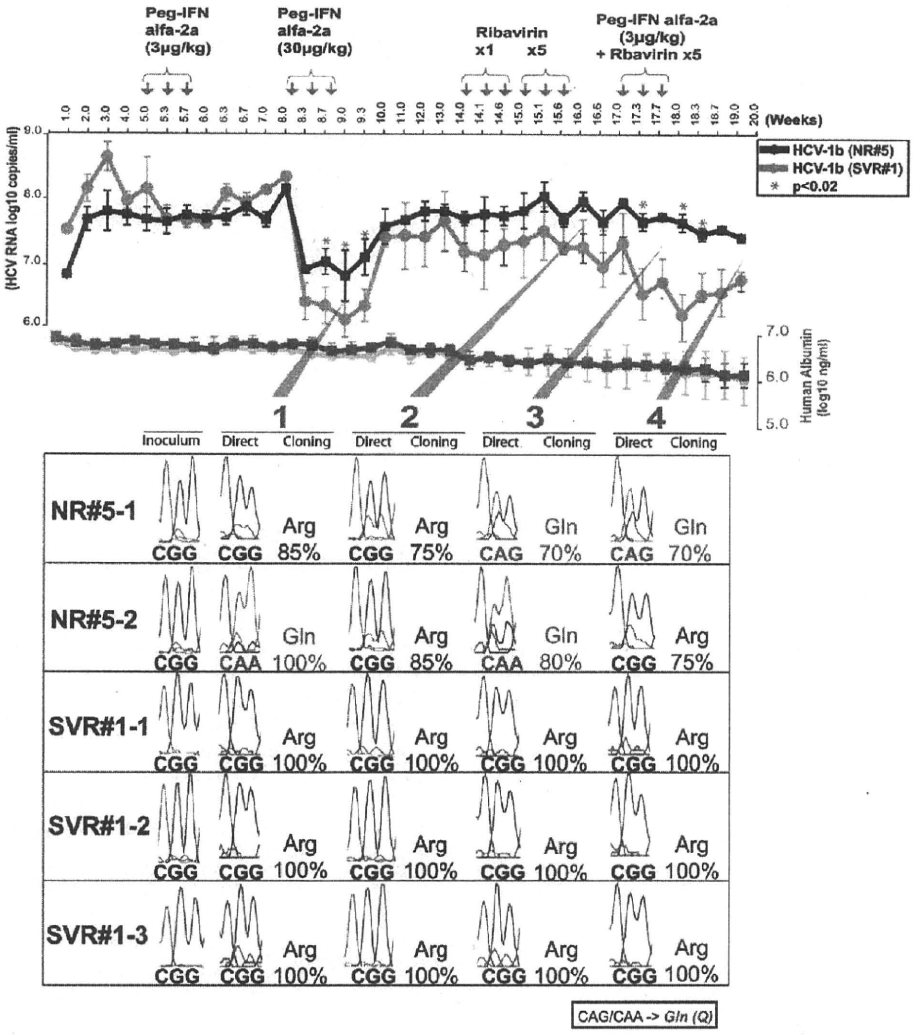

Figure 3. Changes in the proportion of core protein 70Q to 70R coding clones in chimeric mice infected with hepatitis C virus (HCV) by inoculation of serum samples from patient 5, who experienced nonresponse (NR; 2 mice), or from patient 1, who achieved sustained virologic response (SVR; 3 mice), as determined by both direct sequencing and cloning at 4 different time points during the course of postinfection follow-up. The changes in the mean HCV RNA levels estimated for mice grouped according to inoculation source are graphed on the top (*left scale*), and corresponding human albumin levels are graphed on the bottom (*right scale*). Times of administration of drugs are indicated by arrows. Each row summarizes findings for a single mouse, identified by inoculation source patient ID. The direct sequencing results are demonstrated by 5′–3′ nucleotide sequence electropherograms. The cloning results are given as the translated amino acid (glutamine or arginine) from the dominant clone, and the proportion of the dominant clone is evaluated by the percentage of the total clones (15–20) sequenced at the respective time point.

weeks after the treatment (point 4) the 70R clone majority was restored again in mouse 5-2. The last sample available for mouse 5-1 was taken 2 days after the last administration of medication; 70Q was detected in 70% of the clones sequenced at that time point (data not shown). No clone with 70Q was identified at any of the 4 points in the 3 mice infected with the SVR serum sample.

No changes were observed in the core 91 region (leucine)

and ISDR of the nonresponse group (resistant type; 0 substitutions) and SVR group (mutant type; 8 substitutions). These results suggest that the 70Q genotype is positively selected during pegylated interferon with ribavirin treatment and that detectable 70Q at baseline can be an important predictive marker of potential treatment failure.

Statistical evaluation of the 70Q substitution. We performed univariate analysis including the following variables: greater age (cutoff at median, 56 years), female sex, HCV RNA level of >1500 kIU/L, no early virologic response (EVR; absence of a reduction of 2 log copies/mL by week 12 of treatment), alanine transaminase level of ≥48 IU/L, γ glutamyl transferase level of >40 IU/L, low-density lipoprotein cholesterol level of >86.0 mg/dL, platelet count of $>14.6 \times 10^4$ platelets/μL, glucose level of ≥95 mg/dL, presence of ISDR, presence of the 70Q substitution (including patients in whom it was detected as a minor clone), and core 91M substitution. The analysis indicated that non-EVR and presence of the 70Q substitution were statistically significant factors associated with treatment failure (data not shown).

Statistical significance of the 70Q substitution was further assessed by logistic regression analysis. Non-EVR was not included, and only baseline factors were analyzed. Sixteen patients with nonresponse were compared with 50 patients with response by means of the backward stepwise likelihood ratio logistic regression method; estimated odds ratio (OR) coefficients, their 95% confidence interval ranges, and *P* values are summarized in Table 3 for the variables that remained in equitation at the last step. The predictive value in the multivariate analysis was assessed for the parameters detected at baseline. Detectable 70Q-possessing clones were counted. It was found that the 70Q substitution was statistically significantly associated with nonresponse (OR, 8.7; $P = .007$). High γ glutamyl transferase levels (>40 IU/L) and low alanine aminotransferase levels (<48 IU/L) also had weak association with nonresponse (OR for high γ glutamyl transferase levels, 7.08; $P = .043$; OR for low alanine aminotransferase levels, 0.12; $P = .035$). The positive predictive value of detecting 70Q for nonresponse was 50% (10 of 20 patients) in this cohort. The positive predictive value of detecting 70Q for failure to achieve SVR was 75% (15 of 20 patients). The positive predictive value of detecting 70Q together with a resistant ISDR sequence for failure to achieve SVR was 87.5% (14 of 16 patients).

Table 3. Logistic Regression Analysis of Variables Contributing to Resistance to Treatment with Pegylated Interferon Alfa-2b Plus Ribavirin

Variable	OR (95% CI)	*P*
Core substitution 70Q[a]	15.11 (2.436–93.702)	.004
Age, ≥56 years	4.94 (0.973–25.087)	.054
Viral load, >1500 kIU/L	4.37 (0.798–23.962)	.089
Platelet count, $>14.6 \times 10^4$ platelets/μL	5.396 (0.77–38.08)	.091
γ-GTP level, >40 IU/L	7.27 (1.11–47.648)	.039
Glucose level, ≥95 mg/dL	0.182 (0.33–1.01)	.052
ALT level, ≥48 IU/L	8.711 (1.21–62.816)	.032

NOTE. For each variable except the core substitution, the median value was used as a grouping cutoff. ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ glutamyl transferase; OR, odds ratio.

[a] Including patients with a detected minor clone.

DISCUSSION

A substantial proportion of HCV-infected patients do not have an optimum response to current pegylated interferon with ribavirin treatment regimens [11]. Individualization of therapy would offer the possibility of tailoring treatment to particular patients and selecting the treatment duration that ensures the best chance of achieving SVR while preventing overtreatment. Effective identification of potentially nonresponding patients would be very useful in the context of the rather costly treatment with pegylated interferon–ribavirin, which is accompanied by many adverse effects [2]. A few recent studies reported an arginine-to-glutamine amino acid substitution at position 70 of the HCV core protein (70Q) is associated with poor response in patients infected with HCV-1b [6, 12]. In the present study, comparison between the nonresponse and the response groups also indicated statistically significant differences for the ISDR and core amino acid patterns. Interestingly, the 70Q substitution was detected in the studied cohort at a higher rate during and after treatment, compared with baseline. Patients in whom there was heterogeneity of nucleotide sequence coding of the amino acid at position 70 of the core protein were further investigated for quasispecies composition at different time points. Selection of 70Q-coding clones during treatment was indicated in 4 (25%) of 16 patients with nonresponse. Additionally, a sample obtained from a patient during treatment, who had a minority of core 70Q clones before, during, and after treatment, was inoculated into 2 chimeric mice that were consecutively treated with various regimens of pegylated interferon and ribavirin. After treatment with pegylated interferon and ribavirin, 70Q was detected in a majority of clones retrieved from both mice. The discrepancy between the clinical case and the mouse model may be associated with different dosages, different regimens, different drug metabolisms, and/or different contributions of host factors, which require further study. This study, by demonstrating the positive selection of the 70Q viral strains, provides additional evidence for an association between the amino acid substitution in the core protein of the infecting HCV and the response to antiviral treatment. Hence, outlined findings indicate that the presence of a 70Q clone at baseline (including patients with a detectable minor quasispecies population) may indicate potential failure in achieving virologic response or SVR in patients with chronic HCV-1b infection. In the present study, a 70Q clone was de-

Table 4. Prevalence of the Core 70Q Substitution in Hepatitis C Virus Core Protein among DDBJ, EMBL, and GenBank Entries

This table is available in its entirety in the online version of the *Journal of Infectious Diseases*

tected at baseline in 43.8% of patients who had nonresponse and 17.5% of patients who achieved SVR (similar to the rates of 47.7% and 18.2%, respectively, that were found in another study [13]), indicating that the positive predictive value of detecting 70Q for nonresponse was 50%. The positive predictive values of detecting 70Q alone or together with resistant ISDR for failing to achieve SVR was 75% and 87.5%, respectively. Furthermore, the difference in the proportion of 70Q detected in 2 chimeric mice infected with a nonresponse strain indicated the contribution of host genetic factors [14].

Other reports investigating the baseline prevalence of core amino acid patterns indicated that both 70Q and 91M were associated with nonresponse among treated patients [6, 7]; however, in the cohort studied here, the baseline prevalence of 91M was relatively higher in the SVR group (44.8%) than in the nonresponse and posttreatment relapse groups (25% and 23.8%, respectively). Furthermore, the prevalence of 91M during treatment and at the end of treatment was stable in the studied cohort, which suggests that it was not associated with selection of resistant strains. A study investigating the evolution of the NS5A region during treatment (with pegylated interferon and ribavirin) in patients with HCV genotype 1 infection indicated that an absence of changes in the ISDR pattern was associated with treatment outcome [15]. In the present study, samples from only 1 patient (who was in the nonresponse group) demonstrated a change in the amino acid pattern from sensitive to resistant (from 2 mutations before treatment to 1 mutation after treatment). Although the number of patients was relatively small in this study, the prevalence of the 70Q substitutions and resistant ISDR patterns concurred with those in other published studies [4, 7, 15–17]; 18 (27%) of 66 patients had a sensitive ISDR pattern, and the positive predictive value of presence of the sensitive ISDR pattern for achieving SVR was 89% (16 of 18 patients). Among the remaining 48 (73%) patients, who had the resistant ISDR pattern, 16 (33%) had detectable 70Q in the core protein. Presence of the resistant-type ISDR and detectable 70Q together had a positive predictive value for failing to achieve SVR of 87.5% (14 of 16 patients) in this cohort.

Table 5. Geographical Distribution of Hepatitis C Virus Genotype 1b Strains with the 70Q Substitution

This table is available in its entirety in the online version of the *Journal of Infectious Diseases*

Inspection of 4933 HCV-1b strains currently available in the DDBJ, EMBL, and GenBank genetic databases indicated that the 70Q substitution is present in 54% of HCV-1b isolates; other genotypes in which 70Q is common are 5a (84.4%) and 6 (58.3%), whereas only 8.6% of genotype 3a and none of genotype 2 isolates had this amino acid in this position (Table 4). The geographical distribution of genotype 1b strains with 70Q was substantially different, with 44.3% of strains in Asia, 58.9% in Europe, and 63.7% in America ($P < .001$) (Table 5). Because complete clinical background data were not available for all entries in the database, further studies are needed to confirm the clinical impact of core 70Q in each geographic region.

In conclusion, in this study both clinical material and an in vivo model were used to provide further evidence of an association between the 70Q substitution in the HCV core protein and treatment response by detecting positive selection by treatment with pegylated interferon–ribavirin treatment. Further studies are required to investigate the mechanism of this association.

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MAJOR ARTICLE

Positive Selection of Core 70Q Variant Genotype 1b Hepatitis C Virus Strains Induced by Pegylated Interferon and Ribavirin

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Background. Approximately 20% of patients with hepatitis C virus (HCV) genotype 1b infection have nonresponse to the most current treatment, pegylated interferon with ribavirin. Mutations in the HCV core region were recently proposed to be associated with nonresponse. Our aim was to evaluate the viral factors associated with treatment failure.

Methods. HCV variants were determined directly and after cloning in 66 HCV-1b–infected Japanese patients and in 5 urokinase-type plasminogen activator transgenic severe combined immunodeficiency mice with human hepatocytes (chimeric mice), at baseline, during treatment, and after treatment.

Results. At baseline, glutamine at position 70 of the HCV core protein (70Q) was detected by direct sequencing in 20% of patients with virologic response and in 43.8% of patients with nonresponse. Among patients with nonresponse, who were examined during and after treatment, the prevalence of the 70Q substitution increased to 56.3%, which indicates that treatment-induced selection occurred in all patients with nonresponse who had 70Q quasispecies detectable by cloning. This observation was reinforced by the results from experimentally infected chimeric mice. Logistic regression analysis indicated that detection of 70Q quasispecies was associated with a statistically significantly increased risk of nonresponse (odds ratio, 15.1; $P = .004$) in the studied cohort.

Conclusion. Presence of the 70Q quasispecies at baseline was associated with an increased risk of treatment failure, as indicated by the positive selection of the 70Q clones induced by treatment with pegylated interferon with ribavirin. These results urge further investigation of the mechanisms of this association.

Hepatitis C is a global health problem that affects a significant portion of the world's population. There are ~170 million hepatitis C virus (HCV) carriers in the world, and 3–4 million new cases of infection are diagnosed each year [1]. The estimated burden of HCV infection exceeds 4 million persons in the United States, where hepatitis C represents the leading cause of cirrhosis and hepatocellular carcinoma, as well as the leading indication for liver transplantation [1]. The most effective of the currently available treatments for chronic hepatitis C, a combination of pegylated interferon alfa with ribavirin, does not provide sustained virologic response (SVR) in all patients who receive this treatment. Nonresponse and posttreatment relapse are the main forms of the treatment failure. Posttreatment relapse is often associated with a number of well-described adverse effects (such as hematologic abnormalities and neuropsychiatric symptoms) that require dose reduction and premature withdrawal from therapy [2]. It is thus important to predict individual response to the rather costly treatment with pegylated interferon–ribavirin, which is accompanied by many adverse effects. Factors that contribute to nonresponse have been extensively studied; of these, infection with HCV genotype 1 (vs 2 or 3), high

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Table 1. Primers Used for Polymerase Chain Reaction and Sequencing of Hepatitis C Virus Genotype 1b Genes

Primer	Sequence, 5′–3′	Gene	Nucleic acid position
HCV-264_F	AGGCCTTGTGGTACTGCCTGATAGG	Core	276–300
HCV-852_R	GAAGATAGAAAAGGAGCAACC	Core	872–852
HCV-6879_F	TCCATGCTCACCGAYCCCTCCC	NS5A	6861–6879
HCV-7203_R	AGTGGAGGGTTGTAATCCGGGCGTGCCCA	NS5A	7228–7203
HCV-7061_F	GGCAGGAGATGGGCGGTAACATC	Pre-V3	7042–7064
HCV-7563_R	GTAGACCAAGACCCGTCG	V3	7558–7544

NOTE. NS5A, nonstructural protein 5A; V3, variable region 3.

baseline viral load (vs low), viral kinetics characterized by late response to treatment (vs rapid), amino acid substitutions in nonstructural protein 5A (NS5A), and putative interferon sensitivity–determining region (ISDR; wild type or 1 substitution vs ⩾2 substitutions) [3] have been found to be statistically significantly associated with nonresponse in a number of independent studies [4, 5]. A few recent studies have also indicated that amino acid substitutions in the HCV core protein (70Q and 91M vs 70R and 91L) are associated with treatment failure [6, 7].

The present study targeted HCV genomic regions that have been previously found to be associated with pegylated interferon–ribavirin treatment outcome. With the aim to investigate retrospectively whether treatment-related selection occurred in those genomic regions, we enrolled 66 patients with HCV genotype 1b infection whose treatment outcome had been assessed at the end of 6 months of posttreatment follow-up. Stored serum samples from all the patients were used for determining changes in HCV genetic heterogeneity at baseline, during treatment, at the end of treatment, and during posttreatment follow-up. Selection of the 70Q substitution was further investigated using an in vivo model of HCV infection in chimeric mice with livers repopulated by human hepatocytes.

MATERIALS AND METHODS

Patients. Sixty-six patients in Japan with chronic HCV-1b infection were enrolled in this study from a group of patients at 3 hospitals (Nagoya City University Hospital, Social Insurance Chukyo Hospital, and Nagoya City Johoku Hospital, Nagoya) who received therapy with pegylated interferon alfa-2b (1.5 μg/kg of body weight by subcutaneous injection once per week) plus ribavirin (600–1000 mg daily, according to body weight). Patients with other hepatitis virus infection or human immunodeficiency virus coinfection were not included in this study. The mean age of the population was 57 years, and 37 (57%) were men. The mean (± standard deviation [SD]) value for the alanine transaminase level was 66.3 (± 41.4) IU/L and for the platelet count was 17.6×10^4 ($\pm 17.3 \times 10^4$) platelets/μL. The mean HCV RNA level was 2041 (± 1557) kIU/mL, as measured by means of a Cobas Amplicor HCV Monitor test (version 2.0; Roche). The doses of pegylated interferon alfa-2b and ribavirin were individually reduced during the treatment whenever needed to lessen adverse effects, and these dose reductions were performed according to the labeling. Informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institutions' human research committees.

Definition of response. This is a retrospective study of a cohort of patients who underwent a standard protocol of 48 weeks of treatment and consecutive 24 weeks of follow-up. All the patients underwent HCV RNA testing at weeks 4, 8, 12, 16, 20, 24, and 48 of the combination therapy. Follow-up testing was performed at week 72. SVR, posttreatment relapse, and nonresponse were defined on the basis of test results (Cobas Amplicor HCV Monitor test, version 2.0; Roche). SVR was defined as a serum HCV RNA level that was undetectable 6 months after the end of therapy. Posttreatment relapse was defined as reappearance of HCV RNA in serum after treatment was discontinued in a patient whose HCV RNA level was undetectable during or at the completion of therapy. Nonresponse was defined as a decrease in HCV RNA level of <2 log copies/mL at week 12 and a detectable HCV RNA level at week 24 during combination therapy.

Determination of core and NS5A amino acid sequence. Total HCV RNA was extracted from serum by means of a SepaGene RV-R nucleic acid extracting kit (Sanko Junyaku) and reverse transcribed to complementary DNA by means of SuperScript II RNAse H^- reverse transcriptase (Invitrogen Tech-Line), with a random hexamer primer (Takara Shuzo). Target sequences were amplified by means of polymerase chain reaction with specific primers (Table 1). The nucleotide sequences of the amplicons were determined directly or after cloning in the thymine-adenine (TA) vector (Topo TA cloning kit; Invitrogen) in both forward and reverse directions, using the ABI Prism BigDye Terminator cycle-sequencing ready reaction kit on a fluorescent model 3100 DNA sequencer (Applied Biosystems). The amino acid sequences were deduced and aligned using Win software (version 7.0; Genetyx). Throughout this article, the amino acids

Table 2. Characteristics of Patients with Chronic Hepatitis C Virus (HCV) Genotype 1b Infection Who Received Pegylated Interferon Alfa-2b Plus Ribavirin

Characteristic	Nonresponse (n = 16)	Response (n = 50)	P
Age, years	60.4 (7.4)	52.5 (10.3)	.006
Male sex, no. (%) of patients	9 (56.3)	28 (56.0)	.9
ALT level, IU/L	57.1 (42.1)	69.4 (41.2)	.38
γ-GTP level, IU/L	60.4 (37.2)	58.0 (53.8)	.87
LDL cholesterol level, mg/dL	84.7 (26.4)	98.2 (26.4)	.19
Glucose level, mg/dL	102.4 (12.1)	96.5 (21.3)	.35
Platelet count, $10^4/\mu L$	15.2 (8.1)	15.6 (4.7)	.31
HCV RNA level, kIU/mL	2196.3 (1539.6)	1991.2 (1574.1)	.65

NOTE. Data are mean (standard deviation) values, unless otherwise indicated. ALT, alanine aminotransferase; γ-GTP, γ glutamyl transferase; LDL, low-density lipoprotein.

are numbered according to the full-length genome sequence of isolate H77 (GenBank and DDBJ accession number AF009606) [8].

HCV infection of chimeric urokinase-type plasminogen activator transgenic severe combined immunodeficiency mice. The chimeric mice were purchased from Phoenix Bio [9]. Human hepatocytes were imported from BD Biosciences. The human serum albumin level was measured in murine serum samples by means of commercial enzyme-linked immunosorbent assay kits (Eiken Chemical). The serum levels of the human albumins and the body weight were required to be identical among all of the mice to provide reliable comparison. All mice were infected successfully with HCV recovered from serum samples of patients. Four protocols for administration of the medication were consecutively applied to the same mice: monotherapy with interferon alfa-2a (Chugai) in doses of 3 μg/kg (standard treatment dose), monotherapy with interferon in doses of 30 μg/kg (10-fold treatment dose), monotherapy with ribavirin in doses of 10 mg/kg (standard clinical treatment dose) followed by 50 mg/kg (5-fold treatment dose), and finally combined therapy with interferon (standard treatment dose) and ribavirin (5-fold treatment dose) [10].

Statistical analyses. Statistical differences were evaluated by use of the Fisher exact test or the χ^2 test with the Yates correction. Contribution to the treatment outcome was evaluated for host and viral factors in multivariate and logistic regression models by use of Stata software (version 8.0; StataCorp) and SPSS software (version 16; SPSS).

RESULTS

Virologic response. Among the 66 patients, 16 (24.2%) did not respond to the treatment (nonresponse), 29 (43.9%) achieved SVR, and 21 (31.8%) had achieved virologic response but experienced relapse after completion of the therapy, during the posttreatment follow-up (posttreatment relapse). Patients in the nonresponse group were older than those in the SVR group (mean age ± SD, 60.4 ± 7.4 years vs 50.6 ± 9.8 years; P = .002). Except for age, no statistically significant differences were observed among the 3 groups for comparisons of age, sex, alanine transaminase level, γ glutamyl transferase level, low-density lipoprotein cholesterol level, triglyceride level, total cholesterol level, blood sugar level, platelet count, erythrocyte count, hemoglobin level, leukocyte count, or HCV load. To evaluate factors associated with the virologic nonresponse to therapy, patients who achieved SVR and those who were responsive to therapy but experienced relapse during the posttreatment follow-up were grouped together into a "response" category. Characteristics of the patients in the nonresponse (n = 16) and response (n = 60) groups are summarized in Table 2. Patients in the nonresponse group were statistically significantly older than those in the response group; however, there was no statistically significant difference observed between the groups in clinical laboratory characteristics.

Prevalence of amino acid substitutions in HCV polyprotein before, during, and after therapy. HCV polyprotein amino acid substitutions that have been reported to be associated with outcome of interferon or combination interferon-ribavirin therapies (HCV core amino acids 70 and 91 and NS5A amino acids 2209–2248) were examined in the cohort under study.

Core amino acids 70 and 91. Direct sequencing of HCV in pretreatment baseline samples revealed the presence of glutamine at position 70 of the core protein (70Q) in 5 (17.2%) of the patients with SVR, 5 (23.8%) of the patients with posttreatment relapse, and 7 (43.8%) of the patients with nonresponse. In 2 of the 7 patients with nonresponse and in 1 of the 5 patients with posttreatment relapse, an ambiguous amino acid (glutamine or arginine) at position 70 was identified by means of direct sequencing; these patients were considered to have the 70Q (mutant) clone. When samples obtained during treatment were examined, 70Q was detected in 9 (56.3%) patients in the nonresponse group, whereas all samples from the posttreatment relapse and SVR groups showed negative results

after a polymerase chain reaction was performed. The prevalences of the 70Q clone in posttreatment samples from patients with nonresponse and from patients with posttreatment relapse were 56.3% (9 patients) and 23.8% (5 patients), respectively, whereas all patients who achieved SVR had negative polymerase chain reaction results. The prevalence of HCV with the methionine substitution (91M) was relatively higher in the SVR group (44.8%) than that in the nonresponse group (25%) and that in the posttreatment relapse group (23.8%). The prevalence of HCV with 91M in the nonresponse and posttreatment relapse groups remained constant when posttreatment samples were sequenced.

The prevalence of the 70Q substitution tended to be higher in the nonresponse group than in the response group ($P = .1$; Yates corrected χ^2); however, the difference was more pronounced when the prevalence among patients in the response group was compared to the posttreatment prevalence among patients in the nonresponse group ($P = .01$) (Figure 1).

Amino acid patterns of ISDR. The amino acid sequences spanning the ISDR were compared with the reference HCV-J strain (DDBJ, EMBL, and GenBank accession number D90208), and the number of amino acid substitutions was calculated for each patient. The wild type (no substitutions) and intermediate type (1 substitution) were considered as markers predisposing to resistance (resistant type), whereas the mutant type (≥2 substitutions) was considered as a marker predisposing to virologic response to antiviral therapy [4]. At baseline, the prevalence of HCV with resistant type (<2 substitutions in the ISDR) was 55.2% in the SVR group, 93.8% in the nonresponse group, and 81.0% in the posttreatment relapse group. After treatment, the prevalence was constant in the posttreatment relapse group (81.3%), whereas HCV from 1 patient in the nonresponse group had shifted from 2 substitutions (at baseline) to 1 substitution (after therapy), which resulted in a relative increase of the resistant ISDR prevalence in the nonresponse group in this study (from 93.8% at baseline to 100% after therapy; $P = .99$). A comparison of the prevalence of HCV with the resistant-type ISDR alone or in combination with core 70Q or 91M is shown in Figure 1.

Changes in HCV quasispecies during and after treatment. Direct nucleotide sequence coding amino acid 70 in the HCV core region was carefully revised in all 66 pretreatment baseline samples to reveal the presence of minor electrophoregram "picks" indicating the presence of heterogeneous quasispecies. Samples from 2 patients in the nonresponse group (patients 4 and 10) showing obvious ambiguity (glutamine or arginine) and samples from 3 other nonresponse patients (patients 1, 2, and 5) showing minor "picks" were subjected to TA cloning and sequencing of samples taken at baseline, during treatment, at the end of treatment, and at the end of follow-up. A total of 360 clones (15–20 for each time point) were picked, sequenced, and analyzed. The result is summarized in Figure 2. In samples from 4 of the 5 patients (patients 1, 2, 4, and 10), 70Q was found in the majority of clones picked during and at the end of treatment. However, during the posttreatment follow-up, the prevalence of 70Q was found to be decreased in all of the 4 patients, in 2 of which (patients 2 and 4) the wild-type sequence (70R) became detectable in a majority of clones. These results suggest that in these 4 patients HCV quasispecies possessing the 70Q substitution in the core protein were positively selected during treatment with pegylated interferon–ribavirin. Interestingly, another patient in whom 70Q was detected in a minority of strains at baseline (patient 5) did not show substantial changes in the prevalence of 70Q strains during treatment. The sample obtained from this patient during treatment was injected into chimeric mice.

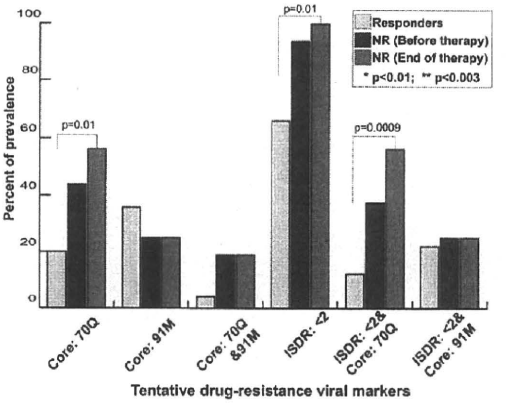

Figure 1. Independent or combined occurrence of the 70Q and 91M substitutions in hepatitis C virus (HCV) core protein and the resistant interferon sensitivity–determining region (ISDR; <2 substitutions), as detected by direct sequencing among patients with nonresponse (NR) to pegylated interferon plus ribavirin ($n = 16$) and patients with sustained ($n = 29$) or transient ($n = 21$) response to the therapy. The nonresponse group was assessed both at baseline and at the end of treatment; the response group was assessed at baseline. All patients had been infected with hepatitis C virus genotype 1b.

Minor clone changes in HCV-infected chimeric mice. To further investigate the effect of the presence of 70Q as a minor clone, a serum sample obtained from patient 5 (who experienced nonresponse) during treatment was injected into 2 chimeric mice with human hepatocytes for analysis. Because all patients with SVR in this study had achieved an undetectable viral load during and after treatment, the drug-selection effect was not available for estimation in this group. Therefore, 3 additional mice were infected with a baseline sample obtained from a patient who had achieved SVR. Serum samples were collected from each mouse and HCV RNA was quantified on