

approximately 10 mg were homogenized in 100 μ L of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30 μ g of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti- β -actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon²⁰ were used for a positive control for cleaved IPS-1.

Definitions of Response to Therapy. A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFN α -2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

Statistical Analysis. Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney *U* test for two groups. All tests of significance were two-tailed and *P* < 0.05 was considered statistically significant.

Results

Patient Characteristics and IL28B Genotype. Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report

of the rarity of a minor homozygote in Japanese patients.³ *IL28B* minor patients were significantly associated with a higher γ -glutamyl transpeptidase (γ -GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

Gene Expression Involving Innate Immunity and IFN λ in the Liver. Hepatic expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFN λ* (*IL28A/B*) expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFN λ* based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the *ISG15/USP18* system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFN δ* was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the *RIG-I/IPS-1* and *ISG15/USP18* systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

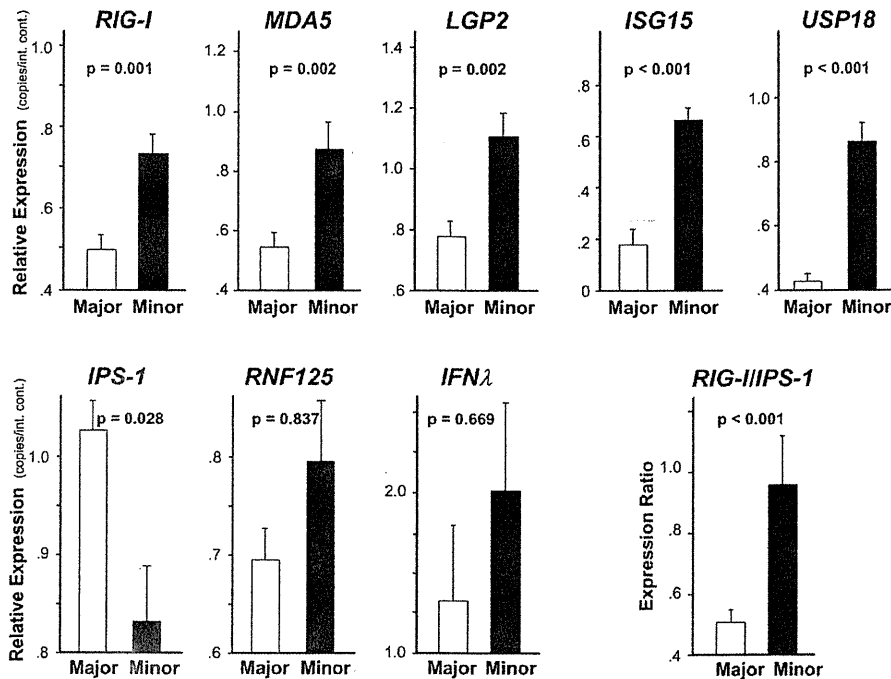


Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, $n = 54$) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, $n = 34$). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The P -values were determined by the Mann-Whitney U test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

Receiver Operator Characteristic (ROC) Analysis. To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

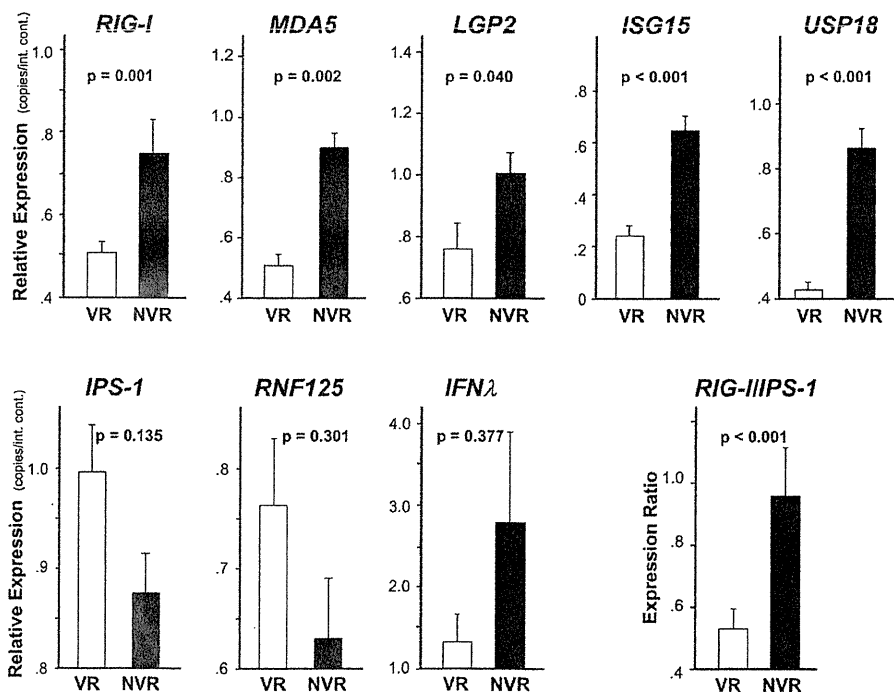


Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, $n = 60$) and nonvirological responders (NVR, $n = 28$). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The P -values were determined by the Mann-Whitney U test.

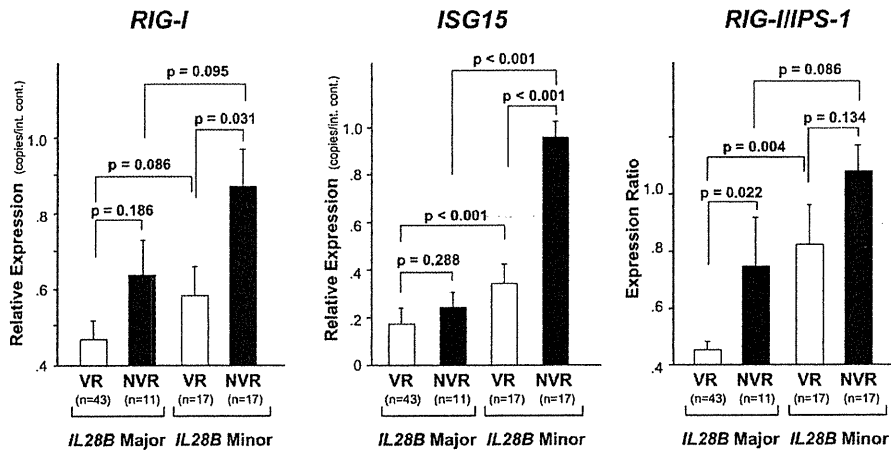


Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-IIIPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIG-IIIPS-1* ratio, no statistically significant difference was found in

NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

Factors Associated with NVR. In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic expressions of *RIG-I*, *MDA5*, *LGP2*, *ISG15*, and *USP18*, and *RIG-IIIPS-1* ratio were significantly

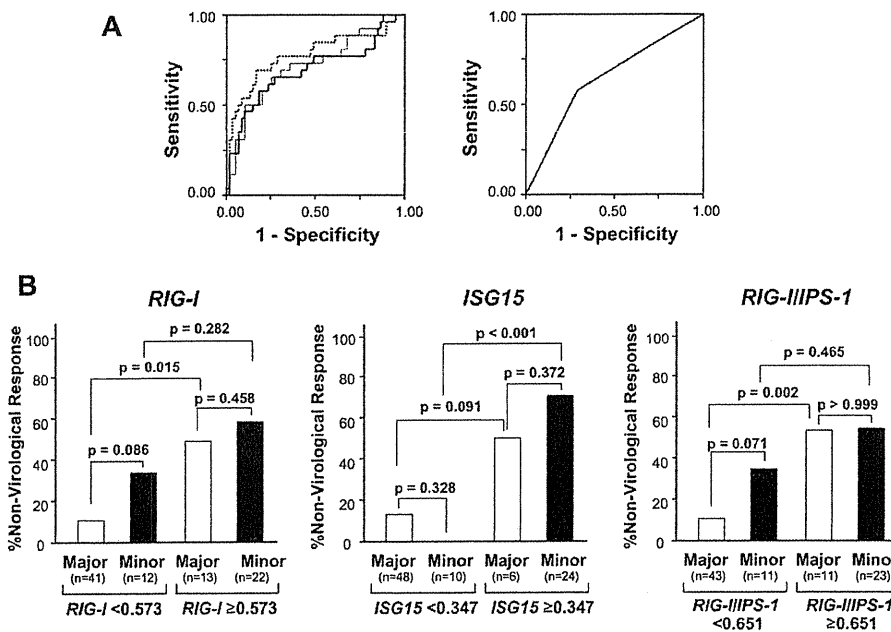


Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/ISG15* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
<i>RIG-I</i> (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
<i>ISG15</i> (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
<i>RIG-I/IPS-1</i> (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
<i>IL28B</i> genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

*Genotype at rs8099917.

†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

IPS-1 and RIG-I Protein Expression in the Liver. Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

Table 3. Factors Associated with Nonvirological Response

Factors	Univariate Analysis		Multivariate Analysis*	
	Risk Ratio (95% CI)	P-value	Risk Ratio (95% CI)	P-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m ²)	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10 ⁴ /μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
<i>IL28B</i> genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/int. control)				
<i>RIG-I</i>	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
<i>MDA5</i>	1.53 (1.12-2.00)	0.001		
<i>LGP2</i>	1.34 (1.04-1.74)	0.026		
<i>IPS-1</i>	0.90 (0.78-1.04)	0.143		
<i>RNF125</i>	0.93 (0.83-1.04)	0.204		
<i>ISG15</i>	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
<i>USP18</i>	1.67 (1.27-2.20)	<0.001		
<i>IFNλ</i>	1.02 (0.99-1.05)	0.170		
<i>RIG-I/IPS-1</i> ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.

*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5*, *LGP2*, *USP18*, and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.

†rs8099917 TT and rs12979860 CC.

‡rs8099917 TG and rs12979860 CT.

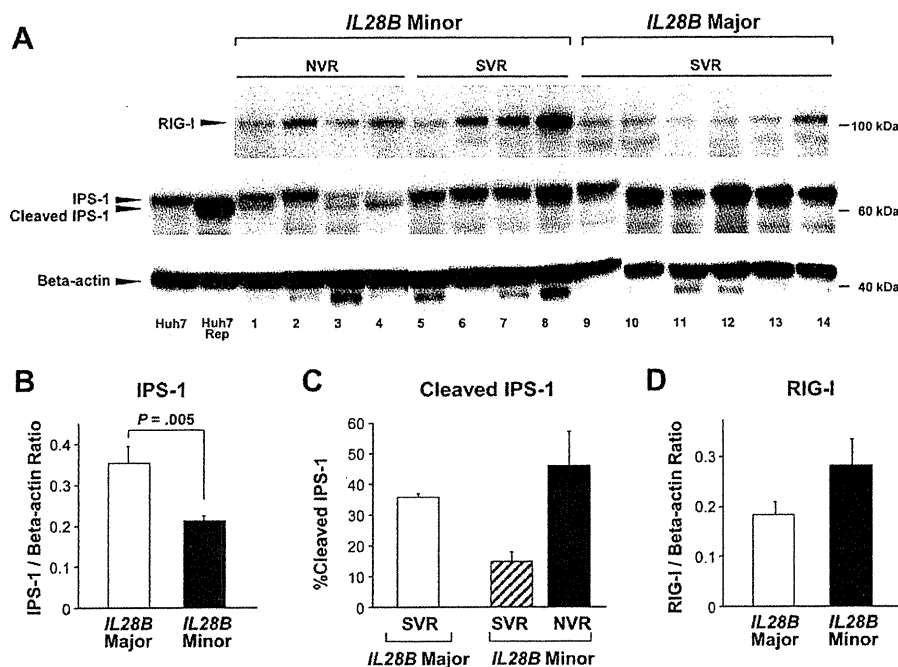


Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β -actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β -actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β -actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFN α /RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFN α /RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a

pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.^{7,8} Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,¹⁹ suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFN α /RBV response as well as with *IL28B* genotype.²¹⁻²³

In contrast to cytoplasmic viral sensor (*RIG-I*, *MDA5*, and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,^{9,18}

transcriptional and translational *IPS-1* expression are probably suppressed by HCV with resistant phenotype, which may be more adaptive in *IL28B* minor patients than in *IL28B* major patients. When we analyzed the proportion of full-length or cleaved IPS-1 to the total IPS-1 protein in a subgroup of *IL28B* minor patients, cleaved IPS-1 product was less dominant in SVR than in NVR, whereas uncleaved full-length IPS-1 protein was more dominant in SVR than in NVR. Therefore, the ability of HCV to evade host innate immunity by cleaving IPS-1 protein and/or host capability of protection from IPS-1 cleavage is probably responsible for the variable treatment responses in *IL28B* minor patients.

Our results indicated a close association between *IL28B* minor patients with higher γ -GTP level and higher frequency of HCV core double mutants, which are known factors for NVR. In contrast, no significant association was observed between *IL28B* genotype and age, gender, or liver fibrosis, which are also known to be unfavorable factors for virological response to PEG-IFN α /RBV. Therefore, certain factors other than the *IL28B* genotype may independently influence virological response. To elucidate whether gene expression involving innate immunity independently associates with a virological response from the *IL28B* genotype, we performed further analysis in a subgroup and conducted a multivariate regression and ROC analyses. Our multivariate and ROC analyses demonstrate that higher expressions of *RIG-I* and *ISG15* as well as a higher ratio of *RIG-I/IPS-1* are independently associated with NVR, and quantification of these values is more useful in predicting final virological response to PEG-IFN α /RBV than determination of *IL28B* genotype in each individual patients. However, the SVR rates in our patients were similar among *IL28B* genotypes, which suggests more SVR patients with the *IL28B* minor allele were included in the present study than those in the general CH-C population. Hence, our data did not necessarily exclude the possibility of the *IL28B* genotype in predicting NVR, although our multivariate analysis could not identify the *IL28B* minor allele as an independent factor for NVR. Interestingly, an association between *IL28B* genotype and expressions of *RIG-I* and *ISG15* as well as *RIG-I/IPS-1* expression ratio is still observed even in patients with the same subgroup of virological response (Fig. 3).

In the present study, although hepatic *IFN λ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of

IL28B from that of *IL28A*, and moreover, we could not specify which cell expresses *IFN λ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFN λ* in relation to treatment response need further clarification by specifying type of *IFN λ* and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I/IPS-1* ratio was significantly higher in NVR with HCV genotype 2.¹⁹ However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.²⁴ The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

Differences in allele frequency for *IL28B* SNPs among the population groups has been reported. The frequency of *IL28B* major allele among patients with Asian ancestry is higher than that among patients with European and African ancestry.²⁵ Because *IL28B* polymorphism strongly influences treatment responses within each population group,⁵ our data obtained from Japanese patients can be applied to other population groups. However, the rate of SVR having African ancestry was lower than that having European ancestry within the same *IL28B* genotype.⁵ Hence, further study is required to clarify whether this difference among the population groups with the same *IL28B* genotype could be explained by differences in expression of genes involved in innate immunity.

In a recent report, an SVR rate of telaprevir with PEG-IFN α /RBV was only 27.6% in *IL28B* minor patients.²⁶ Because new anti-HCV therapy should still contain PEG-IFN α /RBV as a platform for the therapy, our findings regarding innate immunity in addressing the mechanism of virological response and predicting NVR remain important in this new era of directly acting anti-HCV agents, such as telaprevir and boceprevir.

In conclusion, this clinical study in humans demonstrates the potential relevance of the molecules involved in innate immunity to the genetic variation

of *IL28B* and clinical response to PEG-IFN α /RBV. Both the *IL28B* minor allele and higher expressions of *RIG-I* and *ISG15* as well as higher *RIG-I/IPS-1* ratio are independently associated with NVR. Innate immune responses in *IL28B* minor patients may have adapted to a different equilibrium compared with that in *IL28B* major patients. Our data will advance both understanding of the pathogenesis of HCV resistance and the development of new antiviral therapy targeted toward the innate immune system.

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Analysis of the Complete Open Reading Frame of Genotype 2b Hepatitis C Virus in Association with the Response to Peginterferon and Ribavirin Therapy

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Abstract

Background and Aims: Patients infected with genotype 2b hepatitis C virus (HCV) generally can achieve favorable responses to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV). However, a proportion of patients show poorer responses and the correlation between viral sequence variation and treatment outcome remains unclear.

Methods: The pretreatment complete open reading frame (ORF) sequences of genotype 2b HCV determined by direct sequencing were investigated for correlation with the final outcome in a total of 60 patients.

Results: In this study group, 87.5% (14/16) of non-sustained virological response (non-SVR) patients (n = 16) were relapsers. Compared to sustained virological response (SVR) patients (n = 44), non-SVR patients were older and could not achieve prompt viral clearance after the therapy induction. Comparing each viral protein between the two groups, viral sequences were more diverse in SVR patients and that diversity was found primarily in the E1, p7, and NS5A proteins. In searching for specific viral regions associated with the final outcome, several regions in E2, p7, NS2, NS5A, and NS5B were extracted. Among these regions, part of the interferon sensitivity determining region (ISDR) was included. In these regions, amino acid substitutions were associated with the final outcome in an incremental manner, depending upon the number of substitutions.

Conclusions: Viral sequences are more diverse in SVR patients than non-SVR patients receiving PEG-IFN/RBV therapy for genotype-2b HCV infection. Through systematic comparison of viral sequences, several specific regions, including part of the ISDR, were extracted as having significant correlation with the final outcome.

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Introduction

Worldwide, 180 million people are estimated to be infected with hepatitis C virus (HCV), a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN)-based therapy can result in viral clearance as well as biochemical and histological improvements [2]. In this IFN-based therapy, HCV genotype is the most significant factor affecting treatment responses [3,4].

In genotype 2b HCV infection, 80% of patients with high viral titers can achieve a sustained virological response (SVR) to the regimen of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks [5,6]. This response is high considering that much

lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1 [1]. However, in other words, 20% of patients infected with genotype 2b HCV still cannot clear the virus and remain at risk of developing HCC. On the other hand, although various studies have been undertaken to clarify the factors contributing to the response to IFN-based therapy in genotype 1 infection, it remains poorly understood which patients with genotype 2b HCV infection will show unfavorable responses. Recently, the significance of IL28B single nucleotide polymorphisms (SNPs) in determining the response to PEG-IFN/RBV therapy was demonstrated in genotype 1 HCV infection [7,8]. However, the significance of IL28B SNPs was rather weak in genotype 2 HCV infection [9].

In terms of the association between HCV sequence variation and treatment responses, previous studies have reported that

Table 1. Baseline Characteristics of Studied Patients.

Characteristic	SVR (n = 44)	non-SVR (n = 16)	P value
Gender (Male/Female)	26/18	9/7	NS [†]
Age (yrs)	56 (22–72) [*]	59 (30–80)	0.04 [‡]
BMI	23.5 (16.6–30.3)	24.7 (18.5–31.7)	NS [†]
ALT (IU/l)	51 (19–380)	41 (17–390)	NS [†]
GGTP (IU/l)	36 (11–133)	40 (17–292)	NS [†]
T.Chol (mg/dl)	169 (119–225)	178 (145–217)	NS [†]
WBC (/μl)	4600 (2620–7200)	5080 (3270–8600)	NS [†]
Hb (g/dl)	14.2 (11.5–17.3)	14.6 (11.8–16.4)	NS [†]
Platelet (×10 ⁶ /mm ³)	19 (7.1–31.8)	17.8 (8–36.7)	NS [†]
Fibrosis score (0–2/≥3) [§]	38/5	7/3	NS [†]
HCV RNA (KIU/ml)	2050 (100–16000)	1800 (140–6300)	NS [†]
IFN dose (≥80%/60–80%)	36/8	13/3	NS [†]
Ribavirin dose (≥80%/60–80%)	32/12	10/6	NS [†]
RVR rate (%)	55.8	6.3	0.0008 [†]
EVR rate (%)	97.7	68.8	0.004 [†]
ETR rate (%)	100	87.5	NS [†]

§: SVR : n = 43, non-SVR : n = 10.
 *: median (range).
 †: Fisher's exact probability test.
 ‡: Mann-Whitney's U test.
 doi:10.1371/journal.pone.0024514.t001

amino acid variation in the NS5A-ISDR [10], NS5A-IRRDR [11], NS5B [12], PKR-eIF2 phosphorylation homology domain (PePHD) of E2 [13], and Core [14] correlate with the clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy for genotype 1b HCV infection. In the meantime, these viral sequence studies have been controversial regarding their true clinical importance, because the results of different studies were not always coincident [15,16,17]. On this background, recent studies trying to analyze the correlation of complete HCV open reading frame diversity, clinical characteristics, and the response to PEG-IFN/RBV therapy for genotype 1 HCV infection, in the most comprehensive approach yet attempted, have clarified that viral amino acid variation is associated with treatment responses, with consideration of racial background [18,19]. In genotype 2 infection, however, only a few studies have investigated the association of HCV sequence variation and treatment response [20,21] and the clinical significance has been yet established. We reported recently that variation of amino acid (aa) 110 in Core and amino acids (aa) 2258–2308 in NS5A were significantly associated with treatment outcome of the PEG-IFN/RBV therapy for genotype 2a HCV infection, through the analysis of the complete HCV ORFs in Japanese patients [22].

In this study, to assess comprehensively the influence of viral sequence variation on the response to the PEG-IFN/RBV therapy in genotype 2b HCV infection, we determined the complete pretreatment HCV ORFs from Japanese patients and investigated amino acid variation and its correlation with the response to combination therapy with PEG-IFN plus RBV.

Methods

Patients

A total of 77 adult Japanese patients infected with genotype 2b HCV, who received the combination therapy with PEG-IFN

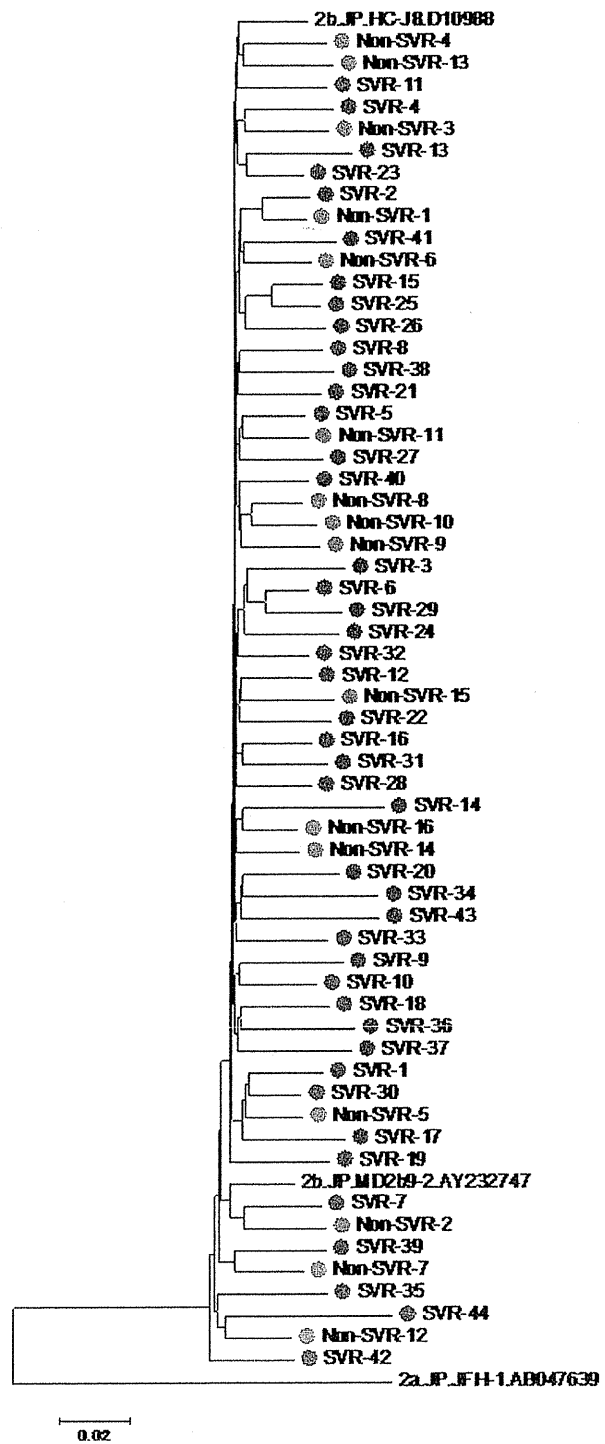


Figure 1. Phylogenetic analysis of the genotype-2b polyprotein sequences. In order to perform the phylogenetic analysis, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients along with reference sequences (2b.HC-J8.D10988, 2b.JP.MD2b9-2, and 2a.JP.JFH-1.AB047639), using the ClustalW program, and constructed the phylogenetic tree using the Neighbor-Joining method with MEGA version 4 software. Blue circles indicate SVR patients and red circles indicate non-SVR patients.
 doi:10.1371/journal.pone.0024514.g001

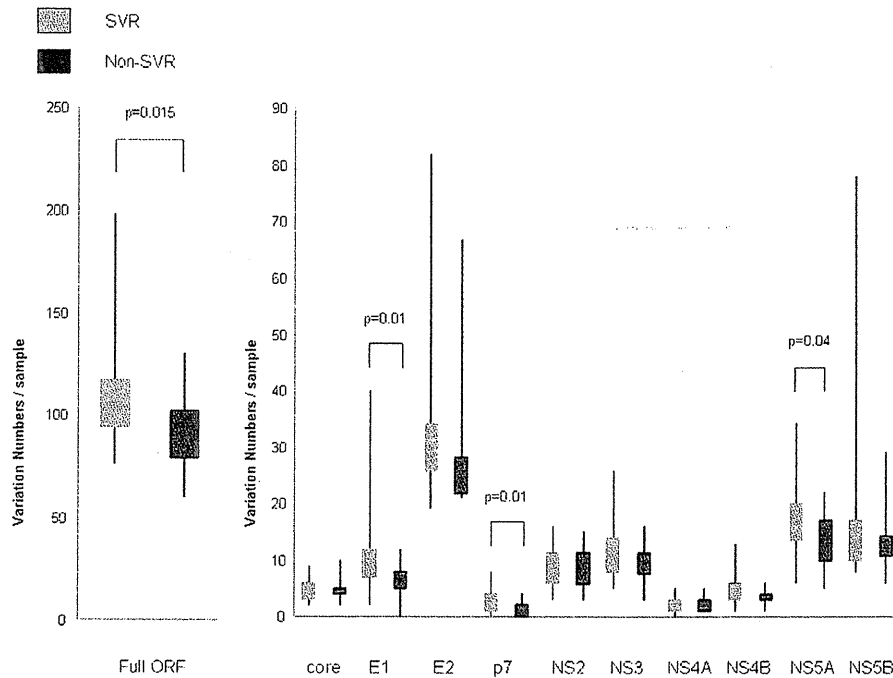


Figure 2. Number of amino acid substitutions per sample in the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) group. The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the complete open reading frame (ORF) (Fig. 1, left) and for each HCV protein (Fig. 1, right). doi:10.1371/journal.pone.0024514.g002

(PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2009 at University of Yamanashi, Tokyo Medical and Dental University,

and related institutions were first included in the study. They all fulfilled following criteria: (1) negative for hepatitis B surface antigen, (2) high viral load (≥ 100 KIU/ml), (3) absence of

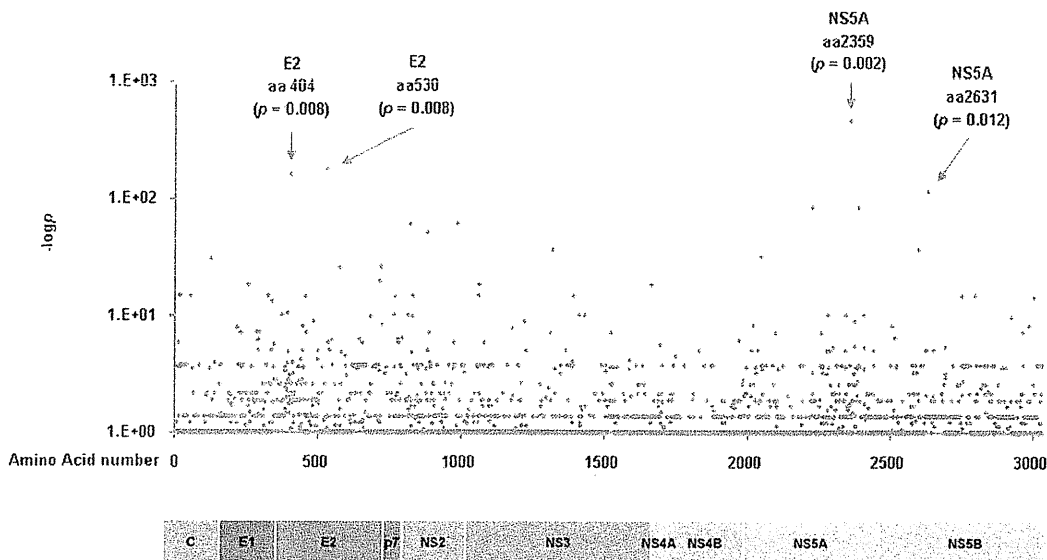


Figure 3. Different amino acid usage at each viral amino acid position between the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) patients. (a) Different amino acid usage at each viral amino acid position between the SVR and the non-SVR patients was analyzed by Fisher's exact probability test. The longitudinal axis shows the $-\log P$ value. (b) Sequence alignment in the Core region is demonstrated. Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes. doi:10.1371/journal.pone.0024514.g003

Table 2. Variation at each Amino Acid Position and SVR rate.

	E2 aa 404 non T	E2 aa 530 non T	NS5A aa 2359 N	NS5B aa 2631 non P
SVR rate	86.1% (31*/36**, p = 0.008)	87.9% (29/33, p = 0.008)	82% (41/50, p = 0.002)	94.7% (18/19, p = 0.012)

*SVR number in patients fulfilling the criteria.
 **Number of patients fulfilling the criteria.
 doi:10.1371/journal.pone.0024514.t002

hepatocellular carcinoma, (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease, (5) free of co-infection with human immunodeficiency virus. To clearly disclose the non-SVR viral characteristics, we have considered only those patients who achieved total drug administration of 60% or more for both PEG-IFN and RBV, with the completion of the standard treatment duration. Moreover, although we excluded patients with extended therapy to make the studied population uniform, we have included non-SVR patients with extended therapy to clarify the specific characteristics of non-SVR patients, a minor population group. As a result, 17 patients were excluded for the following reasons: 1 patient received insufficient dose, 4 patients were discontinued from the therapy within 12 weeks, and 12 SVR patients received extended therapy. Finally, 60 patients were considered as eligible for the study. During the combination therapy, blood samples were obtained at least once every month before, during and after treatment and were analyzed for blood count, ALT and HCV RNA levels. Liver biopsy specimens were obtained from most of the patients. All patients gave written informed consent to the study. The study was approved by the ethics committees of University of Yamanaishi,

Tokyo Medical and Dental University, and related institutions. The therapy was performed according to the standard treatment protocol of PEG-IFN/RBV therapy for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan (PEG-IFN α -2b 1.5 μ g/kg body weight, once weekly subcutaneously, and RBV 600–800 mg daily per os for 24 weeks).

Complete HCV-ORF Sequence Determination by Direct Sequencing from Pretreatment Sera

HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the following protocol. Briefly, 150 μ l of serum were mixed with 700 μ l of Isogen, and an aqueous phase was extracted with 150 μ l of chloroform. RNA was precipitated with 600 μ l of isopropanol and with 2 μ l of Glyco Blue (Ambion, Tokyo, Japan) as a carrier. The purified RNA was washed once with ethanol and finally dissolved in 15 μ l of distilled water and stored at -70° C until use.

Complementary DNA was synthesized according to the following protocol: 30 μ l of the reverse transcription mixture were adjusted to contain 3 μ l of the RNA solution, 300 U of Superscript

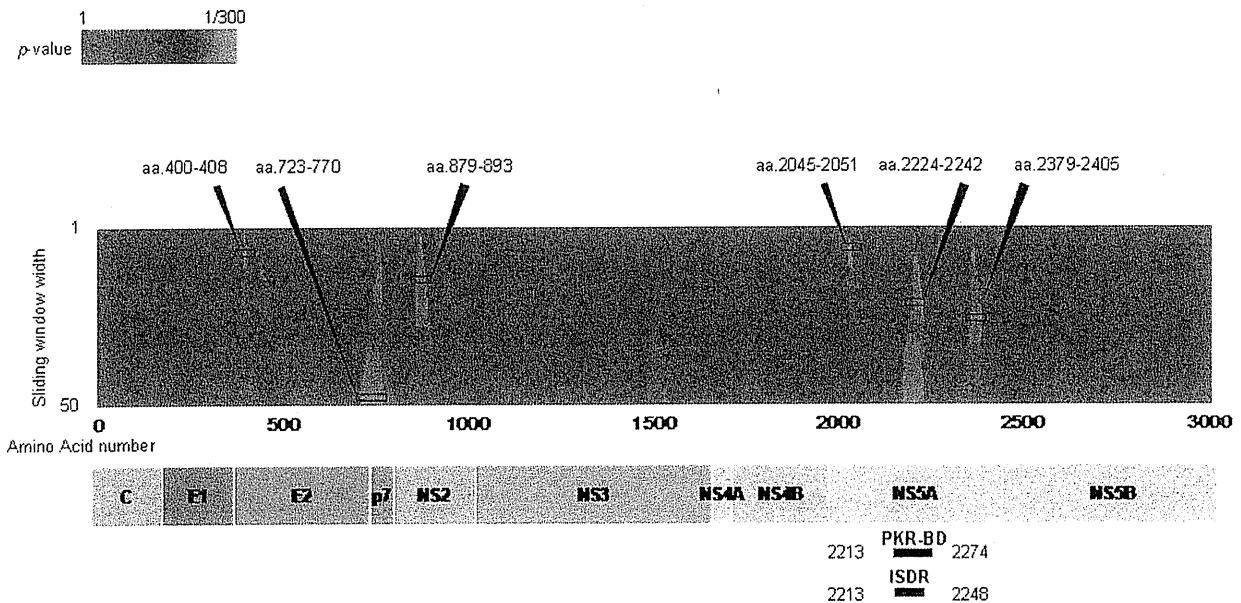


Figure 4. Sliding window analysis. (a) Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis was performed. Viral regions affecting treatment outcome are shown as red areas. There are six hot areas: amino acid 400–408 and 723–770 in the E2 region, amino acid 879–893 in the NS2 region and, amino acid 2045–2051, 2224–2242 and 2379–2405 in the NS5A region. (b) Sequence alignment in the nonstructural (NS)5A around amino acids 2213 to 2274 is demonstrated. Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.
 doi:10.1371/journal.pone.0024514.g004

Table 3. Number of Amino Acid Substitutions in each Region and SVR rate.

	E2 aa 400–408 mutation ≥ 4	E2 aa 723–770 mutation ≥ 2	NS2 aa 879–893 mutation ≥ 2	NS5A aa 2045–2051 absence of mutation	NS5A ISDR (aa 2213–2248) mutation ≥ 1	NS5A aa 2224–2242 mutation ≥ 1	NS5A aa 2379–2405 mutation ≥ 2
SVR rate	86.5% (32*/37**) p = 0.006	100% (18/18) p = 0.001	94.7% (18/19) p = 0.01	89.7% (35/39) p = 0.0002	86.1% (31/36) p = 0.008	90.9% (30/33) p = 0.001	90.9% (20/22) p = 0.03

*SVR number in patients fulfilling the criteria.

**Number of patients fulfilling the criteria.

doi:10.1371/journal.pone.0024514.t003

II (Invitrogen, Tokyo, Japan) with an accompanied buffer according to the manufacturer's instructions, 60 units of RNase inhibitor (Promega Corp., Madison, WI), and 300 pg of random primers (Invitrogen). The mixture was incubated at 37°C for 30 min. The HCV genome was amplified with 24 partially overlapping primer (Table S6) sets, designed specifically for this study, to perform two-step nested PCR. As previously reported, a M13 forward primer (5'-TGTA AACGACGGCCAGT-3') and a M13 reverse primer (5'-CAGGAAACAGCTATGACC-3') were attached to the 5' termini of the sense and antisense second-round PCR primers, respectively, to facilitate direct sequencing. All samples were initially denatured at 95°C for 7 min., followed by 40 cycles with denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 45 seconds with BD AdvantageTM 2 PCR Enzyme System (BD Biosciences Clontech, CA, USA). PCR amplicons were sequenced directly by Big Dye Terminator Version 3.1 (ABI, Tokyo, Japan) with universal M13 forward/M13 reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen) and base-calling errors were corrected following visual inspection of the chromatogram. When several peaks were observed at the same nucleotide position in the chromatogram, the highest chromatogram peak was read as the dominant nucleotide. In sequence analysis, multiple sequence alignment was performed with ClustalW, and the mean genetic distance was calculated using the p-distance algorithm in the MEGA version 4 DNA software. As a result, 60 genotype-2b HCV full open reading frame sequences were determined. In Table S1, obtained GenBank accession numbers for these sequences determined in this study are listed.

Table 4. Multivariate Logistic Regression Analysis.

Factor	odds (95% CI)	p value
Age	0.94 (0.85–1.04)	0.20
E2 aa 530 non T	4.33 (0.48–39.3)	0.19
NS5A aa 2359 N	3.22 (0.18–57.7)	0.43
NS5B 2631 non P	5.14 (0.29–91.2)	0.26
NS2 aa 879–893 mutations ≥ 2	9.77 (0.52–182)	0.13
NS5A aa 2045–2051 no mutations	4.46 (0.39–50.6)	0.23
NS5A aa 2224–2242 mutations ≥ 1	11.0 (1.13–107)	0.04
NS5A aa 2379–2405 mutations ≥ 1	7.03 (0.62–79.8)	0.12

To evaluate the optimal threshold of amino acid variations for SVR prediction in each viral region extracted, a receiver operating characteristic curve was constructed and the most optimal cut off value was determined for each region.

doi:10.1371/journal.pone.0024514.t004

Sliding Window Analysis

A sliding window analysis was introduced to search through HCV amino acid "regions", rather than single amino acid positions, related to the final outcome of PEG-IFN/RBV therapy. Briefly, the total number of amino acid substitutions compared to the consensus sequence within a given amino acid length were counted at each amino acid position in each HCV sequence. The consensus sequence was generated from these 60 patients. Then the relation of substitution numbers and the final outcome was compared statistically between the SVR and non-SVR groups by Mann-Whitney's U test for each amino acid position. In this study, we changed the window length from 1 to 50 to search for those HCV regions. To visualize the result, significantly lower p-values were colored in red and non-significant p-values were colored in green using Microsoft Excel software to generate a "heat map" appearance. In the present study, p-value of 1/300 or lower was colored in the maximum red.

Statistical Analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, and virological data such as sequence variation factors, were determined between the various groups by Mann-Whitney's U test for numerical variables and Fisher's exact probability test for categorical variables. To evaluate the optimal threshold of variations for SVR prediction, a receiver operating characteristic curve was constructed and the area under the curve as well as the sensitivity and specificity were calculated. Variables that achieved statistical significance ($p < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All p values of < 0.05 by the two-tailed test were considered significant.

Results

Characteristics of the patients studied

The SVR rate of the patients analyzed was 75.9% (44/58) with the standard therapy (two non-SVR patients received extended therapy). The baseline characteristics of the patients classified according to achievement of SVR are shown in Table 1. Rapid virological response (RVR; undetectable serum HCV RNA within 4 weeks) and early virological response (EVR; undetectable serum HCV RNA within 12 weeks) rates were significantly higher in SVR patients ($p = 0.0008$ and 0.004). In addition, patients with non-SVR were older ($p = 0.04$). Pretreatment HCV RNA titer, which is known to affect the treatment outcome in genotype 1 and 2a HCV infection, did not differ significantly between two groups. Achievement of RVR reached 42.4% when all patients were included, and this rate was high compared to achievement of RVR in patients with genotype 1b infection (~10%) observed in

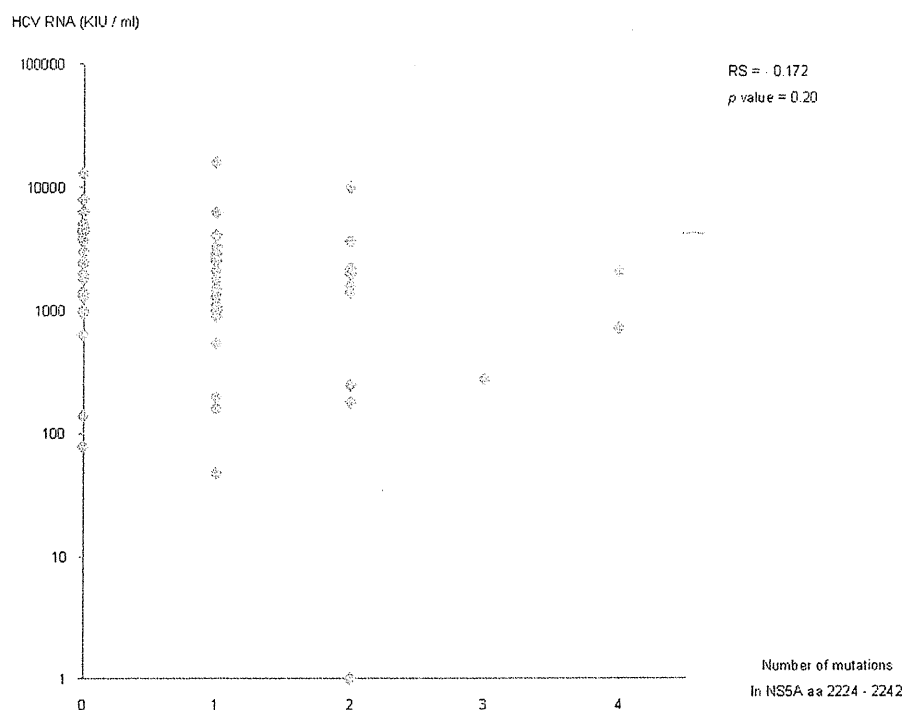


Figure 5. Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2224 to 2242. Spearman's correlation coefficient by rank test is demonstrated. doi:10.1371/journal.pone.0024514.g005

University of Yamanashi (data not shown). The early virological response (EVR) rate was equally high in the SVR (97.7%) and non-SVR (68.8%) groups. Interestingly, most of the non-SVR patients (14/16, 87.5%) in genotype-2b HCV infection showed end-of-treatment response (ETR; undetectable serum HCV RNA at the end of therapy), demonstrating that the main cause of non-SVR was relapse (reappearance of hepatitis C viremia during the follow-up period after stopping therapy in patients with an ETR,

$n = 14$), and not null response (detectable serum HCV RNA at the end of therapy, $n = 2$).

Phylogenetic analysis of SVR and non-SVR patients using the complete HCV amino acid sequence

To determine the viral sequence characteristics in the SVR and non-SVR groups, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients' pretreatment sera along

Table 5. Baseline Characteristics of patients with NS5A aa 2224–2242 variations none or 1≤.

Characteristic	Variation 1≤ (n = 33)	No variation (n = 27)	P value
Gender (Male/Female)	17/16	18/9	NS [†]
Age (yrs)	57 (29–72) [‡]	57 (22–80)	NS [‡]
ALT (IU/l)	72 (19–380)	47 (17–390)	NS [‡]
Platelet ($\times 10^4/\text{mm}^3$)	19.3 (7.1–31.8)	17.5 (10.4–36.7)	NS [‡]
Fibrosis score (0–2/≥3) [§]	26/5	19/3	NS [‡]
HCV RNA (KIU/ml)	1600 (100–16000)	2450 (140–13000)	NS [‡]
IFN dose (≥80%/60–80%)	26/7	23/4	NS [‡]
Ribavirin dose (≥80%/60–80%)	24/9	19/8	NS [‡]
RVR rate (%)	53.1	29.6	NS [†]
EVR rate (%)	96.9	81.5	NS [†]
SVR rate (%)	90.9	51.9	0.001 [†]
Relapse rate (%)	40.7	9.1	0.006 [†]

[§]: 1≤ : n = 31, 0 : n = 22.

[‡]: median (range).

[†]: Fisher's exact probability test.

[‡]: Mann-Whitney's U test.

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with reference sequences (2b.HC-J8.D10988, 2JP.MD2b9-2, and 2a.JP.JFH-1.AB047639 obtained from the Los Alamos HCV Database as representative sequences for genotype 2b and genotype 2a HCV) and constructed a phylogenetic tree (Fig. 1). As demonstrated in the tree, no evident clustering was apparent according to the difference of responses.

Comparison of amino acid variation between the SVR and non-SVR in the complete HCV polyprotein and each HCV protein

Next, we compared amino acid variations that were unique, relative to a population consensus, to either the SVR or non-SVR patients for the complete HCV polyprotein and each HCV protein. The number of amino acid variations in the sequences from the SVR patients was significantly higher than in those from the non-SVR patients, when the entire HCV polyprotein was analyzed (Fig. 2, left). These differences were especially significant in E1, p7 and NS5A (Fig. 2, right). This result demonstrated that HCV sequences from patients with SVR comprised a heterogeneous population, while HCV sequences from patients with non-SVR comprised a rather homogeneous population, indicating the existence of unique non-responsive HCV sequences in those regions in E1, p7, and NS5A.

Comparison of HCV sequence variation between the SVR and non-SVR patients at each amino acid position

Each amino acid position in the HCV ORF was compared to detect any differences between the SVR and non-SVR patients. In Fig. 3a, differences in amino acid residues at each position are shown as dots demonstrating $-\log P$ values. As shown in Table 2, four points were extracted: amino acid (aa) 404 in the E2 region ($p = 0.008$), aa 530 in the E2 region ($p = 0.008$), aa 2359 in the NS5A region ($p = 0.002$) and aa 2631 in the NS5B region ($p = 0.012$). Among them, the residue at aa 2359 in the NS5A region differed most frequently between the SVR and non-SVR patients. Amino acids 4 and 110 in the Core region, residues that have been reported to vary according to the virological responses in genotype 2a infection [22,23], did not differ significantly in this genotype 2b HCV study. Meanwhile, amino acids 70 and 91, which have been reported to vary according to virological response to PEG-IFN/RBV therapy in genotype 1b infection, were conserved irrespective of the outcome (Fig. 3b).

Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis

Fig. 4a and Table 3 shows the result of sliding window analysis. This approach was used to detect differing HCV amino acid "regions", rather than single amino acid positions, between the SVR and the non-SVR patients. According to the result, six regions were associated with the final outcome (p-values less than 1/20): aa 400-408 in the E2 region ($p = 0.006$), aa 723-770 in the E2 and the N-terminus of p7 region ($p = 0.001$), aa 879-893 in the NS2 region ($p = 0.01$), aa 2045-2051 in the NS5A region ($p = 0.0002$), aa 2224-2242 in the NS5A region ($p = 0.001$) and aa 2379-2405 in the NS5A region ($p = 0.03$). Interestingly, aa 2224-2242 in the NS5A was located in the interferon sensitivity determining region (ISDR). Fig. 4b shows the aligned sequences of amino acids around 2213-2274 of HCV NS5A. Among these 6 regions, aa 723-770, aa 879-893, aa 2224-2242, and aa 2379-2405 were correlated with the final outcome in an incremental manner according to the number of substitutions in those regions (Table S2, S3, S4, S5). The number of substitutions in the ISDR

was also correlated to the final outcome in an incremental step-wise manner (data not shown).

Multivariate analysis to detect independent predictive factors contributing to the SVR

Next, multivariate analysis was undertaken to identify pretreatment variables correlated with the final outcome. To evaluate the optimal threshold of amino acid variations for SVR prediction in each viral region extracted, a receiver operating characteristic curve was constructed and the most optimal cut off value was determined for each region. E2 aa404-408 was excluded from the analysis because we considered that the region was unlikely to be truly associated to the outcome as it is located in the hypervariable region, the region of the highest mutation rate in the HCV genome as a result of host's immune attack. E2 aa 723-770 was excluded from the analysis because all the patients above the cut-off value in the region achieved SVR and an odds calculation was not possible. The ISDR was also excluded because NS5A aa2224-2242 was completely contained in the ISDR. In addition, variables of EVR and RVR were excluded because they were post treatment variables. The multivariate analysis revealed that only NS5A aa 2224-2242 (odds ratio 11.0, $p = 0.039$) was finally identified as the independent variable predicting the final outcome (Table 4).

Biological relevance of variation in NS5A in this study group

Because NS5A aa 2224-2242 is located within the ISDR, for which the amino acid substitution numbers have been reported to be correlated with the HCV RNA titer in genotype 1 and 2a HCV infection [13], we analyzed the relationship between amino acid variations in that region and pretreatment HCV RNA titers. Contrary to our expectation, no evident relationship was found between variations in the NS5A region aa 2224-2242 and HCV RNA titer (Fig. 5). On the other hand, as shown in Table 5, although the initial viral responses (RVR or EVR) did not show evident association with the amino acid variations in the region, treatment relapse was significantly correlated with the amino acid variations in the region. In addition to NS5A aa 2224-2242, there was no evident relationship between HCV RNA level and variations in the other regions found in this study (data not shown).

Discussion

In this study, we showed that genotype 2b HCV sequences from Japanese patients who achieved SVR were more diverse than the sequences from patients with non-SVR. The result that SVR patients were more diverse in their HCV sequences than non-SVR patients is in accordance with previous studies of genotype 1 HCV infection, although the diverse viral genes varied according to genotype [18,19]. We found that these diversities were primarily found in E1, p7 and NS5A.

In systemic searching for single amino acid positions or consecutive amino acid regions in the HCV ORF associated with the treatment outcome, several regions were extracted in E2, p7, NS2, NS5A and NS5B. Among those identified regions, E2 aa 723-770, NS2 aa 879-893, NS5A aa2224-2242, and NS5A aa2379-2405 were correlated with the final outcome in an incremental manner according to the number of amino acid substitutions. Specifically, the sequences of those regions in non-SVR patients were almost homogeneous, while the sequences of the region in SVR patients were significantly diverse and multiple amino acid substitutions were found compared to the consensus sequence. Interestingly, among those regions, aa 2224-2242 was completely included in the ISDR, in which the number of amino acid substitutions is known to show significant correlation with

the treatment response to IFN-based therapy in genotype 1b, and also in genotype 2 [21,24].

In recent studies of genotype 1b infection, amino acid variation of residues 70 and 91 in the Core were reported to be associated with the treatment response to IFN-based therapy. The correlation of amino acid variation in the Core (residues 4 and 110) with the response to PEG-IFN/RBV therapy was also identified in genotype 2a infection [22,23]. In genotype 2b infection, however, we could not find such associations between amino acid variation in the core region and the response to PEG-IFN/RBV therapy (Fig. 3b). Amino acid residues of aa 70 and 91 were conserved irrespective of differences in the PEG-IFN/RBV responses. On the other hand, although amino acid variations were also sometimes found at residues 4 and 110 in genotype 2b HCV, their frequency was low, and no evident association between the variation and the treatment response was found. Although the reason of the lack of association between the Core and the PEG-IFN/RBV treatment response in genotype-2b HCV infection is unknown, it suggests that a different mechanism affecting the treatment response might exist, depending on genotype-specific viral features.

In genotype 1 HCV, variations within the PKR-binding region of NS5A, including those within the ISDR, were reported to disrupt the NS5A-PKR interaction, possibly rendering HCV sensitive to the antiviral effects of interferon [25]. Clinically, the number of substitutions within the ISDR has been reported to correlate with the serum HCV RNA level in genotype 1 and 2a infections [13]. In addition, a recent study reported that mutations in the ISDR also show the correlation with the relapse in the PEG-IFN/RBV therapy in genotype 1b infection [26]. Because NS5A aa2224–2242, part of ISDR, was extracted as one of those regions related to the treatment response in genotype 2b infection, we undertook further analysis to investigate the correlation between amino acid variation numbers and serum HCV RNA level. Though the reason is unknown, we could not find evidence of a relationship between variation in the NS5A aa 2224–2242 and HCV RNA titer in genotype 2b infection, unlike genotypes 1 and 2a. Of note, a high SVR rate in genotype 1 and genotype 2a infection is known to be closely correlated with a low HCV RNA level and multiple substitutions in ISDR. However, in genotype 2b infection in our study, there was no significant difference in the HCV RNA level between SVR and non-SVR patients, as shown in Table 1. Previously, the role of the ISDR in the contribution to SVR in genotype 1 and 2a has been discussed in detail in the context of serum HCV RNA level, and multiple substitutions in the ISDR are related to a low HCV RNA level and high SVR rate. However, it is not known which of these two factors is directly associated with viral clearance. Consideration of this three-sided relationship of ISDR, HCV RNA level and SVR rate in genotype-2b infection leads to the suggestion that amino acid variation in ISDR to be more direct contributor for SVR.

In spite of these findings, there were still limitations in our study. First, because genotype 2b infection only accounts for 10% of all HCV infection in Japan, the number of studied patients was rather small, especially non-SVR patients. In addition, because genotype 2b HCV contains as many as 3033 amino acids, it is possible that incorrect amino acids or regions were judged as significant in the complete HCV ORF comparison study as a result of type I errors.

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Therefore, if more patients were available for the analysis, the statistical power detecting the meaningful differences would be greater. Secondly, we could not include the IL28B SNP analysis in this study. If we could have combined the information of IL28B SNPs with the full HCV ORF information, a more comprehensive analysis would have been achieved.

In conclusion, we have shown that viral sequences were more diverse in SVR patients infected with genotype 2b HCV. Through systematic comparison between SVR and non-SVR patients, we have also shown that several localized regions were extracted as hot spots whose amino acid substitutions were closely related to the final outcome by affecting the relapse rate in the PEG-IFN/RBV therapy.

Supporting Information

Table S1 GenBank Accession Numbers. Obtained GenBank accession numbers for 60 genotype-2b HCV full open reading frame sequences are listed. (DOC)

Table S2 Substitutions in NS5A aa 2224–2242 Amino Acid Regions and SVR rate. SVR rate increased with the number of substitutions in this region. (DOC)

Table S3 Substitutions in NS5A aa 2379–2405 Amino Acid Regions and SVR rate. SVR rate increased with the number of substitutions in this region. (DOC)

Table S4 Substitutions in NS2 aa 879–893 Amino Acid Regions and SVR rate. SVR rate increased with the number of substitutions in this region. (DOC)

Table S5 Substitutions in E2 aa 723–770 Amino Acid Regions and SVR rate. SVR rate increased with the number of substitutions in this region. (DOC)

Table S6 PCR Primer List. Primers designed to perform two-step nested PCR for this study are listed. Dominant genotype-2b HCV full open reading frame sequences was determined by the 24 partially overlapping amplicons amplified by these primers. (XLS)

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Author Contributions

Conceived and designed the experiments: MK SM NE. Performed the experiments: MK. Analyzed the data: MK SM NE. Contributed reagents/materials/analysis tools: RS MM HS KK. Wrote the paper: MK SM NE. Critical revision of the manuscript for important intellectual content: FA TU TI MS MN NS MW.

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Analysis of Interferon Signaling by Infectious Hepatitis C Virus Clones with Substitutions of Core Amino Acids 70 and 91^{∇§}

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Substitution of amino acids 70 and 91 in the hepatitis C virus (HCV) core region is a significant predictor of poor responses to peginterferon-plus-ribavirin therapy, while their molecular mechanisms remain unclear. Here we investigated these differences in the response to alpha interferon (IFN) by using HCV cell culture with R70Q, R70H, and L91M substitutions. IFN treatment of cells transfected or infected with the wild type or the mutant HCV clones showed that the R70Q, R70H, and L91M core mutants were significantly more resistant than the wild type. Among HCV-transfected cells, intracellular HCV RNA levels were significantly higher for the core mutants than for the wild type, while HCV RNA in culture supernatant was significantly lower for these mutants than for the wild type. IFN-induced phosphorylation of STAT1 and STAT2 and expression of the interferon-inducible genes were significantly lower for the core mutants than for the wild type, suggesting cellular unresponsiveness to IFN. The expression level of an interferon signal attenuator, SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type. Interleukin 6 (IL-6), which upregulates SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type, suggesting interferon resistance, possibly through IL-6-induced, SOCS3-mediated suppression of interferon signaling. Expression levels of endoplasmic reticulum (ER) stress proteins were significantly higher in cells transfected with a core mutant than in those transfected with the wild type. In conclusion, HCV R70 and L91 core mutants were resistant to interferon *in vitro*, and the resistance may be induced by IL-6-induced upregulation of SOCS3. Those mechanisms may explain clinical interferon resistance of HCV core mutants.

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality. Approximately 3% of the worldwide population is infected with HCV, which represents 170 million people, and 3 million to 4 million individuals are newly infected each year (33, 47, 62). There is no therapeutic or prophylactic vaccine available for HCV. Antiviral treatment has been shown to improve liver histology and decrease the incidence of hepatocellular carcinoma in chronic hepatitis C (CHC) (17, 64). Current therapies for CHC consist of treatment with pegylated interferon (peg-IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism (16, 31). However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained virological response (SVR) or a cure of the infection (14, 16). Given this situations, gaining a detailed understanding of the molecular mechanisms of interferon (IFN) resistance has been a high priority in academia and industry.

The response to peg-IFN-plus-RBV treatment is affected by

several viral and host factors, including age, gender (22, 23), grade of liver fibrosis (21, 42), HCV genotype, and serum viral load (14, 59). Several viral genetic factors influence treatment outcomes, including mutations in NS5A-interferon sensitivity determining region (ISDR) (13, 38) and the core region (4, 6). Akuta et al. reported that HCV-core amino acid substitutions at positions 70 and 91 are significantly correlated with poor responses to peg-IFN-plus-RBV therapy (6) and with increased hepatocarcinogenesis (2, 3). Furthermore, it was reported recently that the core amino acid 70 and amino acid 91 substitutions are associated with a poor response to peg-IFN, RBV, and telaprevir combination therapy, respectively (1). However, the underlying molecular mechanisms of such distinct biological properties of the core 70/91 mutations are poorly understood.

In this study, we have analyzed virus infection and replication kinetics and response to interferon treatment using the HCV-JFH1 cell culture system (HCVcc) (60, 65). We constructed HCVcc expressing virus with substitutions of core amino acid 70 and amino acid 91 (R70Q, R70H, and L91M). The core mutant HCV clones were compared in terms of intracellular replication, infectious virus production, and sensitivity to alpha interferon (IFN- α). Here we have shown that the differences in sensitivity to IFN are attributable to upregulated overexpression of the cellular interferon signal attenuator SOCS3 and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

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MATERIALS AND METHODS

Reagents. Recombinant human IFN- α 2b was from Schering-Plough (Kenilworth, NJ). Beta-mercaptoethanol was from Wako (Osaka, Japan). Antibodies used were SOCS3 and SOCS1, which were from Cell Signaling (Beverly, MA), HCV core (Abcam, Cambridge, MA), NSSA (BioDesign, Saco, ME), GRP78, GADD153/CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), disulfide isomerase (PDI) (Stressgen Biotechnologies, Victoria, British Columbia, Canada), and beta-actin antibody (Sigma). Secondary antibodies were peroxidase-labeled anti-mouse, anti-rabbit antibody (GE Healthcare, Connecticut), donkey anti-goat IgG-horse radish peroxidase (HRP) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Alexa 405-labeled goat anti-mouse and Alexa 568-labeled donkey anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA).

Cells and cell culture. Huh7 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) (Sigma Chemical Co, St. Louis, MO) supplemented with 2 mmol/liter L-glutamine and 10% fetal bovine serum at 37°C under 5.0% CO₂.

Sequence analyses. Nucleotide sequences were read from both strands using BigDye Terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems).

Establishment of mutant HCV clones. In order to introduce various mutations into the core region of JFH1, plasmid pJFH1full was digested with EcoRI and BsiWI, and then the DNA fragment encompassing nucleotides 1 to 456 was subcloned into the pGEM-T Easy vector (Promega, Madison, WI). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Change II site-directed mutagenesis kit; Stratagene): R70Q, R70H, L91M, and GKPG77-80KKKK. Finally, the EcoRI-BsiWI fragments were subcloned back into the parental plasmid, pJFH1full.

In vitro RNA synthesis and transfection. Full-length HCV expression plasmids were as follows: pJFH1full, which encodes the full-length HCV-JFH1 sequence (60), pR70Q, pR70H, pL91M, and p7780K. These plasmids were linearized at their 3' ends and used as templates for HCV RNA synthesis using the RiboMax large-scale RNA production system (Promega, Madison, WI). After DNase I (RQ-1 RNase-free DNase; Promega) treatment, the transcribed HCV RNA was purified using Isogen reagent (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7 cells were washed twice in phosphate-buffered saline (PBS), and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μ g of HCV RNA, transferred into a 4-mm electroporation cuvette, and finally subjected to an electric pulse (1,050 μ F and 270 V) using the Easy Jet system (EquiBio, Middlesex, United Kingdom). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish. Forty-eight hours after transfection, the levels of HCV replication and viral protein expression were detected by real-time PCR and Western blotting.

HCVcc infection analyses. Huh7 cells were plated on 12-well plates at a density of 1.2×10^4 cells per well. Supernatants from HCV RNA-transfected cells were inoculated onto each well at a titer of 8×10^5 copies/well (quantified by real-time reverse transcriptase PCR [RT-PCR]). Forty-eight hours after infection, various amounts of interferon were added, and the cells were harvested after 72 h of the interferon treatment (48).

RNA extraction, cDNA synthesis, and real-time RT-PCR analysis. For the detection of HCV RNA in culture supernatant, the supernatant was passed through a 0.45- μ m filter (Millex-HA, Millipore, Bedford, MA) and stored at -80°C until use. Protocols and primers for the real-time RT-PCR analysis of HCV RNA have been described previously (48). For the detection of endogenous mRNAs, total cellular RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Expression of mRNA was quantified using the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA).

Luciferase assays. Luciferase activities were measured using a luminometer (Lumat LB9501; Promega) using the Dual-Luciferase reporter assay system (Promega). Assays were performed in triplicate.

Western blot analysis. Western blotting was carried out as described previously (24, 53, 63). Briefly, 10 mg of total cell lysate was separated using NuPAGE 4%-12% Bis-Tris gels (Invitrogen) and blotted onto a polyvinylidene fluoride (PVDF) Western blotting membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom).

Immunohistochemistry. HCV-transfected Huh7 cells were cultured on 18-mm round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, lipid droplet, and endoplasmic reticulum (ER), cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 h at 37°C. The fluorescent secondary antibodies were Alexa 405 goat anti-mouse and 568 donkey anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA). Lipid droplets (LDs) were visualized by using Bodipy 493/503 dye (Invitrogen). Cells were mounted with Vecta Shield mounting medium and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and visualized by using a confocal laser scanning microscope (FV10i; Olympus, Tokyo, Japan).

Calculation of 50% effective concentrations (EC₅₀). The EC₅₀ was calculated as the concentration of IFN required for 50% reduction in HCV RNA expression. We used the probit regression analysis to obtain values.

Statistical analyses. Statistical analyses were performed by using Welch's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

HCV core 70/91 mutants show resistance to IFN treatment.

First, we investigated sensitivity to IFN treatment of the HCV core mutant R70Q, R70H, and L91M virus clones and compared them to the wild type. The wild type and core mutants were transfected into Huh7 cells, which were cultured in the presence of various concentrations of IFN- α for 48 h. RNA was extracted from the cells and culture supernatant, and the level of HCV RNA was quantified by real-time RT-PCR. Although the levels of supernatant HCV RNA did not differ between the wild type and core mutants (Fig. 1A), the levels of cellular HCV RNA showed that all three core mutants were significantly resistant to IFN compared to the wild type, with EC₅₀s of 5.0 IU/ml, 48 IU/ml, 32 IU/ml, and 47 IU/ml for the R70Q, R70H, L91M, and mutants and the wild type, respectively (Fig. 1B). To exclude the possible effects on interferon signaling by the input HCV RNA, we performed interferon sensitivity analyses by HCVcc infection. As shown in Fig. 1C, the interferon sensitivities of HCV core mutants and the wild type were consistent with the results of HCV RNA transfection. Similarly, according to Western blotting, the core mutants were more resistant to IFN treatment than the wild type (Fig. 1D).

Core mutants show decreased secretion of viral particles.

To determine the mechanisms underlying the resistance to interferon, we compared baseline virus expression levels in cells and culture supernatants. The three core mutants, carrying R70Q, R70H, and L91M, expressed significantly higher levels of intracellular HCV RNA than the wild type, as well as the 7780K clone. (Fig. 2A). 7780K was a negative-control clone that lacked virus particle secretion (37). On the contrary, these core mutants released significantly smaller amounts of HCV RNA into the culture supernatant than the wild type, as well as the negative-control 7780K clone. (Fig. 2B). Consistent with the HCV RNA data, Western blotting showed that cellular HCV core protein levels were higher for the core amino acid 70/91 mutants than the wild type (Fig. 2C). These results suggested that the core 70/91 mutant clones were partially defective in the secretion of infectious virus particles.

Subcellular localization of wild-type and mutant core proteins and lipid droplets. It has been reported that HCV core protein localizes on the cellular LD membrane and may mediate encapsidation of viral genomic RNA and subsequent virus assembly (35, 36). Therefore, we visualized the subcellular localization of wild-type and mutant core proteins in rela-

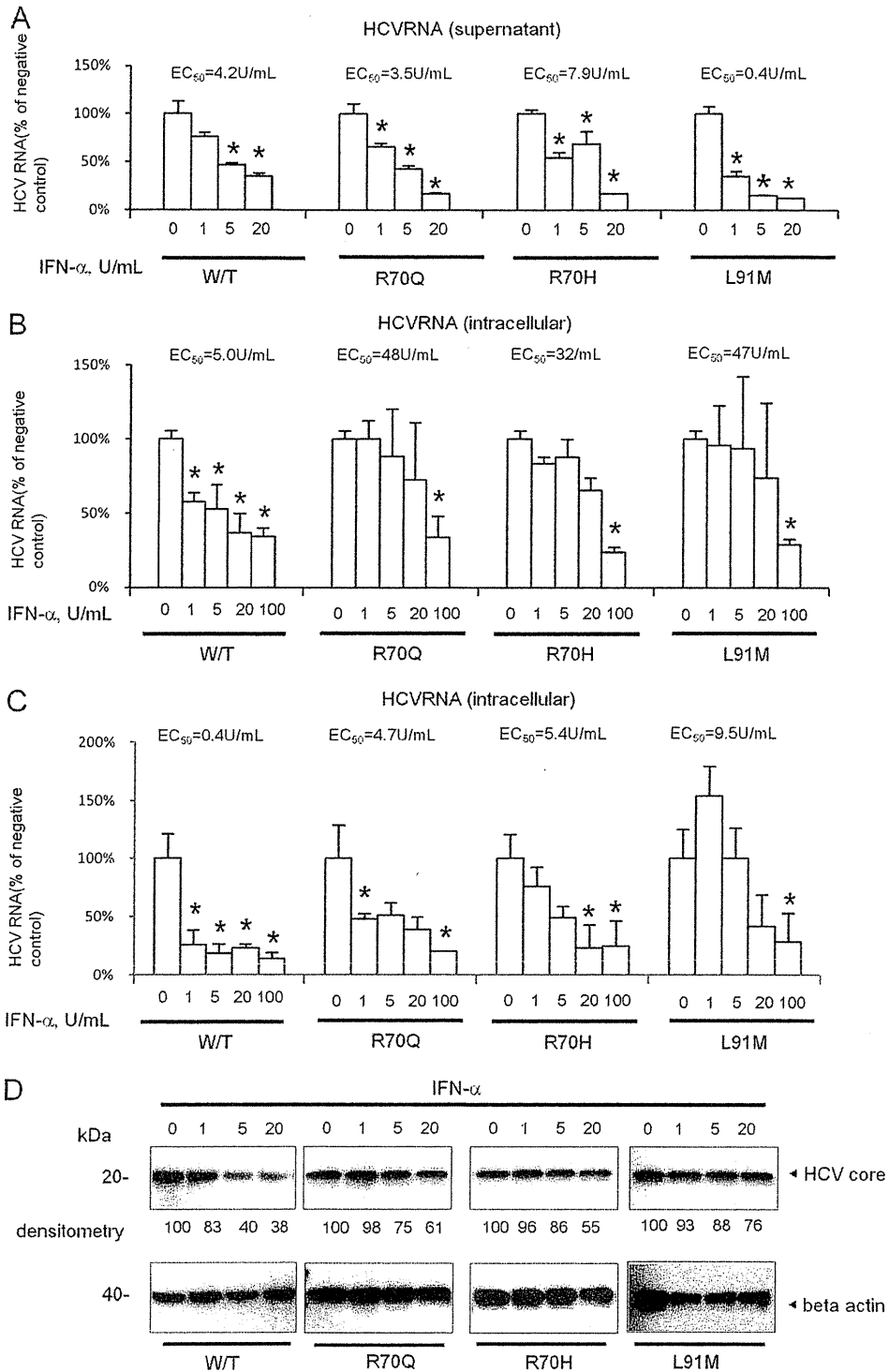


FIG. 1. Comparison of interferon sensitivity between HCV wild type and core mutant clones. The wild type and core mutants were transfected into Huh7 cells and cultured in the presence of IFN- α 2b at concentrations ranging from 0 to 100 U/ml. (A) The culture supernatant of HCV-transfected Huh7 cells was collected 72 h after transfection, and the levels of HCV core antigen in the culture supernatant were measured. The values are displayed as percentages of those for the IFN-untreated control. The experiments were repeated three times, and representative results are shown. (B) Expression of intracellular HCV RNA. Cellular RNA was harvested at 72 h posttransfection. HCV RNA was quantified by real-time RT-PCR. The values are displayed as percentages of those for the IFN-untreated control. (C) Expression of intracellular HCV RNA. Cellular RNA was harvested at 72 h postinfection. HCV RNA was quantified by real-time RT-PCR. The values are displayed as percentages of those for the IFN-untreated control. In panels A through C, asterisks indicate *P* values of less than 0.05, compared to results for the interferon-negative control. (D) Western blotting was performed to assess intracellular suppression of HCV core protein. Ten micrograms of harvested cell lysates were subjected to Western blotting using anti-HCV core antibodies. Densitometry of core protein was performed, and results are shown as percentages of the results for an IFN-negative sample.

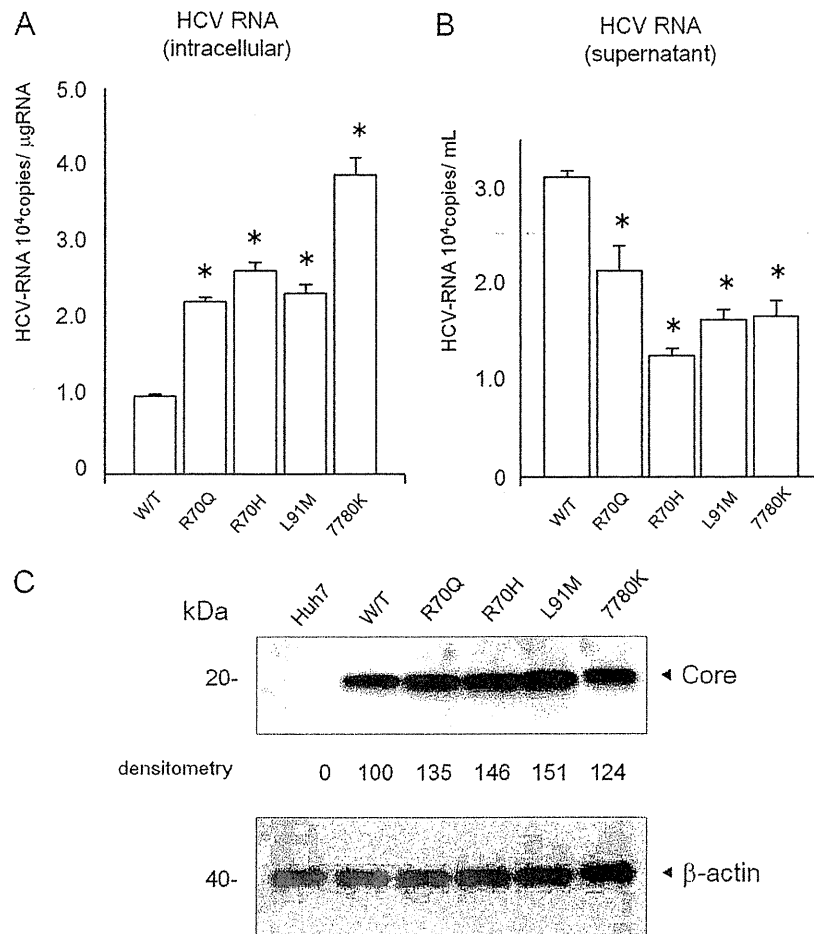


FIG. 2. Analysis of intracellular and supernatant HCV RNA levels in core 70/91 mutants. *In vitro*-transcribed mutant and wild-type RNAs were transfected into Huh7 cells. Three days after transfection, RNA was extracted from cells (A) or culture supernatant (B) and quantified by real-time RT-PCR. Asterisks indicate *P* values of less than 0.05 compared to results for the wild type. (C) Western blotting. Expression of core proteins in HCV-transfected cells. Total cellular protein was prepared from HCV RNA-transfected cells, and Western blotting was performed using anticore and anti-beta-actin antibodies. Densitometry was performed, and results are shown as percentages of that for an HCV-negative sample.

tion to that of LDs and the ER by indirect immunofluorescence and confocal microscopy. Consistent with previous reports, core proteins were colocalized with LDs but not with an ER-located protein, PDI, in the HCV-transfected cells (see the figure in the supplemental material). There were no obvious differences in colocalization of core and LDs or core and ER between the wild type and mutant core proteins.

Induction of interferon-stimulated genes following treatment of HCV-transfected cells with interferon. To investigate the mechanism of the relative IFN resistance of the core 70/91 mutants, as demonstrated in Fig. 1, we analyzed the cellular IFN signaling pathway. First, we assessed the expression and IFN-mediated induction of the mRNA transcripts of the IFN-stimulated genes (ISGs), encoding P56, double-stranded RNA-dependent protein kinase R (PKR), and 2',5'-oligoadenylate synthetase (25AS), which mediate direct antiviral effects on HCV expression (24, 25). Cellular expression of PKR, P56, and 25AS was substantially increased in HCV-transfected cells, as well as naive cells, following IFN treatment. However, the levels of induction were significantly lower

in the three HCV core mutant-transfected cells than in wild-type-transfected cells (Fig. 3A, B, and C). We next detected IFN-induced phosphorylation of STAT1 and STAT2 in the mutant and wild-type HCV-expressing cells. Our previous experiments showed that the levels of phosphorylated STAT1 and STAT2 (pSTAT1 and pSTAT2, respectively) increased within minutes of the addition of IFN and decreased subsequently at 8 h (25). Therefore, we detected pSTAT1 and pSTAT2 levels before and at 15 min after the addition of IFN. As shown in Fig. 3D and E, levels of pSTAT1 and pSTAT2 were lower in core mutant-transfected and -infected cells after IFN treatment than in wild-type-transfected cells and naive cells. These findings indicate that the differences in sensitivity to interferon of core mutant clones and the wild type were associated with attenuation of the cellular IFN signaling pathway.

SOCS3 is upregulated in core mutant clones-transfected, IFN-resistant cells. We examined next the effects of HCV replication on the expression of SOCS1 and SOCS3, proteins that suppress IFN receptor-mediated signaling (50, 58). There was no significant difference in expression levels of SOCS1