creates a novel gene, designated as *IFNL4*, that encodes the interferon-λ4 protein (IFNL4) having moderate similarities to IFNL3. *IFNL4* is transcribed and translated in primary hepatocyte cell lines by the double-stranded RNA virus analog polyinosinic:polycytidylic acid, causing upregulation of interferon-stimulated genes in these cells and replication reduction of an HCV subgenomic replicon (10). In addition, ss469415590 is strongly associated with HCV clearance in African-Americans (9), but this polymorphism has not yet been investigated in the Japanese population. The objective of this study was to determine the prevalence of this *IFNL4* SNP among the Japanese and assess whether it influenced the treatment outcome of PEG-IFN and RBV therapy in patients with chronic hepatitis C.

We recruited 213 treatment-naïve patients with chronic hepatitis C and 176 healthy subjects. Patients were seen at Shinshu University Hospital or affiliated hospitals. Controls were hospital staff volunteers who had indicated the absence of any major illness on a standard questionnaire. The racial background of all subjects was Japanese. Diagnosis of chronic hepatitis C was based on the following criteria, as reported previously (11): (1) presence of serum HCV antibodies and detectable viral RNA; (2) absence of detectable hepatitis B surface antigen and antibody in the human immunodeficiency virus; and (3) exclusion of other causes of chronic liver disease. No patient had a history of, or developed, decompensated cirrhosis or HCC. The baseline characteristics of the patients are shown in Table 1. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine (No. 302) and all participants provided written informed consent.

Antibodies to HCV were measured in serum samples via third-generation Abbott HCV EIA-3 assays (Abbott Laboratories, Abbott Park, IL). Serum levels of HCV RNA were determined using Cobas Amplicor assays (sensitivity: 50 IU/ml; Roche Diagnostic Systems, Tokyo, Japan). All patients in our test cohort were infected with genotype 1b. Relevant biochemical tests were performed using standard methods (12).

Patients received bodyweight-adjusted doses of PEG-IFNα-2b (PegIntron, MSD K.K., Tokyo, Japan) and RBV (Rebetol, MSD K.K.) for 48 weeks, as reported previously (13). Response to therapy was categorized as follows: an SVR was defined as undetectable serum HCV RNA 24 weeks after completing therapy. Relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A nonresponse was defined as a decrease in HCV RNA of <2 log copies/ml at week 12 and detectable HCV RNA during the treatment course.

Genomic DNA was isolated from whole blood samples by phenolic extraction of sodium dodecyl sulfate-lyzed and proteinase K-treated cells, as described previously (14). Genotyping of the rs8099917 SNP (T/G) was performed using an ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) (8). Exon 1 of the *IFNL4* gene was amplified by the polymerase chain reaction (PCR) in the presence of Takara Taq™ (Takara Bio Inc., Otsu, Japan) with the sense primer (5′-CATTGCCTTCCCTGGGATCCTAAC-3′) and the antisense primer (5′-GGACCCCTTGGGACAGGAAC-3′). The sizes of the amplified DNA fragments (333 or 334 bp) were confirmed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. PCR products were directly sequenced with a BigDye Terminator Cycle Sequencing Reaction Kit using an ABI 3100 DNA sequencer. We assessed the rs4803221 (C/G), ss469415590 (TT/ΔG), rs73555604 (G/A), and rs150891559 (G/C) SNPs in this study.

Statistical analyses were performed using PASW Statistics 21.0J software (IBM, Tokyo, Japan). The Mann–Whitney *U*-test was used to analyze continuous variables, whereas the chi-squared test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was <5, Fisher's exact test was employed. The Hardy–Weinberg equilibrium test was performed for each SNP between control and patient groups. Pairwise linkage disequilibrium pattern, haplotype block structure, and haplotype frequency analysis were assessed for all SNPs by the block definition by Gabriel

Table 1 Demographic and clinical characteristics of patients with chronic hepatitis C

Characteristic	All ($n=213$)	VR ($n=162$)	NVR ($n=51$)	*P* value
Age (years)[a]	60 (24–80)	60 (24–80)	59 (39–75)	0.859
Male, *n* (%)	66 (58)	50 (58)	16 (57)	0.926
White blood cell count (/μl)[a]	4300 (1870–8610)	4490 (1870–8610)	4055 (2000–8240)	0.076
Hemoglobin (g/dl)[a]	14.3 (9.0–18.2)	14.4 (9.0–18.2)	13.9 (10.9–16.4)	0.049
Platelets (10^4/μl)[a]	15.4 (7.7–33.6)	15.7 (7.7–33.6)	13.3 (7.7–29.2)	0.236
ALT (IU/l)[a]	46 (14–389)	45 (14–389)	48 (19–323)	0.353
AST (IU/l)[a]	43 (17–246)	41 (17–231)	43 (19–246)	0.297
HCV RNA (10^5 IU/ml)[a]	18 (1.1–51)	19 (1.1–51)	14 (1.5–51)	0.411
rs8099917 allele (TT/TG/GG)	144/64/5	124/36/2	20/28/3	<0.001
ss469415590 allele (TT/TT TT/ΔG ΔGΔG)	142/65/6	122/37/3	20/28/3	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; VR, virological response; NVR, null virological response.
[a]Median (range).

et al. (15) and were based on a 95% confidence interval (CI) of D' with HAPLOVIEW version 4.2 software (16). We plotted r^2 values. A P value of ≤ 0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% CI.

Of the 213 patients receiving PEG-IFN and RBV therapy, 105 (49%) achieved an SVR. Among the 108 patients not reaching an SVR, 57 relapsed and 51 did not respond to therapy. Pre-treatment values for median hemoglobin were significantly higher in the virological response (VR) group compared with the null virological response (NVR) group (Table 1).

We genotyped the rs8099917 SNP and four other SNPs in *IFNL4* (rs150891559, rs73555604, ss469415590, and rs4803221) in 213 patients with chronic hepatitis C and 176 healthy subjects. As rs150891559 and rs73555604 were homozygous for the major allele in all patients and controls, we focused on the rs8099917, ss469415590, and rs4803221 SNPs in this study. The observed genotype frequencies for patients and controls were all in Hardy–Weinberg equilibrium. Among the 213 patients ($2n = 426$), the three SNPs (rs4803221, ss469415590, and rs8099917) showed strong linkage disequilibrium (LD) ($r^2 = 0.88–0.96$) with each other and exhibited haplotypes as follows: haplotype 1: G/TT/T; $n = 348$ (81.7%); haplotype 2: C/ΔG/G; $n = 73$ (17.1%); haplotype 3: G/ΔG/T; $n = 4$ (0.9%); and haplotype 4: G/TT/G; $n = 1$ (0.2%). Among the 176 healthy subjects ($2n = 352$), haplotypes 1 and 2 were seen in 323 (91.8%) and 29 (8.2%) of cases, respectively. The frequencies of haplotypes 1 and 2 showed significant differences between patients with chronic HCV infection and healthy subjects ($P = 0.049$ and $P = 0.025$, respectively). The statistical power of this study was 0.90 and therefore sufficient for analysis.

The G allele frequency of rs8099917 (33.3% *vs* 12.3%; $P = 0.011$, OR = 3.55) and the ΔG allele frequency of ss469415590 (37.3% *vs* 13.3%; $P = 0.044$, OR = 3.27) were significantly higher in the NVR group than in virological responders (Table 2). The overall frequency of the TG or GG genotype for rs8099917 was 60.8% and more common in subjects with an NVR than in those showing a virological response (60.8% *vs* 23.5%; $P = 0.068$, OR = 5.06). TT/ΔG or ΔG/ΔG was also significantly associated with an NVR (60.8% *vs* 24.7%; $P = 0.019$, OR = 4.73). Interestingly, 5 of 213 patients had a rare linkage between rs8099917 and ss469415590 (haplotypes 3 and 4), but showed differing responses to PEG-IFN and RBV therapy. Among the four patients with haplotype 3 (T/ΔG/G), three achieved an SVR and one experienced an NVR. The other patient with haplotype 4 (G/TT/C) exhibited an NVR.

We next evaluated several factors apparently associated with an NVR to PEG-IFN and RBV therapy for independence by multivariate analysis. Only the rs8099917 SNP (TG or GG) was found to be an independent risk factor (OR = 5.28, 95% CI: 2.53–11.01, $P = 0.009$).

In this study, we investigated the frequency of the ss469415590 SNP in Japanese patients with type 1 chronic hepatitis C and analyzed its association with the outcome of PEG-IFN and RBV therapy. As it has been suggested that ss469415590 ΔG/TT is in complete linkage disequilibrium with the minor allele [G] of rs8099917 in individuals of Chinese ancestry, our data were able to confirm that the correlation between ss469415590 and rs8099917 was extremely high ($r^2 = 0.92$, $D' = 0.98$), such that only 5 (2.3%) of 213 individuals were ruled out.

Our data showed that the frequency of haplotype 1 (rs4803221, ss469415590, and rs8099917: G/TT/T) was higher in healthy subjects than in those chronic hepatitis C. These frequencies were very similar to those in published (6) and HapMap data from Japan. Since the major IL28B alleles have been associated with spontaneous clearance of HCV infection (17), carriers of haplotype 1 may have a decreased risk of chronic HCV infection.

As proposed by Prokunina-Olsson et al., ss469415590 [ΔG] was highly associated with treatment outcome in Japanese patients (OR = 4.73, $P = 0.019$). We observed a stronger association for rs8099917 (OR = 5.06) than for ss469415590 with an NVR, although this difference did not reach statistical significance. In multivariate analysis of our cohort,

Table 2 Association of rs8099917 and ss469415590 SNPs with response to PEG-IFN and RBV therapy[a]

SNP	NVR ($n = 51$)	VR ($n = 162$)	OR (95% CI)	P	Controls ($n = 176$)
rs8099917					
G allele	33.3	12.3	3.55 (2.09–6.02)	0.011	8.2
T allele	66.7	87.7			91.8
T/G or G/G	60.8	23.5	5.06 (2.59–9.88)	0.068	16.5
T/T	39.2	76.5			83.5
ss469415590					
ΔG allele	33.3	13.3	3.27 (1.94–5.51)	0.044	8.2
TT allele	66.7	86.7			91.8
TT/ΔG or ΔG/ΔG	60.8	24.7	4.73 (2.43–9.20)	0.019	16.5
TT/TT	39.2	75.3			83.5

CI, confidence interval; NVR, null virological response; OR, odds ratio; SNP, single nucleotide polymorphism; VR, virological response.
[a]All values are expressed as percentages.

rs8099917 [G] was an independent factor related to an NVR in Japanese patients treated with PEG-IFN and RBV therapy, and treatment response was correlated with the rs8099917 SNP, but not ss469415590, in five patients with varying SNPs. Hence, our results indicate that although rs8099917 remains a powerful predictor of PEG-IFN and RBV therapy in the Japanese, ss469415590 may be an effective marker as well.

Although our data support that ss469415590 [ΔG] is strongly associated with treatment failure in chronic hepatitis C, the mechanism remains unknown by which ss469415590 [ΔG] and the IFN-λ4 protein might cause impaired HCV clearance. Prokunina-Olsson et al. (9) showed that IFN-λ4 may pre-active the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway and limit further activation by type-I and type-III IFNs. Furthermore, Bibert et al. (18) have attributed the effect of IFNL4ΔG to decreased induction of IFNL3 and IP-10 mRNA, which rely on the ss469415590 genotype.

In summary, this report demonstrates an association between the dinucleotide variant ss469415590 and treatment response to PEG-IFN and RBV therapy for HCV genotype 1 infections in the Japanese population. Further studies on this polymorphism will more clearly elucidate the mechanism of antiviral response.

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Conflict of Interest

Drs TU and ET are currently conducting research sponsored by MSD. All other authors have declared no conflicting interests.

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KIR, *HLA*, and *IL28B* Variant Predict Response to Antiviral Therapy in Genotype 1 Chronic Hepatitis C Patients in Japan

Yuichi Nozawa[1☯], Takeji Umemura[1*☯], Satoru Joshita[1], Yoshihiko Katsuyama[2], Soichiro Shibata[1], Takefumi Kimura[1], Susumu Morita[1], Michiharu Komatsu[1], Akihiro Matsumoto[1], Eiji Tanaka[1], Masao Ota[3*]

1 Division of Hepatology and Gastroenterology, Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, **2** Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan, **3** Department of Legal Medicine, Shinshu University School of Medicine, Matsumoto, Japan

Abstract

Natural killer cell responses play a crucial role in virus clearance by the innate immune system. Although the killer immunoglobulin-like receptor (KIR) in combination with its cognate human leukocyte antigen (HLA) ligand, especially *KIR2DL3-HLA-C1*, is associated with both treatment-induced and spontaneous clearance of hepatitis C virus (HCV) infection in Caucasians, these innate immunity genes have not been fully clarified in Japanese patients. We therefore investigated 16 KIR genotypes along with *HLA-B* and *-C* ligands and a genetic variant of interleukin (IL) 28B (rs8099917) in 115 chronic hepatitis C genotype 1 patients who underwent pegylated-interferon-α2b (PEG-IFN) and ribavirin therapy. *HLA-Bw4* was significantly associated with a sustained virological response (SVR) to treatment (P = 0.017; odds ratio [OR] = 2.50,), as was the centromeric A/A haplotype of *KIR* (P = 0.015; OR 3.37). In contrast, SVR rates were significantly decreased in patients with *KIR2DL2* or *KIR2DS2* (P = 0.015; OR = 0.30, and P = 0.025; OR = 0.32, respectively). Multivariate logistic regression analysis subsequently identified the *IL28B* TT genotype (P = 0.00009; OR = 6.87, 95% confidence interval [CI] = 2.62 - 18.01), *KIR2DL2/HLA-C1* (P = 0.014; OR = 0.24, 95% CI = 0.08 - 0.75), *KIR3DL1/HLA-Bw4* (P = 0.008, OR = 3.32, 95% CI = 1.37 - 8.05), and white blood cell count at baseline (P = 0.009; OR = 3.32, 95% CI = 1.35 - 8.16) as independent predictive factors of an SVR. We observed a significant association between the combination of *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* in responders (P = 0.0019), whereas *IL28B* TT along with *KIR2DL2-HLA-C1* was related to a non-response (P = 0.0067). In conclusion, combinations of *KIR3DL1/HLA-Bw4*, *KIR2DL2/HLA-C1*, and a genetic variant of the *IL28B* gene are predictive of the response to PEG-IFN and ribavirin therapy in Japanese patients infected with genotype 1b HCV.

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* E-mail: tumemura@shinshu-u.ac.jp (TU); otamasao@shinshu-u.ac.jp (MO)

☯ These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection often develops into chronic hepatitis, which may progress to liver cirrhosis and/or hepatocellular carcinoma (HCC)[1]. HCC is a leading cause of death from malignant neoplasms in Japan[2]. Since approximately 70% of Japanese HCC patients are infected with HCV, the successful eradication of this virus, defined as a sustained virological response (SVR), is considered important to decrease the incidence of HCC.

Natural killer (NK) cells are key components of the innate antiviral immune response that are controlled by a balance of activation and inhibitory receptors. NK cell activation receptors include C-type lectin-like receptors (NKG2C, NKG2D, and NKG2E), natural cytotoxicity receptors (NKp30, NKp44, and NKp46), and CD16, while known inhibitory receptors include killer cell immunoglobulin-like receptors (KIRs) and the CD94/NKG2 family, which also contains a C-type lectin-like receptor (NKG2A) [3,4]. Sixteen *KIR* genes and pseudogenes have been identified that are encoded by a family of genes located on human chromosome 19q13.4. One particular feature of *KIRs* is their substantial genetic diversity. Some inhibitory *KIRs*

recognize human leukocyte antigen (HLA) class I molecules as their ligands; *KIR2DL1* recognizes *HLA*-C group 2 (*HLA-C2*) allotypes having lysine at amino acid position 80, whereas *KIR2DL2* and KIR2DL3 recognize *HLA-C* group 1 (*HLA-C1*) allotypes having asparagine at amino acid position 80 [5]. *KIR2DL2* and *KIR2DL3* also recognize HLA-B*4601 acquiring the-C1 epitope by gene conversion [6]. Furthermore, *KIR3DL1* recognizes subsets of *HLA*-A and *HLA*-B allotypes having the -Bw4 epitope determined by amino acid positions 77-83 [7].

It has been well documented that certain KIR-HLA receptor-ligand combinations are associated with susceptibility to infectious diseases, such as HCV, as well as with disease progression and treatment response [8-15]. Recent reports have also identified a relationship between interleukin (IL) 28B gene polymorphisms and treatment and spontaneous resolution of HCV infection[16-19]. Dring et al. observed that the presence of *IL28B* gene polymorphisms and *KIR* genotypes synergized to increase the risk of chronic HCV infection[20], although this finding is under debate[21]. Suppiah et al. [22] recently reported that genotyping for *IL28B*, *HLA*-C, and *KIR* genes was useful for predicting HCV treatment response in patients of European descent. As these gene associations have not yet been studied in the Japanese population, we evaluated whether HLA-KIR interactions, in addition to an *IL28B* polymorphism, would influence the outcome of pegylated-interferon-α (PEG-IFN) and ribavirin therapy in Japanese patients with chronic hepatitis C.

Materials and Methods

Ethics statement

This study was approved by the ethical committee of Shinshu University School of Medicine, Matsumoto, Japan, and written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Subjects

One hundred and fifteen consecutive IFN-treatment-naïve patients with chronic hepatitis C were enrolled in this study. All subjects were seen at Shinshu University Hospital or one of its affiliated hospitals. The clinical and demographic characteristics of our cohort are shown in Table 1. Diagnosis of chronic hepatitis C was based on previously reported criteria [23]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) absence of detectable hepatitis B surface antigen and antibody to the human immunodeficiency virus; and 3) exclusion of other causes of chronic liver disease or a history of decompensated cirrhosis or HCC. Serum levels of HCV RNA were determined using Cobas Amplicor assays (sensitivity: 50 IU/mL; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II kits (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods[24]. Liver fibrosis was assessed using the AST to platelet ratio index (APRI) in this study. APRI has been recognized as a noninvasive test to estimate the degree of liver fibrosis in chronic liver disease with HCV infection[25]. APRI was calculated for all study subjects as follows: AST/upper limit of normal (45 IU/L) × 100/platelet count (10^9/L). Patients received PEG-IFN-α2b (Pegintron; MSD KK, Tokyo, Japan; 1.5 μg/kg of body weight by subcutaneous injection once per week) and ribavirin (Rebetol; MSD KK; 600-1000 grams daily, according to body weight) for 48 weeks, as described previously[26]. Patients achieving a sustained HCV response were defined as those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Patients who did not meet this criterion, who included non-responders and relapsers, were regarded as treatment failures.

Table 1. Clinical features of sustained and non-sustained virological response patients with chronic hepatitis C.

Characteristic	All	SVR	Non-SVR	*P*
	(n = 115)	(n = 56)	(n = 59)	
Age (yr)	60 (24 - 80)	59 (25 - 80)	60 (24 - 75)	0.43
Male	66 (57)	34 (61)	32 (54)	0.48
Alanine aminotransferase (IU/L)	46 (17 - 389)	48 (17 - 389)	45 (17 - 309)	0.81
Aspartate aminotransferase (IU/L)	43 (17 - 246)	42 (17 - 231)	43 (17 - 246)	0.49
White blood cells (/μL)	4410 (2280 - 8240)	4740 (2700 - 8170)	4070 (2280 - 8240)	0.011
Hemoglobin (g/dL)	14.4 (9.2 - 18.2)	15.1 (11.0 - 18.2)	13.9 (9.2 - 17.4)	0.002
Platelet count (10^4/μL)	15.9 (6.7 - 33.6)	16.6 (8.3 - 26.2)	15.6 (6.7 - 33.6)	0.30
APRI	0.89 (0.21 - 5.40)	0.59 (0.22 - 5.40)	0.66 (0.21 - 5.06)	0.41
HCV RNA ($\log_{10}$ IU/mL)	6.4 (5.0 - 7.3)	6.1 (5.0 - 6.8)	6.5 (5.0 - 7.3)	< 0.001

Data are expressed as median (range) or n (%) as appropriate. SVR, sustained virological response; HCV, hepatitis C virus

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HLA, KIR, and IL28B (rs8099917) Genotyping

Genomic DNA was isolated from whole blood samples using QuickGene-800 assays (Fujifilm, Tokyo, Japan). We genotyped *HLA*-B, *HLA*-C, and *KIR* using a Luminex multi-analyzer profiling system with a LAB type® HD and KIR SSO genotyping kit (One Lambda, Inc., Canoga Park, CA), which is based on PCR sequence-specific oligonucleotide probes[27]. Subjects were identified as having the B/x or A/A genotype as defined previously[28]. Genotypes for the centromeric (*Cen*) and telomeric (*Tel*) parts of the *KIR* locus were determined according to the presence or absence of one or more B haplotype-defining *KIR* genes. Thus, *Cen-A1* and *Tel-A1* were the centromeric and telomeric motifs, respectively, of the canonical *A KIR* haplotype in the present study, *Cen-B1* and *Cen-B2* were alternative centromeric motifs of common *B KIR* haplotypes, and *Tel-B1* was the common telomeric motif of *B* haplotypes[29]. For much of this analysis, *Cen-B1* and *-B2* were grouped together as *Cen-B*, whereas *Cen-A1* was shortened to *Cen-A* and *Tel-A1* to *Tel-A*, as reported

previously[30,31]. Genotyping of an *IL28B* SNP (rs8099917) was performed using a TaqMan 5' exonuclease assay with primers supplied by Applied Biosystems[32]. Probe fluorescence signals were detected using a TaqMan assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

The Mann-Whitney *U* test was employed to analyze continuous variables. Pearson's chi-squared test was used for the analysis of categorical data. We adopted Fisher's exact test when the number of subjects was less than 5. The Bonferroni correction for multiple testing was applied to our data of KIR-HLA combinations using the number of comparisons performed by our primary factors of interest in Table 2 (i.e., 8 tests = 4 combinations × 2 comparisons between two groups). A P value of < 0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI). Our model was checked by regression diagnostic plots to verify normality, linearity of data, and constant variance. Stepwise logistic regression analysis with a forward approach was performed to identify independent factors associated with an SVR after continuous variables were separated into 2 categorical variables by each median value. Statistical analyses were performed using SPSS software version 21.0J (IBM, Tokyo, Japan). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to determine the reliability of the predictors of therapy response.

Results

Patient Characteristics and Treatment Outcome

All patients in our test cohort were infected with HCV genotype 1b. Of the 115 patients receiving PEG-IFN-α2b and ribavirin therapy, 56 (49%) achieved an SVR. The remaining 59 patients were non-responders, 28 of whom experienced a relapse and 31 who were null responders. The median white blood cell count (P = 0.011) and hemoglobin value (P = 0.002) in the SVR group were significantly higher than those in the non-SVR group prior to treatment. HCV viral load at baseline was significantly associated with treatment outcome (P < 0.001).

Association of HLA and KIR with a Sustained Virological Response

We first determined the frequency of *HLA-Bw* and *HLA-C* alleles in SVR and non-SVR patients (Figure 1). The frequency of *HLA-Bw4Bw6* in responders was significantly higher than that in non-responders (55% [31/56] vs. 36% [21/59]; P = 0.033; OR = 2.24, 95% CI = 1.06 - 4.75). Conversely, patients with the *HLA-Bw6* homozygote had a higher non-SVR rate (32% [18/56] vs. 54% [32/59]; P = 0.017; OR = 0.40, 95% CI = 0.19 - 0.85). Overall, *HLA-Bw4* was associated with an SVR among patients (68% [38/56] vs. 46% [27/59]; P = 0.017; OR = 2.50, 95% CI = 1.17 - 5.35). The frequencies of HLA-C were not statistically significant. We further checked whether particular HLA-Bw or HLA-C alleles were beneficial to treatment outcome. The HLA-B*35:01 allele was more frequently found in patients with an SVR than in those without (13% [15/102] vs. 4% [5/118]; P = 0.014 [Pc = 0.36]; OR = 3.49, 95% CI = 1.23 - 9.97).

Table 2. Frequency of *IL28B* genotype, *KIR3DL1/HLA-Bw4*, and *KIR2DL2/HLA-C1* combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

KIR3DL1/ HLA-Bw4	*KIR2DL2/HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
+/+	+/+	5 (9%)	7 (12%)	0.61	
+/+	Other	31 (55%)	19 (32%)	0.012 (0.1)	2.61 (1.22 - 5.58)
Other	+/+	1 (2%)	10 (17%)	0.014 (0.12)	0.09 (0.01 - 0.72)
Other	Other	19 (34%)	23 (39%)	0.57	
IL28B	*KIR3DL1/ HLA-Bw4*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	+/+	27 (48%)	13 (22%)	0.003 (0.024)	3.29 (1.47 - 7.39)
TT	Other	17 (30%)	14 (24%)	0.42	
TG/GG	+/+	9 (16%)	13 (22%)	0.42	
TG/GG	Other	3 (5%)	19 (32%)	0.00062 (0.0005)	0.12 (0.03 - 0.43)
IL28B	*KIR2DL2/ HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	Other	38 (68%)	18 (31%)	0.000062 (0.0005)	4.81 (2.19 - 10.58)
TT	+/+	6 (11%)	9 (15%)	0.47	
TG/GG	Other	12 (21%)	24 (41%)	0.026 (0.21)	0.40 (0.17 - 0.91)
TG/GG	+/+	0 (0%)	8 (14%)	0.013 (0.1)	-

Data are expressed as n (%).

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The distribution of *KIR* genes and their association with treatment outcome are shown in Figure 2. No statistically significant differences were found for any allele combination apart from *KIR2DL2* and *KIR2DS2*; patients with these genes had significantly decreased SVR frequencies compared with those without (P = 0.015 [Pc = 0.48]; OR = 0.30, 95% CI = 0.11 - 0.82 and P = 0.025 [Pc = 0.8]; OR = 0.32, 95% CI = 0.12 - 0.90, respectively).

KIR genotype profiles were determined by the presence or absence of each *KIR* locus in patients (Figure 3). Since strong linkage disequilibrium is a prominent feature in the *KIR* region, *KIR* gene profiles were classified based on *Cen* and *Tel* motifs. When we evaluated SVR according to genotype and *Cen* and *Tel* frequencies, we observed that virologic clearance with *Cen-A/A* was significantly higher than that without (54% [50/92] vs.

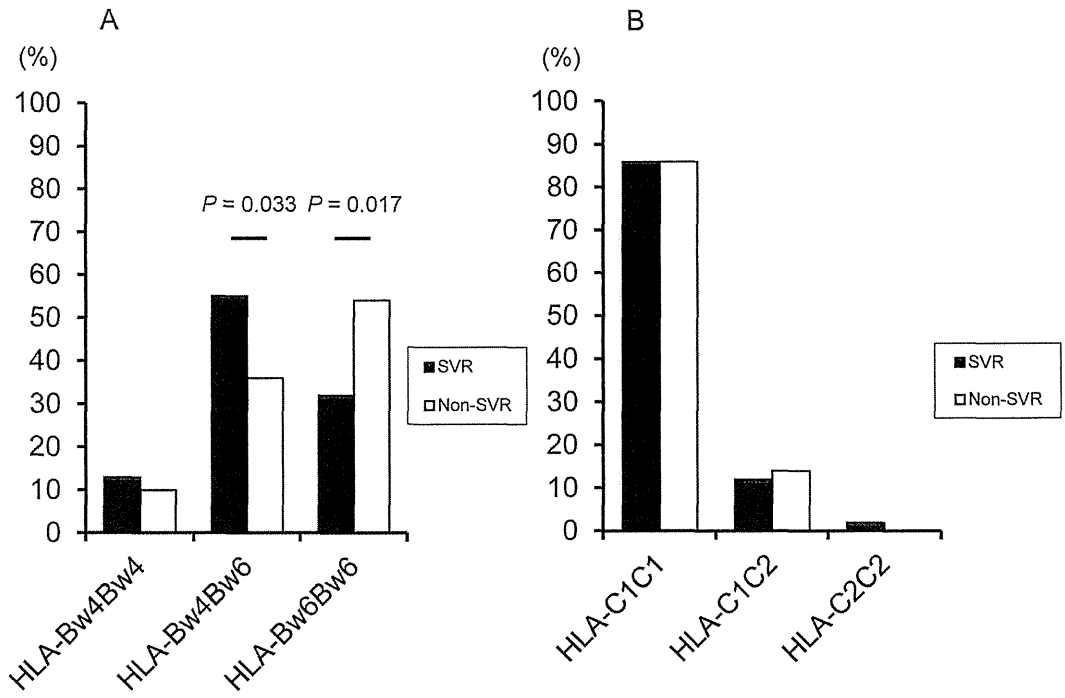

Figure 1. Frequency of *HLA-Bw* and -C alleles in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

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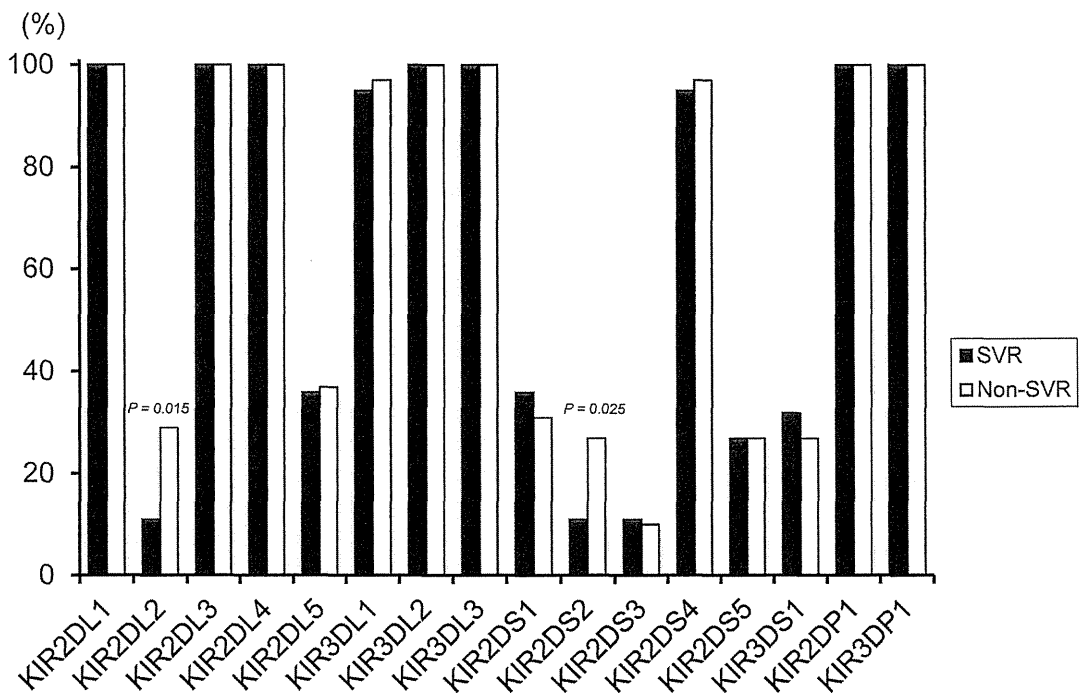

Figure 2. Frequency of each *KIR* gene in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

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26% [6/23], $P = 0.015$; OR = 3.37, 95% CI = 1.22 - 9.33). There were no significant differences regarding *AA* genotype and *Tel*.

We next analyzed combinations of activation/inhibitory *KIR*s and their *HLA* ligands for possible associations with an SVR. Among the combinations of *KIR3DL1-HLA-Bw4*, *KIR2DL2-HLA-C1*, and KIR2DL1-*HLA*-C2, patients who carried the inhibitory *KIR3DL1* receptor and its ligand *HLA-Bw4* had a significantly higher response rate than those without *KIR3DL1* or *HLA-Bw4* (58% [36/62] vs. 38% [20/53]; $P = 0.030$ [$Pc = 0.12$]; OR = 2.29, 95% CI = 1.08 - 4.84). In contrast, the *KIR2DL2-HLA-C1* combination resulted in a significantly lower SVR rate (26% [6/23] vs. 54% [50/92]; $P = 0.015$ [$Pc = 0.06$]; OR = 0.30, 95% CI = 0.11 - 0.82). Although several studies have found that *KIR2DL3-HLA-C1* carriers are associated with treatment-induced and spontaneous clearance of HCV in Caucasians, no such association was found in our cohort (data not shown).

Patients with *KIR3DL1-HLA-Bw4* but without *KIR2DL2-HLA-C1* had a higher SVR rate (55% [31/56] vs. 32% [19/59]; $P = 0.012$ [$Pc = 0.1$]; OR = 2.61, 95% CI = 1.22 - 5.58) (Table 2). Conversely, the frequency of the *KIR2DL2-HLA-C1* positive, but *KIR3DL1-HLA-Bw4* negative condition was significantly higher in non-responders (17% [10/59] vs. 2% [1/56]; P = 0.014 [$Pc = 0.12$]; OR = 0.09, 95% CI = 0.01 - 0.72).

Prediction of a Sustained Virological Response by KIR-HLA and IL28B

Examination of the *IL28B* rs8099917 SNP in our cohort revealed significant differences in SVR frequencies. The SVR rate in patients with the *IL28B* TT genotype was significantly higher in those with TG or GG genotypes (62% [44/71] vs. 27% [12/44), $P = 0.0003$; OR = 4.35, 95% CI = 1.92 - 9.85). In subjects with *IL28B* TT and *KIR3DL1-HLABw4*, virologic clearance was significantly increased over other combinations (68% [27/40] vs. 39% [29/75]; $P = 0.003$ [$Pc = 0.024$]; OR 3.29, 95% CI = 1.47 - 7.39).

We next evaluated several factors found in association with an SVR to PEG-IFN and ribavirin therapy for independence by logistic regression analysis. Fifty-six responders were compared with 59 non-responders by means of a forward stepwise likelihood ratio logistic regression method; estimated OR coefficients, 95% CI, and *P* values are summarized in Table 3 for the variables that remained in equation at the last step. *IL28B* TT genotype ($P = 0.00009$; OR = 6.87, 95% CI = 2.62 - 18.01), *KIR2DL2-HLA*-C1 ($P = 0.014$; OR = 0.24, 95% CI = 0.08 - 0.75), white blood cell count ≥ 4410/μL ($P = 0.009$; OR = 3.32, 95% CI= 1.35 - 8.16), and *KIR3DL1-HLA-Bw4* ($P = 0.008$; OR = 3.32, 95% CI = 1.37 - 8.05) were all identified as independent parameters that significantly influenced an SVR.

The frequency of the *IL28B* TT genotype with *KIR3DL1-HLA-Bw4* in responders was significantly higher than in non-responders (48% [27/56] vs. 22% [13/59]; $P = 0.003$ [$Pc = 0.024$]; OR = 3.29, 95% CI = 1.47 - 7.39) (Table 2). Patients with the *IL28B* TT genotype without *KIR2DL2-HLA-C1* had a significantly higher SVR rate (68% [38/56] vs. 31% [18/59]; $P = 0.000062$ [$Pc = 0.0005$]; OR = 4.81, 95% CI = 2.19 - 10.58). The frequency of a non-SVR was significantly higher in patients with the *IL28B* non-TT genotype both with and without

KIR profile	Geno type	Cen motif	Tel motif	3DL3	2DS2	2DL2	2DL3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5	2DS3	2DS5	2DS1	2DS4	3DL2	SVR (n = 56)	Non-SVR (n = 59)
1	AA	A/A	A/A	+	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	34 (60.7)	28 (47.5)
2	Bx	A/A	A/B	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	9 (16.1)	10 (16.9)
3	Bx	A/B	A/A	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	2 (3.6)	8 (13.6)
4	Bx	A/A	A/B	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	4 (7.1)	2 (3.4)
5	Bx	A/A	B/B	+	-	-	+	+	+	+	+	-	+	+	-	+	+	-	+	3 (5.4)	1 (1.7)
6	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	1 (1.8)	2 (3.4)
7	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	1 (1.8)	2 (3.4)
8	Bx	A/B	A/A	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	0 (0.0)	3 (5.1)
9	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	1 (1.8)	0 (0.0)
10	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1 (1.8)	0 (0.0)
11	Bx	A/B	B/B	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	1 (1.8)	0 (0.0)
12	Bx	A/B	A/A	+	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	0 (0.0)	1 (1.7)
13	Bx	A/A	A/A	+	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	0 (0.0)	1 (1.7)

Figure 3. *KIR* gene profile frequencies in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Numerical data represent the number of individuals (%). The presence of *KIR* genes is indicated by gray shading. Cen, centromeric; Tel, telomeric.

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Table 3. Logistic regression analysis of variables contributing to a sustained virological response to pegylated interferon and ribavirin.

Factor	Odds ratio	95% confidence interval	*P*
IL28B TT genotype	6.87	2.62 - 18.01	0.00009
KIR2DL2/HLA-C1	0.24	0.08 - 0.75	0.014
White blood cells ≥ 4410/μL	3.32	1.35 - 8.16	0.009
KIR3DL1/HLA-Bw4	3.32	1.37 - 8.05	0.008

Only variables achieving statistical significance ($P < 0.05$) in multivariate logistic regression analysis are shown.

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KIR2DL2-HLA-C1 (14% [8/59] vs. 0% [0/8]; $P = 0.013$ [$Pc = 0.1$] and 41% [24/59] vs. 21% [12/56]; $P = 0.026$ [$Pc = 0.21$]; OR = 0.40, 95% CI = 0.17 - 0.91, respectively). The ability to predict an SVR by *IL28B* genotype and *KIR3DL1-HLA-Bw4* and *KIR2DL2-HLA-C1* was next evaluated. Corresponding values for sensitivity, specificity, PPV, and NPV are listed in Table S1 in File S1. A combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* demonstrated high predictive specificity (78%), as did the combination of *IL28B* TT genotype and *KIR2DL2-HLA-C1* (86%).

Lastly, we analyzed combinations of the three factors of *IL28B* genotype, *KIR3DL1-HLA-Bw4*, and *KIR2DL2-HLA-C1* for prediction of treatment outcome (Table S2 in File S1). The frequencies of *IL28B* TT, *KIR2DL2-HLA-C1*-negative, with and without *KIR3DL1-HLA-Bw4* were significantly higher among responders (38% [21/56] vs. 19% [11/59]; P = 0.024 [Pc = 0.29]; OR = 2.62, 95% CI = 1.12 - 6.12 and 30% [17/56] vs. 12% [7/59]; P = 0.015 [Pc = 0.18]; OR = 3.24, 95% CI = 1.22 - 8.57, respectively).

Discussion

The present study examined *HLA*, *KIR*, and *IL28B* gene variant associations with an SVR following PEG-IFN and ribavirin therapy in Japanese patients with chronic hepatitis C. We found a significant association of *HLA-Bw* alleles with treatment outcome, although the frequency of *HLA-C* alleles did not differ significantly between responders and non-responders. Functional analyses have demonstrated that NK cells in *HLA-C1C1* subjects exhibit a more rapid and stronger antiviral response that those in *HLA-C2C2* subjects due to differing responses of *HLA-C*-inhibited NK subsets[33]. *HLA-C2C2* homozygousity is strongly associated with treatment failure in HCV patients of European ancestry [11,22], but we could not assess its role in our study because this genotype was found in only 1 of 115 patients.

We uncovered a significant association between the presence of *KIR2DL2* or *KIR2DS2* and lower SVR rates. Several reports have shown that *KIR2DL3-HLAC1* in Caucasians [11,22] and *KIR2DL5* in Brazilians [34] are associated with treatment outcome of antiviral therapy. Since our results showed no such statistical significances, these conflicting interpretations may reflect differences in patient selection, genetic background, sample size, and/or treatment regimen. Further studies are required to clarify this discrepancy in the Japanese population.

A study by Dring et al. examined *KIR* haplotypes in patients with HCV infection and showed that a centromeric *KIR* haplotype was increased in chronic HCV infection as compared with resolved cases [20]. We therefore determined *KIR* haplotypes and *Cen-A/B* and *Tel-A/B* in our patients as well, and found an interesting association between *Cen-A/A* and an SVR to antiviral therapy (P = 0.015; OR 3.37). Since *Cen-A/B* is determined by *KIR2DL3* and *KIR2DS2* and/or *KIR2DL2*, this finding is consistent with our results demonstrating a relationship between *KIR2DS2* and *KIR2DL2* genotypes and treatment failure.

The most significant finding in this study was the association between KIR-HLA receptor-ligand pairings and treatment outcome in chronic hepatitis C. Among the inhibitory KIR-HLA receptor-ligand pairs, patients with *KIR3DL1-HLA-Bw4* exhibited a significantly higher SVR rate when compared to those without this pair (P = 0.03; OR 2.29). Conversely, virologic clearance in patients with *KIR2DL2-HLA-C1* was significantly lower than in those without (P = 0.015; OR = 0.30). Stratification analysis of the 4 groups of *KIR3DL1-HLA-Bw4* (presence or absence) and *KIR2DL2-HLA-C1* (presence or absence) revealed a higher frequency of responders with *KIR3DL1-HLA-Bw4* presence, *KIR2DL2-HLA-C1* absence compared with those possessing *KIR2DL2-HLA-C1* presence, *KIR3DL1-HLA-Bw4* absence (62% vs. 9%; P = 0.0044; OR =16.32). When these KIR-HLA pairs were both either positive or negative, SVR rates were similar at 42% and 45%, respectively. Together with the results of logistic regression analysis, we clearly showed that *KIR3DL1-HLA-Bw4* was positively associated with an SVR (OR = 3.32) and that *KIR2DL2-HLA-C1* had a negative association (OR = 0.24) with treatment outcome. As almost one half of the Japanese population have the functional *KIR3DL1-HLA-Bw4* combination, this inhibitory receptor-ligand interaction is potentially important in understanding NK cell diversification. The NK-cell surface expression of KIR3DL1 is higher in individuals having Bw4 than in those lacking it [35]. Therefore, these cells might be more weakly controlled by inhibitory signals than other NK cells, more easily activated by viral infection, and more readily promoted for cytolysis and IFN-gamma production.

This study confirmed that the *IL28B* TT genotype is a strong predictor of an SVR in Japanese patients[18,32]. Furthermore, SVR frequencies were positively correlated with a combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* (P = 0.0019) and negatively associated with the *IL28B* TT genotype and *KIR2DL2-HLA-C1* (P = 0.0067). These combinations were also highly specific for virologic response prediction. In light of these findings, patients with poor expected treatment outcome may be advised to wait for the use of combinations of direct acting antiviral agents[36]. Akuta et al. reported that a combination of amino acid substitutions in the core region of HCV and *IL28B* genotype was a useful predictor of PEG-IFN, ribavirin, and telaprevir therapy results in Japan[37]. Since we could not collect sera before treatment for all patients, we were not able to assess the effect of amino acid substitutions in the HCV core region. Furthermore, interferon-free combinations of direct-acting antiviral agents have become an area of considerable clinical interest. Chu et al. have reported that IL28B genotype appears to affect early viral kinetics in patients with chronic hepatitis C receiving interferon-free treatment [38]. Recently, two groups have discovered *IFN* lambda *4* (*IFNL4*), a new gene that may account for associations of spontaneous and IFN-based treatment clearance of HCV [39,40]. The IFN-λ 4 protein is generated by individuals who carry the ΔG allele of the ss469415590 variant, and the presence of this protein is strongly associated with impaired clearance of HCV. Linkage disequilibrium is strong between the *IFNL4-ΔG* allele and the unfavorable rs12979860-T allele (*IL28B*) in subjects of European or Asian ancestry, whereas this linkage disequilibrium is moderate in individuals of African ancestry [39]. We have confirmed that the linkage disequilibrium between the *IFNL4-ΔG* allele and *IL28B* SNP (rs8099917) is high and that the *IFNL4-ΔG* allele is strongly associated with treatment failure of PEG-IFN and ribavirin therapy in patients with Japanese chronic hepatitis C [41]. Hence, the clinical impacts of HLA-KIR genetic variants, IL28B genotype, and the IFNL4 allele should be explored.

In conclusion, the present study showed significant associations of *KIR3DL1-HLA-Bw4, KIR2DL2-HLA-C1*, and *IL28B* combinations with an SVR to PEG-IFN and ribavirin therapy in Japanese patients with genotype 1 HCV. The clinical significance of *IL28B* genotyping combined with HLA/KIR pairs to predict treatment outcome warrants further validation for triple therapy.

Supporting Information

File S1. Table S1, Sensitivity, specificity, and predictive values of IL28B TT genotype and KIR3DL1/HLA-Bw4 or

KIR2DL2/HLA-C1 for a sustained virological response in 115 patients with chronic hepatitis C. Data are expressed as % (n). PPV, positive predictive value; NPV, negative predictive value. Table **S2**, Frequency of ***IL28B*** genotype and ***KIR3DL1/HLA-Bw4*** and ***KIR2DL2/HLA-C1*** combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Data are expressed as n (%).
(DOC)

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Author Contributions

Conceived and designed the experiments: YN TU ET MO. Performed the experiments: YN TU YK MO. Analyzed the data: YN TU YK MO. Contributed reagents/materials/analysis tools: YN TU SJ YK SS TK SM MK AM ET. Wrote the manuscript: TU MO.

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Model Incorporating the *ITPA* Genotype Identifies Patients at High Risk of Anemia and Treatment Failure With Pegylated-Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

Masayuki Kurosaki,[1] Yasuhito Tanaka,[2] Nao Nishida,[3] Naoya Sakamoto,[4] Nobuyuki Enomoto,[5] Kentaro Matsuura,[2] Yasuhiro Asahina,[6] Mina Nakagawa,[6] Mamoru Watanabe,[6] Minoru Sakamoto,[5] Shinya Maekawa,[5] Katsushi Tokunaga,[3] Masashi Mizokami,[7] and Namiki Izumi[1]*

[1]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[2]*Department of Virology, Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[3]*Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[4]*Department of Gastroenterology and Hematology, Hokkaido University, Sapporo, Japan*
[5]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan*
[6]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*
[7]*Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan*

This study aimed to develop a model for predicting anemia using the inosine triphosphatase (*ITPA*) genotype and to evaluate its relationship with treatment outcome. Patients with genotype 1b chronic hepatitis C (n = 446) treated with peg-interferon alpha and ribavirin (RBV) for 48 weeks were genotyped for the *ITPA* (rs1127354) and *IL28B* (rs8099917) genes. Data mining analysis generated a predictive model for anemia (hemoglobin (Hb) concentration <10 g/dl); the CC genotype of *ITPA*, baseline Hb <14.0 g/dl, and low creatinine clearance (CLcr) were predictors of anemia. The incidence of anemia was highest in patients with Hb <14.0 g/dl and CLcr <90 ml/min (76%), followed by Hb <14.0 g/dl and *ITPA* CC (57%). Patients with Hb ≥14.0 g/dl and *ITPA* AA/CA had the lowest incidence of anemia (17%). Patients with two predictors (high-risk) had a higher incidence of anemia than the others (64% vs. 28%, $P < 0.0001$). At baseline, the *IL28B* genotype was a predictor of a sustained virological response [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$]. In patients who achieved an early virological response, the *IL28B* genotype was not associated with a sustained virological response, while a high risk of anemia was a significant negative predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$]. For high-risk patients with an early virological response, giving >80% of the planned RBV dose increased sustained virological responses by 24%. In conclusion, a predictive model incorporating the *ITPA* genotype could identify patients with a high risk of anemia and reduced probability of sustained virological response. ***J. Med. Virol. 85:449–458, 2013.***

KEY WORDS: hemolytic anemia; ribavirin; creatinine clearance; antiviral therapy

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma worldwide [Kim, 2002]. The rate of eradication of HCV by pegylated interferon (PEG-IFN) plus ribavirin (RBV), defined as a sustained virological response, is around 50% in patients with HCV genotype 1 [Manns et al., 2001; Fried et al., 2002]. Failure of treatment is attributable to the lack of a virological response or relapse after completion of therapy. Genome-wide association studies and subsequent cohort studies

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*Correspondence to: Namiki Izumi, MD, PhD, Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. E-mail: nizumi@musashino.jrc.or.jp

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have shown that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are the most important determinant of virological response to PEG-IFN/RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. On the other hand, among patients with a virological response, the probability of a sustained virological response decreases when the patients become intolerant to therapy because of RBV-induced hemolytic anemia and receive a reduced dose of RBV [McHutchison et al., 2002; Kurosaki et al., 2012]. Genome-wide association studies have shown that variants of the inosine triphosphatase (*ITPA*) gene protect against hemolytic anemia [Fellay et al., 2010; Tanaka et al., 2011]. These variants are associated with a reduced requirement for an anemia-related dose reduction of RBV [Sakamoto et al., 2010; Thompson et al., 2010a; Kurosaki et al., 2011d; Seto et al., 2011]. However, factors other than the *ITPA* gene also contribute to the risk of severe anemia or RBV dose reduction [Ochi et al., 2010; Kurosaki et al., 2011d] and the results of studies on the impact of the *ITPA* genotype on treatment outcome are inconsistent [Ochi et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a, 2011; Kurosaki et al., 2011d].

Data mining is a novel statistical method used to extract relevant factors from a plethora of factors and combine them to predict the incidence of the outcome of interest [Breiman et al., 1980]. Decision tree analysis, a primary component of data mining analysis, has found medical applications recently [Averbook et al., 2002; Miyaki et al., 2002; Baquerizo et al., 2003; Leiter et al., 2004; Garzotto et al., 2005; Zlobec et al., 2005; Valera et al., 2007] and has proven to be a useful tool for predicting therapeutic efficacy [Kurosaki et al., 2010, 2011a,b,c, 2012] and adverse events [Hiramatsu et al., 2011] in patients with chronic hepatitis C treated with PEG-IFN/RBV therapy. Because the results of data mining analysis are presented as a flowchart [LeBlanc and Crowley, 1995], they are easily understandable and usable by clinicians lacking a detailed knowledge of statistics.

For the general application of this genetic information in clinical practice, this study aimed to construct a predictive model of severe anemia using the *ITPA* genotype, together with other relevant factors. This study also aimed to analyze the impact of the risk of anemia on treatment outcome, after adjustment for the *IL28B* genotype. These analyses were carried out at baseline and during therapy, when the early virological response became evident.

MATERIALS AND METHODS

Patients

Data were collected from a total of 446 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. The inclusion criteria were: (1) infection by hepatitis C genotype 1b; (2) no co-infection with hepatitis B virus or human immunodeficiency virus; (3) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis; and (4) availability of DNA for the analysis of the genetic polymorphisms of *IL28B* and *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (1.5 μg/kg) subcutaneously every week and a daily weight-adjusted dose of RBV (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg, and 1,000 mg for patients weighing >80 kg) for 48 weeks. Dose reduction or discontinuation of PEG-IFN and RBV was primarily based on the recommendations on the package inserts and the discretion of the physicians at each university and hospital. The standard duration of therapy was set at 48 weeks. No patient received erythropoietin or other growth factors for the treatment of anemia. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Laboratory Tests

Blood samples obtained before therapy were analyzed for hematologic data, blood chemistry, and HCV RNA. Genetic polymorphisms in SNPs of the *ITPA* gene (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) and the DigiTag2 assay, respectively. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [1976]: for males, CLcr = [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl) and for females, CLcr = 0.85 × [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl). The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis), and F4 (cirrhosis). A rapid virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, Pleasanton, CA) at week 4 of therapy and a complete early virological response was defined as undetectable HCV RNA at week 12. A sustained virological response was defined as undetectable HCV RNA at 24 weeks after completion of therapy. Severe anemia was defined as hemoglobin (Hb) <10 g/dl.

Statistical Analysis

Database for analysis included the following variables: age, sex, body mass index, serum aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, creatinine levels, CLcr, Hb, platelet count, serum levels of HCV RNA, and the stage of liver fibrosis

TABLE I. Patients' Baseline Characteristics

Age (years)	58.6	(9.6)
Gender: male (n, %)	185	(42%)
Body mass index (kg/m^2)	23.1	(3.7)
AST (IU/L)	59.9	(53.8)
ALT (IU/L)	69.8	(53.8)
GGT (IU/L)	48.5	(41.6)
Creatinine (mg/dl)	0.7	(0.2)
Creatinine clearance (ml/min)	89.5	(23.0)
Hemoglobin (g/dl)	14	(1.4)
Platelet count (10^9/L)	154.5	(52.1)
HCV RNA > 600,000 IU/ml (n, %)	354	(79%)
Liver fibrosis: F3-4 (n, %)	108	(24%)
Initial ribavirin dose (n, %)		
600 mg/day	300	(67%)
800 mg/day	138	(31%)
1,000 mg/day	9	(2%)
Pegylated interferon (n, %)		
alpha2a 180 mcg	58	(13%)
alpha2b 1.5 mcg/kg	388	(87%)
ITPA rs1127354: CC (n, %)	317	(71%)
IL28B rs809917: TT (n, %)	311	(70%)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.
Data expressed as mean (standard deviation) unless otherwise mentioned.

(Table I). Based on these data set, a model for predicting the risk of developing severe anemia was constructed by data mining analysis using the IBM-SPSS Modeler 13 as described previously [Kurosaki et al., 2010, 2011a,b,c; Hiramatsu et al., 2011]. Briefly, the software was used to explore the database automatically to search for optimal predictors that discriminated most efficiently patients with severe anemia from those without. The software also determined the optimal cutoff values of each predictor. Patients were divided into two groups according to the predictor and each of the two groups was repeatedly divided in the same way until no significant factor remained or 20 or fewer patients were in a group.

The incidence of severe anemia, the total dose of RBV, and treatment outcome were compared between groups with high and low risks of anemia. On univariate analysis, Student's *t*-test was used for continuous variables, and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. *P* values of <0.05 were considered significant. SPSS Statistics 18 was used for these analyses.

RESULTS

Predictive Model of Severe Anemia

The incidence of severe anemia in the whole cohort was 49% (Fig. 1). The best predictor of severe anemia was the baseline Hb concentration. Patients with a low baseline Hb concentration (<14 g/dl) were more likely to develop severe anemia (67%) than those with a higher Hb (>14 g/dl) (34%). The second best predictor for those patients with a baseline Hb <14.0 g/dl was CLcr. Patients with a CLcr below 90 ml/min had the highest incidence of severe anemia (76%). In those with a CLcr above >90 ml/min the incidence of severe anemia was 57% in patients with the CC allele of the *ITPA* gene while it was 37% in patients with the CA or AA allele. On the other hand, the second best predictor for those patients with a baseline Hb concentration above 14 g/dl was the *ITPA* genotype. Patients with the AA or AC allele had the lowest incidence of anemia (17%). For those with the *ITPA* CC allele, CLcr was the third best predictor; the optimal cutoff value was 85 ml/min for this group. The incidence of severe anemia was 49% in patients with a CLcr below 85 ml/min while it was 32% in those with a CLcr above 85 ml/min.

Following this analysis, the patients were divided into six groups, with the incidence of severe anemia ranging from 17% to 76%. Three groups with two predictors, having an incidence of anemia >40%, were defined as the high-risk group and the remainder were defined as the low-risk group. The incidence of severe anemia was higher in the high-risk group than the low-risk group (65% vs. 28%, $P = 0.029$) (Fig. 2). Comparison of the *ITPA* genotype and the predictive model showed that the sensitivity for the prediction of severe anemia was similar (75.9% vs. 76.4%) but the specificity of the predictive model was greater (33.6% vs. 59.3%).

The Risk of Anemia Impacts on Sustained Virological Responses by Patients Who Achieved an Early Virological Response

The impact of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response was studied at baseline and week 12. At baseline, patients with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele (43% vs. 10%, $P < 0.0001$), the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (28% vs. 40%, $P = 0.011$), and the *ITPA* genotype was not associated with a sustained virological response (Fig. 3A–C). At week 4, patients with rapid virological response had a high rate of sustained virological response, irrespective of the *IL28B* genotype (TT vs. TG/GG; 97% vs. 100%, $P = 1.000$), the *ITPA* genotype (CC vs. CA/AA; 95% vs. 100%, $P = 1.000$), and the risk of anemia (high vs. low; 95% vs. 100%, $P = 1.000$). Among the patients who did not achieve a rapid virological response, those with the *IL28B* TT allele had a significantly higher rate of sustained virological response than those with the TG or GG allele (38% vs. 8%, $P < 0.0001$), and the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (24% vs. 35%, $P = 0.015$). At week 12, in patients who achieved a complete early virological response, the *IL28B* genotype was not associated with a sustained virological response, while the high-risk group for anemia had a

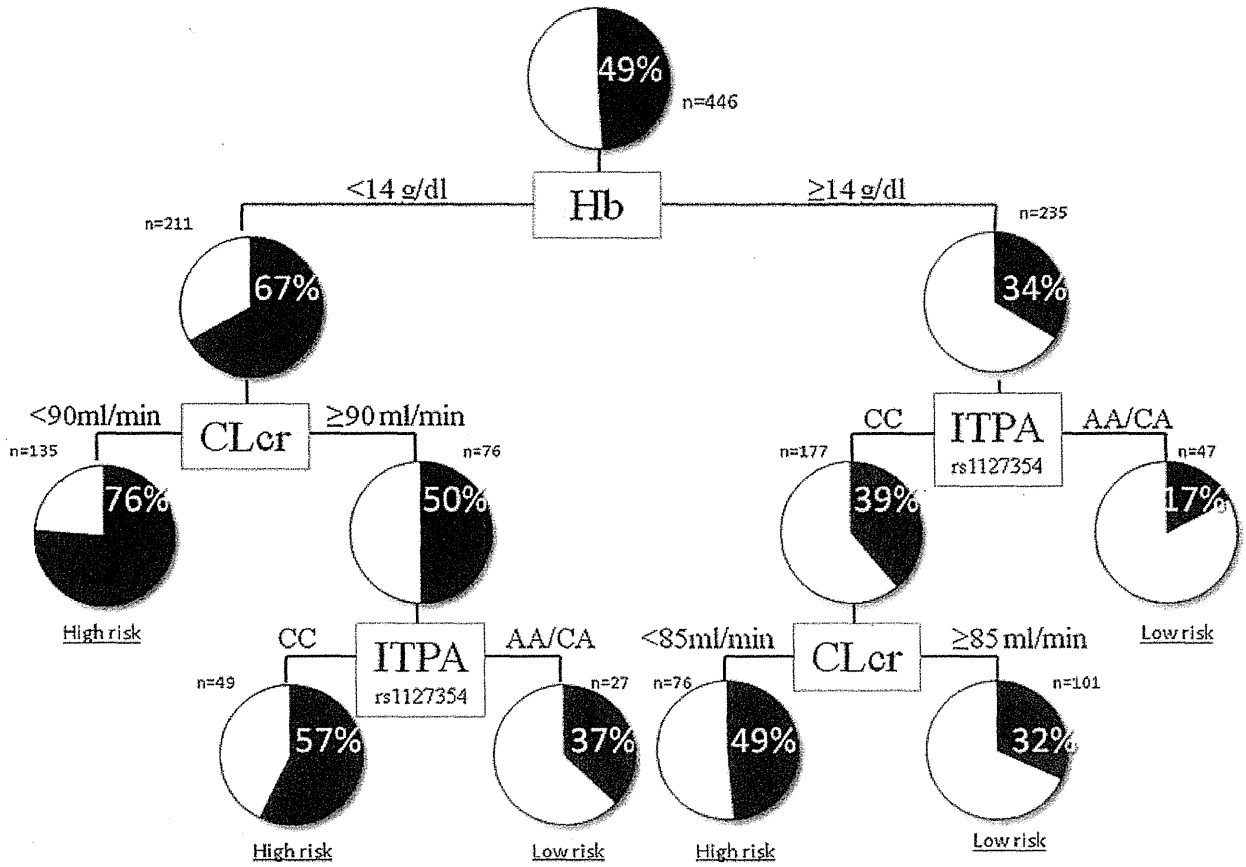

Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients differentiated by analysis are classified as at high risk if the rate is >40% and low risk if the rate is <40%. *ITPA*, inosine triphosphatase; CLcr, creatinine clearance; Hb, hemoglobin.

significantly lower rate of sustained virological response than the low-risk group (59% vs. 76%, $P = 0.013$) (Fig. 3D–F). In patients who did not achieve a complete early virological response, the *IL28B* genotype was a significant predictor of a sustained virological response (TT vs. TG/GG; 14% vs. 2%, $P < 0.0001$) but a high risk for anemia was not (high vs. low; 10% vs. 6%, $P = 0.361$).

From multivariate analysis (Table II), the *IL28B* genotype was the most important predictor of a sustained virological response at baseline [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$], along with female sex [0.42 (0.26–0.68), $P < 0.0001$], platelet count [1.09 (1.04–1.15), $P < 0.0001$], advanced fibrosis [0.49 (0.27–0.91), $P = 0.024$], and baseline HCV RNA load [4.14 (2.27–7.55), $P < 0.0001$]. At week 4, in patients without a rapid virological response, the *IL28B* genotype remained the most important predictor of a sustained virological response [7.16 (3.60–14.25), $P < 0.0001$], along with female sex and platelet count. At week 12, in patients with a complete early virological response, the risk of anemia was an independent and significant predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$], together with the platelet count and HCV RNA load, but the *IL28B* genotype was not associated with a sustained virological response. In patients without a complete early virological response, the *IL28B* genotype was a predictor of a sustained virological response [9.13 (2.02–41.3), $P = 0.004$] along with the platelet count. Thus, *IL28B* was a significant predictor of a sustained virological response at baseline and among virological non-responders at weeks 4 and 12. On the other hand, once a complete early virological response was achieved, the *IL28B* genotype was no longer associated with a sustained virological response but the risk of anemia was an independent predictor of a sustained virological response.

The Risk of Anemia, RBV Dose, and Treatment Outcome in Patients With a Complete Early Virological Response

Patients who achieved a complete early virological response were stratified according to adherence to

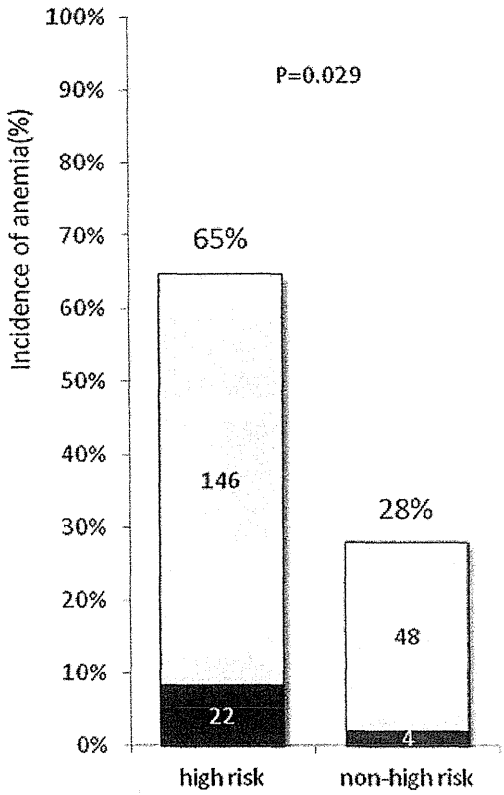

Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV (≤40%, 41–60%, 61–80%, and >80%), which showed that patients with a high risk of anemia were predominantly in subgroups with a lower adherence to RBV (≤40%, 41–60%, and 61–80%), whereas patients with a low risk of anemia were predominantly in subgroups with a higher adherence to RBV (>80%) (Fig. 4, upper panel). The percentage of patients who received >80% of the planned dose of RBV was significantly higher in the low-risk group for anemia than in the high-risk group (74% vs. 55%, $P < 0.0001$).

Within the groups with high and low risks of anemia, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV (Fig. 4, lower panel). The rate of sustained virological response was higher in patients who received >80% of the planned dose of RBV than those who received less, for both high-risk patients (71% vs. 47%, $P = 0.016$) and low-risk patients (81% vs. 60%, $P = 0.072$). Within the same subgroup of RBV adherence, however, the rate of sustained virological response did not differ between patients with a high risk and a low risk of anemia. Taken together, these results suggest that patients with a high risk of anemia have a disadvantage because they are likely to be intolerant to RBV, leading to reduced adherence to RBV throughout the 48 weeks of therapy and a reduced rate of sustained virological response. However, if >80% adherence to RBV could be obtained, the rate of sustained virological response would increase by 24%.

DISCUSSION

This study confirmed previous reports that the *IL28B* genotype is the most significant predictor of a sustained virological response to PEG-IFN plus RBV therapy in chronic hepatitis C patients at baseline [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010; Kurosaki et al., 2011c] and at week 4 [Thompson et al., 2010b], but it had no impact on the rate of sustained virological response among those patients who achieved a complete early virological response [Thompson et al., 2010b; Kurosaki et al., 2011c]. In contrast, the risk of anemia, assessed by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was found to be associated with a sustained virological response in patients who achieved a complete early virological response. Generally, a complete early virological response is the hallmark of a high probability of a sustained virological response, but the rate of sustained virological responses in patients who achieved a complete early virological response and had a high risk of anemia was as low as 59%. This reduced rate of sustained virological response in these patients was attributable to poor adherence to RBV throughout the 48 weeks of therapy. Because administration of >80% of the planned RBV dose increased the rate of sustained virological response by 24%, it may be postulated that personalizing the treatment schedule to achieve a sufficient dose of RBV, such as extension of treatment duration, may improve sustained virological response rates in these patients. Clearly, this postulate needs to be confirmed in future study. Thus, the findings presented here may have the potential to support selection of the optimum, personalized treatment strategy for an individual patient, based on the risk of anemia.

The degree of hemolytic anemia caused by RBV varies among individuals. A reduction of the Hb concentration early during therapy predicts the likely development of severe anemia [Hiramatsu et al., 2008, 2011] but there are no reliable predictors at baseline. A breakthrough came from the results of a genome-wide association study that revealed that variants of the *ITPA* gene are protective against hemolytic anemia [Fellay et al., 2010]. The *ITPA* genotype has been shown repeatedly to be associated with the degree of hemolytic anemia and dose reduction of RBV [Fellay et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a; Seto et al., 2011; Tanaka et al., 2011; Kurosaki et al., 2011d]. However, factors other than the *ITPA* gene, such as baseline Hb concentrations [Ochi et al., 2010; Kurosaki et al., 2011d], platelet counts [Ochi

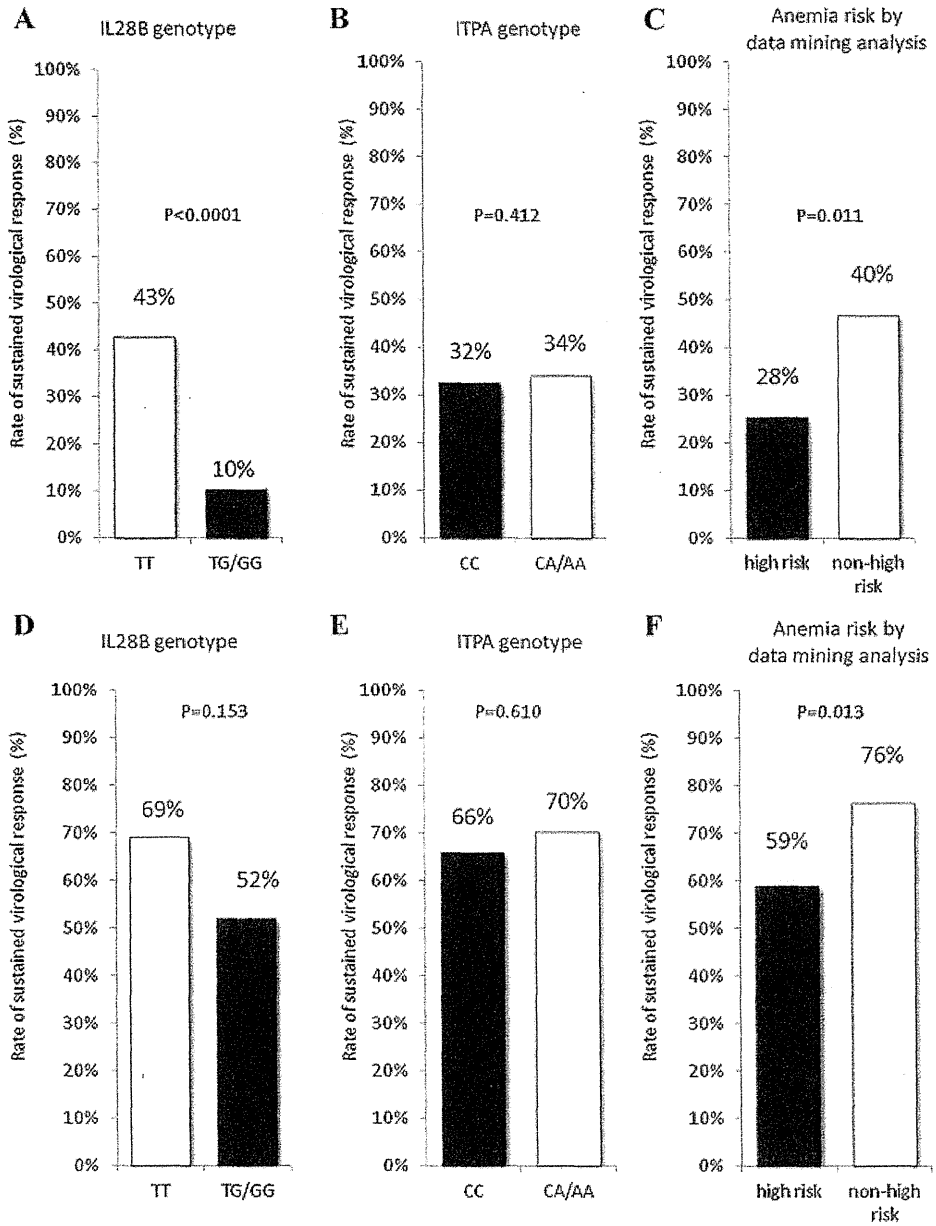

Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (**A–C**) and among those with complete early virological responses (defined as undetectable HCV RNA at week 12) (**D–F**). At baseline, those with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele and the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group. Among patients with complete early virological responses, the *IL28B* genotype was not associated with a sustained virological response, while the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group.

et al., 2010], and CLcr [Kurosaki et al., 2011d], also contribute to the risk of severe anemia or RBV dose reduction. In the present study, the predictive model of anemia based on the data mining analysis selected the *ITPA* genotype, baseline Hb concentration, and baseline CLcr as predictive factors and identified six subgroups of patients with a variable rate of severe anemia, ranging from 17% to 76%. The specificity of the prediction of severe anemia was improved by 25.7% in the predictive model, compared to *ITPA*

TABLE II. Logistic Regression Analysis for Factors Associated With Sustained Virological Response at Baseline, Week 4 and Week 12

	Multi-variable		
	Odds	95% CI	*P*-value
Pre-treatment			
Sex: female	0.42	0.26–0.68	<0.0001
Platelet (10^9/L)	1.09	1.04–1.15	<0.0001
Fibrosis: F3-4	0.49	0.27–0.91	0.024
HCV RNA: <600,000 IU/L	4.14	2.27–7.55	<0.0001
IL28B rs8099917: TT	9.88	5.01–19.48	<0.0001
At week 4			
Non-RVR patients			
Sex: female	0.45	0.28–0.72	0.001
Platelet (10^9/L)	1.10	1.05–1.16	0.000
IL28B rs8099917: TT	7.16	3.60–14.25	<0.0001
At week 12			
cEVR patients			
Platelet (10^9/L)	1.09	1.02–1.17	0.015
HCV RNA: <600,000 IU/L	3.21	1.39–7.55	0.007
High-risk of anemia[a]	0.47	0.24–0.91	0.026
At week 12			
Non-cEVR patients			
Platelet (10^9/L)	1.11	1.02–1.21	0.017
IL28B rs8099917: TT	9.13	2.02–41.3	0.004

RVR: rapid virological response, defined as undetectable HCV RNA at week 4.
cEVR: complete early virological response, defined as undetectable HCV RNA at week 12.
[a]High-risk of anemia defined by decision tree analysis includes the following groups: (1) baseline hemoglobin <14.0 g/dl and creatinine clearance <90 ml/min, (2) baseline hemoglobin <14.0 g/dl, creatinine clearance ≥90 ml/min and ITPA rs1127354 genotype CC, and (3) baseline hemoglobin ≥14.0 g/dl, ITPA rs1127354 genotype CC, and creatinine clearance <85 ml/min.

genotyping alone. Because hemolytic anemia induced by RBV is one of the major adverse events leading to premature termination of therapy [Fried et al., 2002], a method to predict the risk of severe anemia before treatment is important clinically. A predictive model of anemia may have the potential to support individualized treatment strategies; patients at high risk of anemia may be tested intensively for anemia or may be candidates for erythropoietin therapy, whereas those with a low risk of anemia may be treated with a higher dose of RBV. Prediction of anemia will remain important in the era of direct antiviral agents for chronic hepatitis C, because these newer therapies still require RBV and PEG-IFN in combination, and the degree of anemia complicating these therapies may be even greater than with the current combination therapy [McHutchison et al., 2009; Kwo et al., 2010].

Studies of the impact of the *ITPA* genotype on treatment outcome have produced conflicting results. Previous studies of American [Thompson et al., 2010a] and Italian [Thompson et al., 2011] cohorts did not find any association between the *ITPA* genotype and treatment outcome, whereas a marginal difference was observed in a report from Japan [Ochi et al., 2010]. Moreover, with a subgroup analysis of Japanese patients, the variant of the *ITPA* gene was associated with a sustained virological response in patients with the *IL28B* major genotype [Kurosaki et al., 2011d], in patients infected with HCV other than genotype 1[Sakamoto et al., 2010], and in patients with pre-treatment Hb concentrations between 13.5 and 15 g/dl [Azakami et al., 2011]. These inconsistent results may be because the impact of anemia may be greater on a cohort of aged patients, such as in Japan. Another reason may be that the *ITPA* genotype is not the sole determinant of anemia; the *ITPA* genotype alone was not associated with treatment outcome in the present study but a high-risk of anemia, defined by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was associated with sustained virological responses by patients with complete early virological responses, even after adjustment for the *IL28B* genotype and other relevant factors. This is in contrast to the finding that the *IL28B* genotype is an independent and significant predictor at baseline of a sustained virological response by patients without a rapid virological response and those without a complete early virological response, but not those with a complete early virological response. These results indicate that the *IL28B* genotype could be used to predict a sustained virological response at baseline or during therapy in patients in whom HCV RNA has not yet become undetectable, but it has no predictive value in patients in whom HCV RNA has become undetectable. The risk of anemia may be used to predict sustained virological responses in a selected subgroup of patients who achieve a complete early virological response.

Patients who received more than 80% of the planned dose of PEG-IFN or RBV had a higher rate of sustained virological responses than those who received a lower cumulative dose [McHutchison et al., 2002; Davis et al., 2003]. Patients who achieve a complete early virological response usually have a good chance of a sustained virological response and the treatment duration is not extended beyond 48 weeks. However, reduced adherence to drugs in these patients was related to relapse after the completion of 48 weeks of therapy [Hiramatsu et al., 2009; Kurosaki et al., 2012]. In the present study, the rate of sustained virological response was 59% in patients who achieved a complete early virological response but had a high risk of anemia, 17% lower than in patients with a low risk of anemia. However, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV, and the rate of sustained virological response was higher in high-risk patients who received >80% of the planned dose of RBV (71% vs. 47%). This 24% increase in sustained virological response was observed among the patients in the present study who received 48 weeks of treatment. These findings suggest that receiving a sufficient RBV dose is essential for patients with a complete early virological response to attain a sustained virological response and that the treatment strategy should be personalized for patients with a

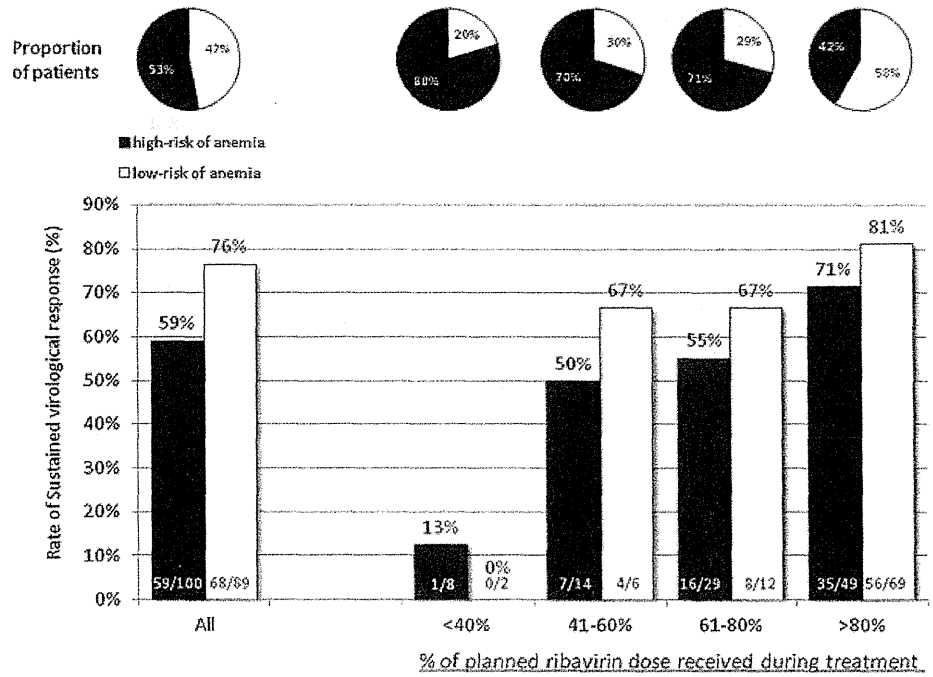

Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of patients with a high risk and a low risk of anemia is shown in the upper panel by pie charts, and the rates of sustained virological responses, stratified by high risk and low risk of anemia, are shown in the lower panel by bar graphs. The black and white bars or charts represent patients with high and low risks of anemia, respectively.

high risk of anemia to extend the duration of treatment, even those patients with a complete early virological response, to obtain >80% adherence to RBV.

In conclusion, the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr could be used as a pre-treatment predictor of anemia. The risk of anemia thus identified is associated with adherence to RBV and impacts on the treatment outcome of patients who achieve a complete early virological response. This is in contrast to the major role of the *IL28B* genotype in the prediction of sustained virological responses at baseline and among non-responders at weeks 4 and 12. Patients who achieve a complete early virological response generally have a high probability of a sustained virological response but those who have a high risk of anemia have a high rate of relapse because of reduced adherence to RBV. To improve the rate of sustained virological responses in these patients, it may be postulated that the treatment schedule may be personalized to obtain >80% adherence to RBV. Clearly, this postulate needs to be confirmed in a future study.

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