

parentheses. Gates 1, 2 and 3 refer to positive negative and background staining, respectively, set using positive, negative and unstained controls. The X-axis refers to fluorescent intensity and the Y-axis refers to the number of phantom contours falling within each designated region.

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Original Article

ITPA gene variant protects against anemia induced by pegylated interferon- α and ribavirin therapy for Japanese patients with chronic hepatitis C

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Aim: Host genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in chronic hepatitis C patients receiving ribavirin. In this study, we evaluated the clinical significance of *ITPA* variants in Japanese hepatitis C patients who were treated with pegylated interferon plus ribavirin.

Methods: In this multicenter retrospective cross-sectional study, 474 hepatitis C patients were enrolled who were treated with pegylated interferon plus ribavirin in four geographically different hospitals in Japan. Patients were grouped according to hemoglobin decline of more than 3 g/dL at week 4. Two single nucleotide polymorphisms (SNP) within or adjacent to the *ITPA* gene (rs6051702, rs1127354) were genotyped.

Results: A functional SNP, rs1127354, within the *ITPA* exon was strongly associated with protection against anemia with only one (0.8%) in 129 patients with the *ITPA* minor variant A

developing severe anemia ($P = 5.9 \times 10^{-20}$). For rs6051702, which had significant association in European-Americans, significant but weak association with severe hemoglobin reduction was found in Japanese ($P = 0.009$). In patients excluding genotype 1b and high viral load, those with the *ITPA* minor variant A achieved significantly higher sustained viral response rate than those with the major variant (CC) (96% vs 70%, respectively, $P = 0.0066$).

Conclusion: *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of ribavirin-induced anemia in Japanese patients. Patients with the *ITPA* minor variant A (~27%) have an advantage in pegylated interferon plus ribavirin-based therapies, due to expected adherence of ribavirin doses, resulting in a higher viral clearance rate.

Key words: *c20orf194*, hemolytic anemia, hepatitis C virus, *ITPA* (inosine triphosphatase), pegylated interferon plus ribavirin therapy

INTRODUCTION

APPROXIMATELY 3% OF the worldwide population is infected with the hepatitis C virus (HCV), which

represents 170 million people, with 3–4 million individuals newly infected each year. Chronic hepatitis C (CHC) has a variable course; although 20–25% of CHC patients maintain persistently normal serum aminotransferases and experience relatively slow histological progression, other patients present a more active biochemical course.^{1–3} Overall, 30% of the CHC patients progress to cirrhosis in their lifetime,³ and 3–8% of cirrhosis patients develop hepatocellular carcinoma (HCC) every year.^{4–6} Among various factors, older age and hepatic steatosis are significant factors accelerating the rate of progression in CHC.^{3,7–9}

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Antiviral treatment has been shown to improve liver histology and decrease incidence of HCC in CHC.^{6,10} Current therapy for CHC consists 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.^{11,12} However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or a cure of the infection.^{11,13} Older patients have showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities.^{14–16} In particular, hematological abnormalities and RBV-induced hemolytic anemia often necessitate dose reduction and premature withdrawal from therapy in 10–14% of patients.^{11,17–20} New drugs and therapeutic approaches for CHC are actively developed and several candidates are in the early trial phase.^{21,22} Given these backgrounds, effective pre-treatment screening for predictor biomarkers with the aim to evaluate possible risks over benefits from currently available treatment would allow avoiding these side-effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of the treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies have demonstrated remarkable associations between single nucleotide polymorphisms (SNP) near or within the region of the *IL28B* gene, which codes for IFN- λ 3.^{23–28} Another recent study indicated that genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in CHC patients receiving RBV.²⁹ The results obtained in one GWAS study need to be evaluated and confirmed in the context of different geographical and racial populations, and independent cohorts. Here, we describe clinical evaluation of two SNP within or adjacent to the *ITPA* gene (6051702 and rs1127354), that was recently highlighted by the GWAS of HCV treatment-induced anemia.²⁹

METHODS

Patients

IN THIS RETROSPECTIVE cross-sectional case-control study, 474 patients with chronic HCV infection treated at Tokyo Medical and Dental University Hospi-

tal, Nagoya City University Hospital, Yamanashi University Hospital, Nagasaki Medical Center and Hyogo University of Health Science Hospital in Japan were enrolled from April 2007 to April 2009. Each patient was treated with PEG IFN- α -2b (1.5 μ g/kg s.c. once a week) or PEG IFN- α -2a (180 μ g/kg once a week) plus RBV (600–1000 mg daily depending on bodyweight). The treatment duration was set at a standard 48 weeks for genotype 1b high viral load (≥ 5 log copies/mL) patients and 24 weeks for genotype 1 low viral load (≤ 5 log copies/mL) and genotypes 2 and 3 patients. On-treatment dose reduction and discontinuation of PEG IFN or RBV were decided based on the recommendations of package inserts or clinical situations in individual patients to avoid possible side-effects. The rates of PEG IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to bodyweight before therapy. Hepatitis B surface antigen (HBsAg) positive and/or anti-HIV positive individuals were excluded from this study. Hemoglobin (Hb) values were measured at baseline and every week until 8 weeks. We considered Hb decline at week 4 to be a clinically important time point, as previously reported.²⁹ The threshold of Hb reduction of more than 3 g/dL was chosen as a clinically significant Hb decline according to the previous reports.^{29–31}

Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

Patient evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, sex, previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pre-treatment biochemical parameters, such as white blood cells, neutrophils, Hb, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/mL). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0, showing no activity; A1, showing mild activity; A2, showing moderate activity; and A3, showing severe activity. Fibrosis was staged on a scale of 0–4: F0, showing no fibrosis; F1, showing moderate fibrosis; F2, showing moderate fibrosis with

few septa; F3, showing severe fibrosis with numerous septa without cirrhosis; and F4, showing cirrhosis.

SNP genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphisms, rs1127354 in *ITPA*, rs6051702 in *C20orf194*, and rs8099917 around the *IL28B* gene were determined by real-time detection polymerase chain reaction with a TaqMan probe or DigiTag2 assay typing one tag SNP located within each locus.²³ Another functional SNP, rs727010 within the *ITPA* gene, was excluded due to no variants in the Asian genetic population as reported in the International HapMap Project database. Our preliminary genotyping of a 100-patient population did not find variants in that SNP.

Outcomes

The primary end-point was Hb decline and dose reduction of PEG IFN or RBV in week 4, the secondary end-point was SVR. An SVR was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. A transient viral response (TVR) meant that HCV RNA became undetectable during treatment but reappeared at the end of follow up. A null response (NR) was defined as persistently positive HCV RNA throughout the treatment. Adverse events and drug adherence were recorded.

Statistical analyses

The association between individual *ITPA* SNP and the incidence of significant Hb decline was tested by a basic allelic test and calculated using the χ^2 -test. Multivariate logistic regression analysis with stepwise forward selection was performed with *P*-values of less than 0.05 as the criteria for model inclusion. These statistical analyses were conducted by using SPSS software package ver. 18J (Chicago, IL, USA) or Microsoft Excel Mac 2008 (Redmond, WA, USA). Discrete variables were evaluated by Fisher's exact probability test. The *P*-values were calculated by two-tailed Student's *t*-tests for continuous data and χ^2 -test for categorical data, and those of less than 0.05 were considered statistically significant.

RESULTS

THE CLINICAL CHARACTERISTICS of the 474 patients are summarized in Table 1. First, we compared baseline clinical and host genetic characteristics of patient groups according to the SNP within the *ITPA* gene, rs1127354, between major homozygote (CC) and

Table 1 Baseline characteristics of participating patients

Total number	474
Age (years)	57.2 ± 10.0
Sex (male/female)	264/210
Bodyweight (kg)	61.1 ± 10.8
HCV genotypes	
1b/2a/2b/3a	416/31/26/1
1b, high viral load/others	387/87
NS5A-ISDR mutations (genotype 1b, <i>n</i> = 334, 0–1/≥2)	285/49
Core mutations (genotype 1b, <i>n</i> = 379)	
C70 (wild/mutant)	240/139
C91 (wild/mutant)	234/145
Histology at biopsy (<i>n</i> = 278)	
Grade of inflammation (A0/1/2/3)	4/97/154/23
Stage of fibrosis (F0/1/2/3/4)	8/102/74/74/20
White blood cells (/μL)†	5707 ± 1495
Neutrophils (/μL)†	2568 ± 1013
Hemoglobin (g/dL)†	14.2 ± 1.4
Platelet count (×10 ⁻³ /μL)†	158 ± 58
ALT (IU/L)†	89 ± 66
Serum HCV RNA (log [IU/mL])‡	6.0 ± 0.9
PEG IFN (PEG IFN-α-2a/PEG IFN-α-2b)	40/434
Hb decline at week 4 (g/dl)	2.4 ± 1.4
Severe anemia, Hb <10 g/dL at week 4	56/474 (11.8%)

†Data are expressed as mean ± standard deviation.

‡Data are shown as median (range) values.

High viral load: HCV RNA ≥ 5 log IU/mL.

ALT, alanine transaminase; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; PEG, pegylated.

a group of heterozygote (CA) and minor homozygote (AA) (Table 2). There were no significant differences in age, sex, blood cell counts, ALT levels, serum viral loads, frequencies of core 70/91 mutations^{32,33} and the numbers of NS5A interferon sensitivity determining region (ISDR) mutations^{34,35} between the two groups. The SNP in the *ITPA* gene did not show significant linkage between the SNP around the *IL28B* gene, rs8099917, which is strongly associated with IFN treatment responses.^{23,25} In contrast, the SNP in the *ITPA* gene showed significant linkage with the SNP in *C20orf194*, rs6051702 (*P* = 7.1 × 10⁻¹⁴).²⁹

Next, we analyzed two SNP, rs6051702 in *C20orf194* and rs1127354 in *ITPA* loci, respectively, for their association with significant Hb decline at 4 weeks of PEG IFN plus RBV treatment using a basic allelic model that compares frequencies of alleles in cases versus controls. The SNP, rs6051702, which showed the strongest association in the European-American population,²⁹ was

Table 2 Clinical and host genetic characteristics of patients according to *ITPA* gene variants

	<i>ITPA</i> SNP, rs1127354		P-value
	CC (n = 345)	CA + AA (n = 129)	
Age (years)†	57.2 ± 10.0	57.1 ± 10.1	0.87
Sex (male/female)	192/153	72/57	0.97
White blood cells (/μL)‡	5312 ± 1537	4995 ± 1388	0.92
Neutrophils (/μL)‡	1696 ± 1415	1803 ± 1516	0.49
Hemoglobin (g/dL)‡	14.2 ± 1.4	14.1 ± 1.4	0.52
Platelet count (×10 ³ /μL)‡	157 ± 54	159 ± 70	0.81
ALT (IU/mL)‡	91 ± 70	83 ± 55	0.46
Serum HCV RNA (log copies /mL)†	6.1 ± 0.7	5.8 ± 1.1	0.093
NS5A-ISDR mutations (genotype 1b, n = 334, 0–1/≥2)	213/31	72/18	0.095
Core mutations (genotype 1b, n = 389)			
aa. 70 (wild/mutant)	179/96	61/43	0.25
aa. 91 (wild/mutant)	172/103	62/42	0.60
<i>IL28B</i> , rs8099917 (TT/TG/GG)	233/94/3	96/29/1	0.23
<i>C20orf194</i> , rs6051702 (AA/AC/CC)	254/85/6	47/72/10	7.1 × 10 ⁻¹⁴ *

*P-values were calculated by student's t-test or by χ^2 analysis.

†Data are show as median (range) values.

‡Data are expressed as mean ± standard deviation.

IL28B SNP, major allele-T and minor allele-G.

C20orf194 SNP, major allele-A and minor allele-C.

ALT, alanine transaminase; HCV, hepatitis C virus; SNP, single nucleotide polymorphisms.

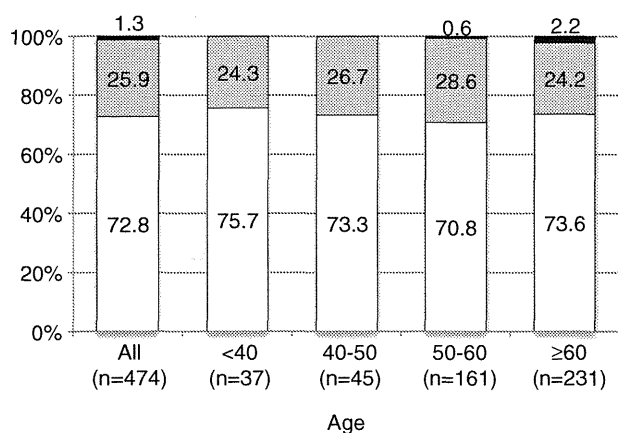


Figure 1 *ITPA* variant, rs1127354, known to be responsible for inosine triphosphatase deficiency and its age-related differences. *ITPA*, inosine triphosphatase. The numbers in parentheses denote numbers of patients.

associated with the Hb decline significantly but with smaller effect size (odds ratio [OR] = 1.40, $P = 9.0 \times 10^{-3}$, Table 3). Notably, another SNP in the *ITPA* gene, rs1127354, showed overwhelming association with the Hb decline (OR = 62.8, $P = 5.9 \times 10^{-20}$). The prevalence of *ITPA* variants is shown in Figure 1. Percentages of *ITPA*, rs1127354, major homozygote (CC), heterozygote (CA) and minor homozygote (AA) were 72.8%, 25.9% and 1.3%, respectively. There was no difference in the frequency of the *ITPA* variants throughout ages and sexes (Fig. 1).

To assess the clinical relevance of these SNP, we analyzed the proportion of patients suffering clinically significant anemia, which we defined as a decline in Hb levels of more than 3 g/dL or Hb levels of less than 10 g/dL, which is the threshold at which RBV dose reduction is recommended. As depicted in Figure 2, in *ITPA*-CC patients, Hb loss of more than 3 g/dL devel-

Table 3 Association of *C20orf194* and *ITPA* gene variants with treatment-induced Hb decline

Gene	SNP	Allele (major/minor)	MAF (%)	OR	P-value*
<i>C20orf194</i>	rs6051702	A/C	19.9	1.40	9.0×10^{-3}
<i>ITPA</i>	rs1127354	C/A	14.2	62.8	5.9×10^{-20}

*The SNP-phenotype associations were analyzed using a basic allelic test.

P-values were calculated by χ^2 analysis.

Hb, hemoglobin; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphisms.

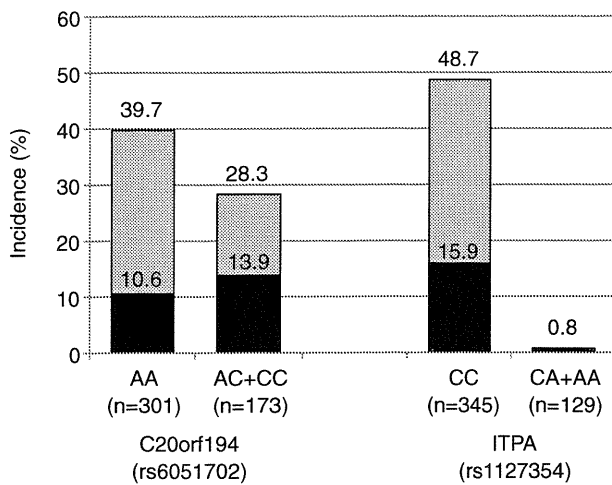


Figure 2 Effects of *c20orf194* and *ITPA* single nucleotide polymorphisms (SNP) on clinically significant anemia induced by pegylated interferon plus ribavirin treatment. Percentages of patients with hemoglobin (Hb) decline of >3 g/dL or Hb levels of >10 g/dL at week 4 of treatment are shown for each SNP in two genes, *c20orf194* (rs6051702) and *ITPA* (rs1127354).

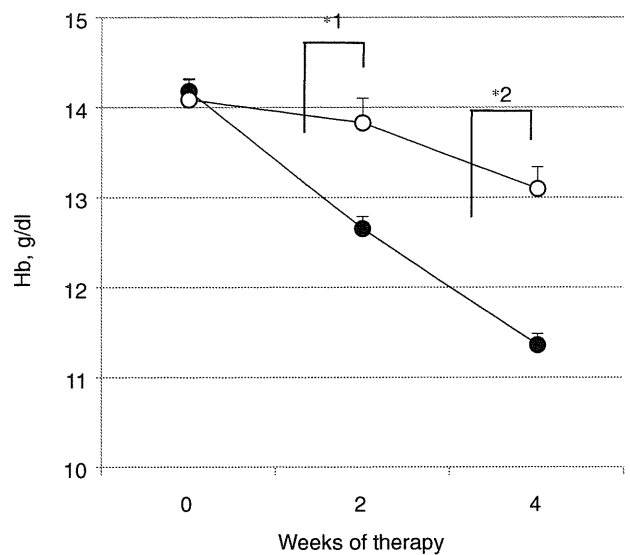


Figure 3 Time-dependent hemoglobin (Hb) decline in *ITPA* major and minor variants. Error bars indicate mean + standard error. Asterisks 1 and 2 indicate statistical significance of $P = 6.6 \times 10^{-13}$ and $P = 3.0 \times 10^{-29}$, respectively.

oped in 48.7% at week 4, and 15.9% of patients achieved Hb levels of less than 10 g/dL. In contrast, only one patient (0.8%) with *ITPA*-CA/AA developed anemia. These differences in the incidence of the treatment-induced Hb decline were consistent throughout ages. The time-dependent Hb decline in patients with *ITPA*-CC and *ITPA*-CA/AA is shown in Figure 3. In patients with *ITPA*-CC, mean Hb drop was 2.9 ± 1.3 g/dL, which was significantly higher than that of patients with *ITPA*-CA/AA (1.1 ± 0.7 g/dL). These results demonstrate that the *ITPA* minor variant A has a protective phenotype for the treatment-induced anemia. The positive predictive value of the *ITPA*-major (CC) for the development of severe anemia was 48.7%, while the negative predictive value of *ITPA*-hetero/minor (CA/AA) was 99.2%. In accordance with the incidence of anemia, there was significant difference in the incidence of RBV dose reduction. At week 4 of treatment, RBV doses were reduced in 27.9% of *ITPA*-CC patients while in only 14.4% of *ITPA*-CA/AA patients ($P = 0.012$, Fig. 4). Similarly to RBV, PEG IFN dose reduction was apparently higher in *ITPA*-CC patients though it did not reach statistical significance.

Knowing that significantly less frequent drug reduction occurred in patients with the *ITPA*-minor variant A, we next investigated if the *ITPA* gene variants affected final treatment outcomes. The treatment outcomes were available in 339 patients with genotype 1b and high viral load

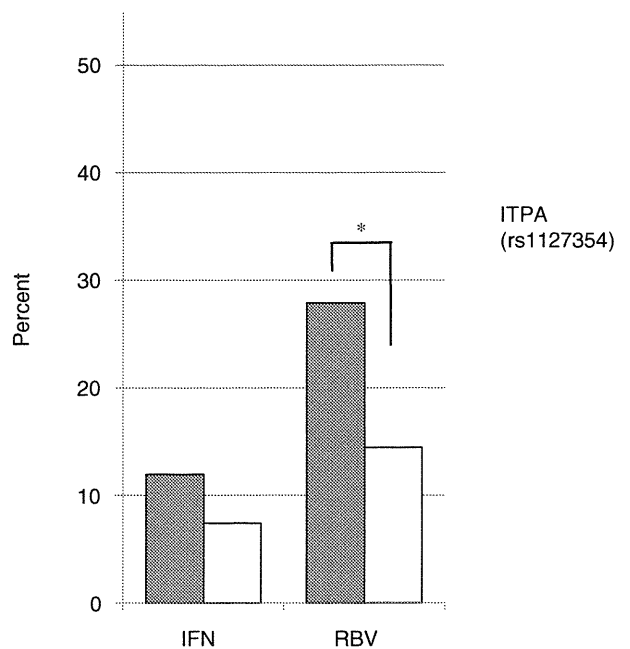


Figure 4 Percentages of patients requiring pegylated interferon (IFN) or ribavirin (RBV) dose reduction at week 4 in *ITPA* major and minor variants. Y-axis indicates percents of patients who required dose reduction. * $P = 0.012$.

Table 4 Sustained viral response rates of each group according to *ITPA* gene variants

<i>ITPA</i> SNP, rs1127354	Genotype 1b, high viral load		Others	
	CC	CA + AA	CC	CA + AA
SVR	92 (37.1%)	39 (42.9%)	41 (70%)	25 (96%)
TVR	90 (36.3%)	34 (37.3%)	15 (25%)	1 (4%)
NR	66 (26.6%)	18 (19.8%)	3 (5%)	0 (0%)
Total	248	91	59	26
<i>P</i> -value	0.33		0.0066	

High viral load; serum HCV RNA ≥ 5 logIU/mL.

"Others" include genotypes genotype 1b, serum HCV RNA <5 logIU/ml, genotypes 2a, 2b and 3a.

P-values were calculated by χ^2 -test analyses of SVR versus TVR plus NR.

HCV, hepatitis C virus; SVR, sustained viral response; TVR, transient viral response; NR, null response.

(HCV RNA ≥ 5.0 log IU/ml) and 85 others, which included genotype 1b, low viral load and genotype 2a, 2b and 3a patients (Table 4). In patients with genotype 1b and high viral load, there was no significant difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA patients (37.1% and 42.9%, respectively). In contrast, there was a striking difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA in the other IFN-sensitive group (non-1b or low viral load); the SVR rate was 70% in *ITPA*-CC patients, while 96% of *ITPA*-CA/AA patients achieved SVR ($P = 0.0066$). These results indicate that the *ITPA* minor variant A is significantly associated with SVR in the IFN-sensitive group excluding genotype 1b and high viral load. Using those subpopulations of patients, we conducted a statistical analysis for association of several host and viral parameters with SVR. As shown in Table 5, univariate analysis identified four significant parameters including age, platelet count, stages of fibrosis and the *ITPA* SNP, rs1127354. Multivariate logistic regression

analysis identified that only age and the *ITPA* SNP were significantly associated with SVR.

DISCUSSION

RECENT GWAS ON HCV infection have identified two important host genetic polymorphisms. One is the SNP in the *IL28B* gene, which is strongly associated with response to therapy of chronic genotype 1 HCV infection,^{23–28} and another is the SNP in the *ITPA* gene, which precisely predicts RBV treatment-associated anemia in the European-American population.²⁹ In our present study, a functional SNP in the *ITPA* locus, rs1127354, is strongly associated with protection against anemia among 474 Japanese patients ($P = 5.9 \times 10^{-20}$, Table 3). Only one of 129 patients (0.8%) who carry the rs1127354 minor allele A had severe anemia (Figs 2,3). These data are consistent with the previous study in the US population²⁹ as well as a recent Japanese study by

Table 5 Univariate and multivariate logistic regression analyses of host and viral characteristics of patients excluding genotype 1b high virus load based on therapeutic responses ($n = 85$)

Variable	<i>P</i> -value (univariate)	<i>P</i> -value (multivariate)	OR	95% CI
Age	0.017	0.047	0.916	0.840–0.999
Sex (male vs female)	0.19	–		
Baseline Hb level	0.17	–		
Baseline platelet count	0.019	0.307	1.092	0.923–1.292
Stage of fibrosis (F0–2 vs 3–4)	0.0020	0.083	4.221	0.827–21.531
PEG IFN adherence ($\geq 80\%$ vs <80%)	0.67	–		
RBV adherence ($\geq 80\%$ vs <80%)	0.30	–		
<i>ITPA</i> SNP rs1127354 (CC vs CA + AA)	0.0066	0.023	12.680	1.386–116.042
<i>IL28B</i> SNP rs8099917 (TT vs TG + GG)	0.29	–		

CI, confidence interval; Hb, hemoglobin; IFN, interferon; *ITPA*, inosine triphosphatase; OR, odds ratio; PEG, pegylated; RBV, ribavirin; SNP, single nucleotide polymorphisms.

Ochi *et al.*³¹ Our data were similar to these two reports; rs1127354 was the most significant SNP that was associated with RBV-induced anemia in Asian genetic populations. Additionally, we have demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected (Fig. 4) and, more importantly, that a significantly higher SVR rate was achieved in *ITPA*-hetero/minor (CA/AA) patients with HCV non-1b or low viral load strains (70% vs 96%, $P = 0.0066$, Table 4). Taken together, our results demonstrate that the *ITPA* minor variant A is not only a protective allele of PEG IFN and RBV treatment-associated anemia in the Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN.

An SNP in *C20orf194*, rs6051702, which showed significant association with Hb reduction in European-Americans ($P = 1.1 \times 10^{-45}$),²⁹ was also significant in our study of a Japanese population, but with smaller effect size on Hb reduction ($P = 0.014$). The discrepancy may be due to the low levels of the linkage disequilibrium (LD) with the functional SNP in the *ITPA* gene in the Japanese/Asian population as compared with the high LD in white subjects. Indeed, it is reported that the predictive values of the *C20orf194* SNP varied between different races including African-Americans ($P = 0.19$) and Hispanics ($P = 9.5 \times 10^{-3}$).²⁹

Ochi *et al.* sequenced the Japanese patient genome including *ITPA* and *DDRGK1* loci, which are located adjacently on chromosome 20. They identified 83 SNP with major allele frequency of more than 0.05, of which four SNP including rs1127354 were significantly associated with RBV-induced anemia and which were in almost absolute LD with each other.³¹ Their report indicates that the *ITPA* SNP, rs1127354, which we genotyped in the present study, represent a dominant variant of *ITPA* deficiency that protects against RBV-induced anemia in Japanese/Asian genetic populations. In our study, however, 51.3% of the *ITPA*-major (CC) patients did not develop significant Hb decline (Fig. 2). This finding suggests that there are other low-frequency *ITPA* variants or SNP in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

The response to PEG IFN plus RBV treatment is affected by several viral and host factors such as age, sex,^{36,37} NS5A-ISDR³⁸ and core region.^{32,33} To maintain good adherence to drugs, especially RBV, it is important to achieve good treatment responses. Increased RBV exposure during the treatment phase was associated with an increased likelihood of SVR in the US³⁹ and Japanese studies.⁴⁰ Because patients with *ITPA* minor variant A are

refractory to RBV-induced anemia, they are advantaged in maintaining good adherence to RBV and may be given even higher doses of RBV, resulting in a higher SVR rate. However, a study by Fellay *et al.* and a very recent replication study by Thompsom *et al.*³⁰ did not observe any significant association between the *ITPA* minor variants and early or late anti-HCV treatment outcomes.²⁹ A possible explanation for the discrepancy is that older and histologically more advanced patients were predominant in our study. Mean age in the US study was 47.5 years while 57.2 years in our present study. The percentage of advanced fibrosis (F3 or F4) was 12.0% in the US study while 34% in our study. It is well known that the incidence of drug dose reduction or discontinuation could increase according to old age as well as advanced stages, that may compromise final treatment outcomes.^{14,15} Importantly, we have additionally demonstrated that in patients with other than genotype 1b HCV, *ITPA* minor variant A was significantly associated with better SVR rates in univariate and multivariate analyses. Because the typical PEG IFN plus RBV treatment period is shorter (24 weeks) in genotype 1 low viral load and genotype 2 patients than in genotype 1 high viral load (48 weeks), early dose reduction of RBV may be more critical to the final treatment outcome.

Ribavirin is a synthetic guanosine analog, and has actions *in vitro* against a wide range of RNA and DNA viruses.⁴¹ Possible antiviral mechanisms of ribavirin include immune modulation by switching the T-cell phenotype from type 2 to type 1,⁴² anti-proliferative effect by inhibition of cellular GTP synthesis,⁴¹ and direct inhibition of virus replication.⁴³ Although monotherapy with RBV clinically showed minimal effect on the viral load and almost no effect on the viral clearance,^{44–47} combinatory use of RBV with IFN elicits strong synergistic effects against HCV *in vitro*⁴⁸ and *in vivo*.^{49,50}

Ribavirin is directly toxic to erythrocytes and is associated with hemolysis, which is usually reversible and dose-related.^{49,50} RBV is incorporated into erythrocytes where it undergoes phosphorylation to its pharmacologically active forms through adenosine kinase. The RBV-phosphate conjugates are unable to cross the erythrocyte cell membrane and are thus accumulated intracellularly and cleared slowly from red cells with a half-life of approximately 40 days.⁵¹ Inosine triphosphatase (ITP) deficiency or low activity variants, in turn, lead to an accumulation of ITP in red blood cells and may compete with RBV triphosphate, and may protect from RBV-induced hemolysis.^{52,53}

There are several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) being tested for

clinical efficacy against hepatitis C.^{21,22} Most experts believe that when new drugs are approved to treat hepatitis C they will be used in combination with PEG IFN and RBV. Moreover, recent clinical trials including NS3 protease inhibitors have shown that PEG IFN plus RBV would be necessary to achieve optimal treatment responses.^{18,19,54} Our present results may give a valuable pharmacogenetic diagnostic tool for the tailoring of RBV dosage to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV chemotherapeutics.

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Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

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Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point

than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

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2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. *J. Med. Virol.* **83:871–878, 2011.** © 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon- λ 3

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon- λ 3 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who

received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN- α 2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μ g/kg subcutaneously once a week) or PEG-IFN- α 2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μ g subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w. 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the IL28B locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann-Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P*-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years) ^a	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m ²) ^a (N = 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N = 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/μl) ^b (N = 94)	5,115 ± 1,630
Neutrophils (/μl) ^b (N = 94)	2,765 ± 1,131
Hemoglobin (g/dl) ^b (N = 95)	14.2 ± 1.3
Platelet count (×10 ³ /μl) ^b (N = 98)	187 ± 95
ALT (IU/L) ^b (N = 95)	82 ± 78
Serum HCV-RNA level (log(IU/ml)) ^{a,c}	6.2 (3.6–7.4)
Treatment duration (>16, ≤24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.

^aData are shown as median (range) values.

^bData are expressed as mean ± SD.

^cData are shown as log(IU/ml).

anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a (*P* = 0.036) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with ≥80% and <80% of RBV dose, *P* = 0.021, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR = 0.170, *P* = 0.019).

TABLE II. Response Rates to Therapy

Character	Number/total number (%)		
Overall			
RVR	68/112 (61)		
ETR	125/129 (97)		
SVR	98/129 (76)		
Genotype	2a	2b	P-value
RVR	46/67 (69)	22/45 (49)	0.036
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response. Bold indicated *P*-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≥80%	<80%	P-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	0.021

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. Bold indicated *P*-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response

rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	P-value
Genotype (2a/2b)	56/42		21/10
IL28B SNPs (rs8099917)			
TT/TG + GG	81/17	19/12	0.024
Age (years) ^a	56 (20–73)	61 (40–72)	0.002
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m ²) ^a	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/ μ l) ^b	5,318 \pm 1,617	4,489 \pm 1,540	0.032
Neutrophils (/ μ l) ^b	2,913 \pm 1,139	2,278 \pm 983	0.021
Hemoglobin (g/dl) ^b	14.2 \pm 1.4	14.1 \pm 1.1	NS
Platelet count ($\times 10^{-3}$ / μ l) ^b	193 \pm 105	171 \pm 54	NS
ALT (IU/ml) ^b	79 \pm 73	94 \pm 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml)) ^{a,c}	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≥80%/<80%)	75/23	21/10	NS
RBV adherence (≥80%/<80%)	61/37	12/19	0.024
RVR/non-RVR	57/24	11/20	0.001

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.

^aData are shown as median (range) values.

^bData are expressed as mean \pm SD.

^cData are shown as log (IU/ml).

Bold indicated *P*-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	P-value
Regression analysis			
RVR	RVR	1	0.019
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≥80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG + GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.
 Bold indicated P-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, $P = 0.002$, sustained virological response: 88% and 44% with IL28B major and hetero/minor, $P = 0.009$).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II). In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, $P = 0.044$), and no one achieved a rapid

virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, $P = 0.630$, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, $P = 0.552$).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	P-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

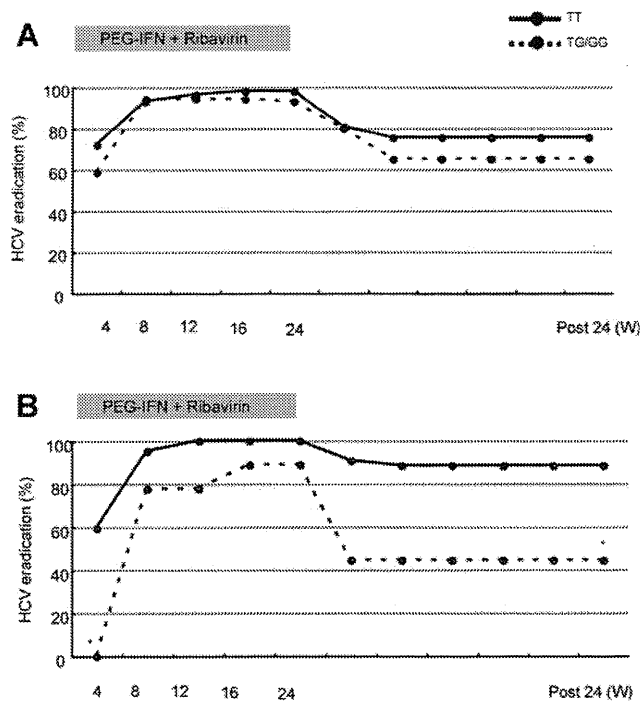


Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. *P*-values were two-tailed and those of less than 0.05 were considered to be statistically significant. **P* < 0.01.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment ($n = 2$), general fatigue ($n = 2$), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B

polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent *in vitro* study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate

analysis (Table IV). Regardless of the drug adherence, end of treatment response rates of patients infected with genotype 2 were around 94–99%, but the sustained virological response rates of the patients who received a total cumulative treatment dose of RBV of <80% was reduced significantly. As reported previously, increased RBV exposure during the treatment phase was associated with an increased likelihood of a sustained virological response [McHutchison et al., 2009] and these results confirm the importance of RBV in order to prevent relapse. Furthermore, host genetic variation leading to inosine triphosphatase (ITPA) deficiency protects against hemolytic anemia in chronic hepatitis C patients receiving RBV as revealed recently [Fellay et al., 2010]. We have reported also that the *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of RBV-induced anemia in Japanese patients and that the incidence of early dose reduction was significantly higher in patients with *ITPA*-major (CC) variant as expected and, more importantly, that a significant higher sustained virological response rate was achieved in patients with the *ITPA*-hetero/minor (CA/AA) variant with non-genotype 1 or low viral loads [Sakamoto et al., 2010].

A rapid virological response was extracted in this study as a factor associated with sustained virological response only by multivariate analysis. It has been reported recently that a rapid virological response is an important treatment predictor and that drug adherence, which is reported to affect the therapeutic efficacy in patients infected with genotype 1, had no impact on the both sustained and rapid virological responses in combination therapy for patients infected with genotype 2 [Inoue et al., 2010]. The reasons why several host factors useful for predicting the response to therapy in patients with genotype 1, such as gender, age, progression of liver fibrosis and IL28B polymorphism had no influence on the efficacy in patients with genotype 2, can be attributed to IFN-sensitive genotypes. Similarly, the other viral factors useful for predicting the response to therapy, such as viral load and amino acid substitutions in the Core and NS5A regions had no influence on treatment outcomes. In this study, patients who achieved a rapid virological response had a high sustained virological response rate, regardless of IL28B polymorphism in patients with genotype 2a but, interestingly, none of the IL28B-TG and -GG patients with genotype 2b achieved a sustained virological response (although there were nine IL28B-TG and -GG patients with genotype 2b, two could not be determined as rapid virological response because the times at which they became HCV-negative were not recorded clearly, being described as 4–8 weeks.) These results also suggest that patients with both genotype 2b and IL28B minor allele are refractory cases.

IL28B encodes a protein also known as IFN- λ 3 [O'Brien, 2009]. *IL28A* (IFN- λ 2) and *IL29* (IFN- λ 1) are found adjacent to *IL28B* on chromosome 19. These three IFN- λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al.,

2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN- λ , and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN- λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN- α induces expression of IFN- λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN- λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN- α and that the anti-HCV activity of either IFN- α or IFN- λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN- α and IFN- λ may play a key role in the suppression of HCV [O'Brien, 2009].

In conclusion, IL28B polymorphism is predictive of PEG-IFN plus RBV treatment outcomes in patients infected with genotype 2, and more remarkably with genotype 2b. These results suggest that IL-28B polymorphism affects responses to IFN-based treatment in more difficult-to-treat subpopulations of HCV patients, and that intersubgenotypic differences between genotype 2a and 2b are revealed by responses to PEG-IFN plus RBV treatment according to IL28B variants.

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Differential interferon signaling in liver lobule and portal area cells under treatment for chronic hepatitis C

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Background & Aims: The mechanisms of treatment resistance to interferon (IFN) and ribavirin (Rib) combination therapy for hepatitis C virus (HCV) infection are not known. This study aims to gain insight into these mechanisms by exploring hepatic gene expression before and during treatment.

Methods: Liver biopsy was performed in 50 patients before therapy and repeated in 30 of them 1 week after initiating combination therapy. The cells in liver lobules (CLL) and the cells in portal areas (CPA) were obtained from 12 patients using laser capture microdissection (LCM).

Results: Forty-three patients were infected with genotype 1 HCV, 20 of who were viral responders (genotype 1-Rsp) with treatment outcome of SVR or TR, while 23 were non-responders (genotype 1-nonRsp) with NR. Only seven patients were infected with genotype 2. Before treatment, the expression of *IFN and Rib-stimulated genes* (IRSGs), apoptosis-associated genes, and immune reaction gene pathways was greater in genotype 1-nonRsp than in Rsp. During treatment, IRSGs were induced in genotype 1-Rsp, but not in nonRsp. IRSG induction was irrelevant in genotype 2-Rsp and was mainly impaired in CLL but not in CPA. Pathway analysis revealed that many immune regulatory pathways were induced in CLL from genotype 1-Rsp, while growth factors related to angiogenesis and fibrogenesis were more induced in CPA from genotype 1-nonRsp.

Conclusions: Impaired IRSGs induction in CLL reduces the sensitivity to treatment for genotype 1 HCV infection. CLL and CPA in the liver might be differentially involved in treatment resistance. These findings could be useful for the improvement of therapy for HCV infection.

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Introduction

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma (HCC). Although interferon (IFN) and ribavirin (Rib) combination therapy has become a popular modality for treating patients with chronic hepatitis C (CH-C), about 50% of patients relapse, particularly those with genotype 1b and high viral load [8]. The reasons for treatment failure are poorly understood. Many studies of IFN and Rib combination therapy for CH-C suggested that patients who cleared HCV viremia early during therapy tended to show favorable outcomes. On the other hand, patients who needed a longer period to clear HCV had poorer outcomes [4,7,17], and those who showed no response (no or minimal decrease in HCV-RNA) to IFN and Rib combination therapy hardly ever achieved a sustained viral response (SVR).

To elucidate the underlying mechanism of treatment resistance, expression profiles in the liver [3,6,20] and peripheral mononuclear cells (PBMC) [10,21] during IFN treatment for CH-C patients have been examined. In chronic viral hepatitis, increased numbers of immune regulatory cells infiltrate the liver. These liver-infiltrating lymphocytes (LLs) might play important roles for virus eradication and are potentially linked to treatment outcome. Previously, we selectively isolated cells in liver lobules (CLL) and cells in the portal area (CPA) from biopsy specimens using laser capture microdissection (LCM) and analyzed their gene expression profiles [11,19]. From these profile analyses, it could be inferred that the majority of CLL were hepatocytes and the majority of CPA were lymphocytes, although other cellular components such as Kupffer cells, endothelial cells, myofibroblasts, and bile duct cells co-existed as well.

To gain further insight into the mechanisms of therapy resistance, we analyzed expression profiles in CLL and CPA in addition to whole liver tissues during IFN therapy for CH-C.

Keywords: HCV; IFN; LCM; Gene expression.

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Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; miRNA, micro RNA; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; HCC-B, hepatitis B-related hepatocellular carcinoma; HCC-C, hepatitis C-related hepatocellular carcinoma; OCT, optimum cutting temperature.



Research Article

Materials and methods

Patients

Patients with CH-C were enrolled in this study at the Graduate School of Medicine, Kanazawa University Hospital, Japan, between 2001 and 2007 (Tables 1 and 2). Prior to the study, we obtained the required approvals, namely: informed consent from all participating patients and ethics approval from the ethics committee for human genome/gene analysis research at Kanazawa University Graduate School of Medical Science. Thirty patients were administered IFN- α 2b (6 MU: every day for 2 weeks, then three times a week for 22 weeks) (Schering-Plough K.K., Tokyo, Japan) and Rib (10–13 mg/kg/day) combination therapy for 24 weeks (Table 1). Twenty patients were administered Peg-IFN- α 2b and Rib combination therapy for 48 weeks (Table 2). The final outcome of the treatment was assessed at 24 weeks after cessation of the combination therapy. In addition, 10 samples of normal liver tissues obtained during surgery for metastatic liver cancer were used as controls.

We defined treatment outcomes according to the decrease in viremia as follows: sustained viral response (SVR), clearance of HCV viremia at 24 weeks after cessation of therapy; transient response (TR), no detectable HCV viremia at 24 weeks but relapse during the follow-up period; and nonresponse (NR), HCV viremia detected at the cessation of therapy. We defined a patient who achieved SVR or TR as a viral responder (Rsp) and a patient who exhibited an NR as a non-responder (nonRsp). As patient 10 stopped treatment at 5 weeks due to an adverse side effect, we grouped this patient as Rsp based on the observed viral decline within 2 weeks (Table 1).

HCV genotype was classified by the methods described by Okamoto et al. [16]. Twenty-three patients were infected with genotype 1b and seven patients were infected with genotype 2 (2a; 6, 2b; 1) (Tables 1 and 2).

Patient serum was aliquoted and stored at -20°C until use. HCV-RNA was serially monitored by quantitative real-time detection (RTD)-PCR (COBAS[®] AmpliPrep/COBAS[®] TaqMan[®] System[®]) [9] before treatment, at 48 h, 2 weeks and 24 weeks after initiation of therapy and at 24 weeks after cessation of therapy.

The grading and staging of chronic hepatitis were histologically assessed according to the method described by Desmet et al. (Table 1) [5].

Table 1. Characteristics of study patients who received IFN and ribavirin combination therapy.

Pt.No.	Sex	Age (yr)	Genotype	ALT (IU/ml)		Liver histology			LCM	HCV-RNA (Log IU/ml)				Viral kinetics		Viral response	Outcome	
				Before therapy	During therapy	Before therapy	During therapy	Before therapy		48 h	2 wk	24 wk	1st phase decline Log/24 h	2nd phase decline Log/week				
1	M	48	1b	83	45	1	1	1	1	+	6.6	4.5	3.5	-	1.1	0.5	Rsp	SVR
2	M	32	1b	192	95	1	1	1	1	-	6.4	3.9	3.2	-	1.3	0.4	Rsp	SVR
3	F	50	1b	57	37	1	1	1	1	-	5.8	2.5	1.5	-	1.7	0.5	Rsp	TR
4	M	36	1b	119	117	1	1	1	1	+	6.1	4.4	4.2	+	0.9	0.1	nonRsp	NR
5	M	54	1b	82	69	1	1	1	1	-	6.6	5.1	3.9	+	0.8	0.6	nonRsp	NR
6	M	43	1b	143	116	1	1	1	1	-	6.3	4.4	4.1	+	1.0	0.2	nonRsp	NR
7	M	48	1b	33	30	1	1	1	1	+	1.5	0.0	0.0	-	>0.8	-	Rsp	SVR
8	M	52	1b	316	374	1	2	1	1	-	4.7	5.1	3.9	+	-0.2	0.6	nonRsp	NR
9	M	45	1b	112	39	1	0	2	0	-	6.2	5.1	5.7	+	0.6	-0.3	nonRsp	NR
10	M	48	1b	48	30	2	2	2	1	+	6.4	4.0	2.6	NA	1.2	0.8	Rsp	NA
11	M	52	1b	114	80	2	2	2	1	-	6.1	3.7	3.0	-	1.2	0.4	Rsp	TR
12	F	63	1b	38	30	2	1	2	1	-	5.2	4.2	4.5	+	0.5	-0.2	nonRsp	NR
13	M	58	1b	90	83	2	2	2	2	+	6.9	4.9	5.6	+	1.0	-0.4	nonRsp	NR
14	F	61	1b	87	43	2	1	2	1	+	6.5	3.9	3.7	+	1.3	0.1	nonRsp	NR
15	F	64	1b	133	111	2	1	3	2	-	6.0	4.4	3.6	+	0.8	0.4	nonRsp	NR
16	F	62	1b	251	159	3	2	3	2	-	4.8	2.7	1.5	-	1.1	0.6	Rsp	SVR
17	M	54	1b	211	205	3	2	3	2	+	6.7	0.0	0.0	-	>3.4	-	Rsp	SVR
18	F	68	1b	153	145	3	2	3	2	+	4.9	4.3	3.5	+	0.3	0.4	nonRsp	NR
19	F	69	1b	64	43	3	2	3	2	-	4.4	1.5	0.0	-	1.5	0.8	Rsp	SVR
20	M	49	1b	91	83	3	2	3	2	+	6.6	4.2	3.8	+	1.2	0.2	nonRsp	NR
21	M	55	1b	187	196	4	1	4	2	-	5.8	5.1	5.6	+	0.4	-0.3	nonRsp	NR
22	F	45	1b	113	75	4	2	3	3	-	5.7	4.2	2.7	-	0.8	0.8	Rsp	TR
23	M	60	1b	86	49	4	2	3	1	-	6.3	3.5	3.5	+	1.4	0.0	nonRsp	NR
24	F	51	2b	98	90	1	1	1	1	-	2.7	1.5	0.0	-	0.6	0.8	Rsp	SVR
25	M	37	2a	241	211	1	0	1	0	-	4.0	1.5	0.0	-	1.3	0.8	Rsp	SVR
26	F	45	2a	91	33	2	1	2	1	-	5.4	2.2	1.5	-	1.6	0.4	Rsp	TR
27	M	46	2a	101	45	2	1	2	1	+	3.6	0.0	0.0	-	>1.8	-	Rsp	SVR
28	M	54	2a	196	177	3	2	2	1	+	4.2	0.0	0.0	-	>2.1	-	Rsp	SVR
29	F	68	2a	234	135	3	1	3	2	+	4.6	3.1	0.0	-	0.8	1.7	Rsp	SVR
30	M	67	2a	155	163	4	2	4	2	-	3.9	1.5	0.0	-	1.2	0.8	Rsp	SVR

First phase decline was determined by subtracting HCV-RNA at 48 h from before therapy.

Second phase decline was determined by subtracting HCV-RNA at 2 wk from 48 h.

NA, not applicable; LCM, laser capture microdissection; ALT, alanine aminotransferase; SVR, sustained viral response; A, activity; NR, nonresponse; F, fibrosis; TR, transient response; Rsp, viral responder, patients with SVR or TR; nonRsp, non-viral responder; patients with NR; HCV-RNA was assayed by COBAS[®] AmpliPrep/COBAS[®] TaqMan[®] System[®] (Log IU/mL).