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

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

In the present study, although hepatic *IFNλ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of *IL28B* from that of *IL28A*, and moreover, we could not specify which cell expresses *IFNλ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFNλ* in relation to treatment response need further clarification by specifying type of IFNλ and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I*/*IPS-1* ratio was significantly higher in NVR with HCV genotype 2.[19] However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I*/*IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.[24] The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

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

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

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

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

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Genome-wide association study identified *ITPA/DDRGK1* variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C

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Hematologic abnormalities during current therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) for chronic hepatitis C (CHC) often necessitate dose reduction and premature withdrawal from therapy. The aim of this study was to identify host factors associated with IFN-induced thrombocytopenia by genome-wide association study (GWAS). In the GWAS stage using 900K single-nucleotide polymorphism (SNP) microarrays, 303 Japanese CHC patients treated with PEG-IFN/RBV therapy were genotyped. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the minor-allele-dominant model with the decrease of platelet counts in response to PEG-IFN/RBV therapy [$P = 8.17 \times 10^{-9}$; odds ratio (OR) = 4.6]. These associations were replicated in another sample set (n = 391) and the combined P-values reached 5.29×10^{-17} (OR = 4.5). Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that rs11697186 at the GWAS stage had a strong linkage disequilibrium with rs1127354, known as a functional variant in the *ITPA* gene. The

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***ITPA*-AA/CA genotype was independently associated with a higher degree of reduction in platelet counts at week 4 (*P* < 0.0001), as well as protection against the reduction in hemoglobin, whereas the CC genotype had significantly less reduction in the mean platelet counts compared with the AA/CA genotype (*P* < 0.0001 for weeks 2, 4, 8, 12), due to a reactive increase of the platelet count through weeks 1–4. Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN/RBV dosing to minimize drug-induced adverse events.**

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide, with ~2.3% of the world population, i.e. more than 120–130 million people, being infected (1). Only 20–30% of HCV-infected individuals recover spontaneously. The remaining 70–80% go on to develop chronic infection, being at significant risk for progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC). Successful treatment of chronic hepatitis C (CHC) leads to a reduction of liver fibrosis stage of patients, and also prevents HCC development (2).

Antiviral treatment has been shown to improve liver histology and decrease incidence of hepatocellular carcinoma in CHC (3,4). Current therapy for CHC consists of treatment with pegylated interferon (IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral pro-drug that interferes with RNA metabolism (5,6). However, <50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or cure of the infection (5,7). Older patients with liver fibrosis showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities (8–10). In particular, hematologic abnormalities often necessitate dose reduction, and premature withdrawal from therapy in 10–14% of patients (5,11–14). New drugs and therapeutic approaches for CHC are actively developed and several candidates are in early trial phase (15,16). Given this background, effective pre-treatment screening for predictive biomarkers with the aim of evaluating possible risks over benefits of currently available treatment will avoid these side effects in patients who will not be helped by treatment, as well as reduce the substantial cost of 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, including our study, have demonstrated marked associations between single-nucleotide polymorphisms (SNPs) within and around *IL28B* gene, which codes for IFN-λ3 (16–21). Another recent study indicated that genetic variants of *ITPA* gene leading to inosine triphosphatase (ITPA) deficiency could protect against hemolytic anemia (HA) in CHC patients receiving RBV (22).

In Japan, HCV-infected patients are relatively old and some of them have had severe fibrosis (9). Thrombocytopenia is one of the critical adverse events by IFN-based therapy among liver cirrhotic patients (23), because low platelet count (PLT), i.e. <30.0 (10^9/l), would be a risk factor for any bleeding, as well as it would lead to poor treatment efficiency due to the initial or early dose reduction of PEG-IFN. Based on its pathogenesis, drug-induced thrombocytopenia is usually due to bone marrow suppression, immune-mediated destruction and platelet aggregation (24). In this study, we firstly found that genetic variants in the *ITPA/DDRGK1* genes were associated with IFN-induced thrombocytopenia, and then examined the correlation between IFN-induced thrombocytopenia and RBV-induced HA in Japanese CHC patients under PEG-IFN/RBV treatment.

RESULTS

Genetic variants associated with IFN-induced thrombocytopenia

In this study, we conducted a GWAS to identify host genes associated with the decrease of platelets in response to PEG-IFN/RBV treatment in 303 Japanese HCV patients (107 patients with the decrease of PLT versus 196 patients without the decrease of PLT based on the criteria described in Materials and Methods), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1. Figure 1 shows a genome-wide view of the single-point association data based on allele frequencies. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 8.17 \times 10^{-9}$) with the decrease of PLT in response to PEG-IFN plus RBV treatment. The association reached genome-wide level of significance [Bonferroni criterion $P < 8.40 \times 10^{-8}$ (0.05/595052)], and another SNP (rs6139030) near *ITPA* gene had a marginal significance ($P = 4.30 \times 10^{-7}$, in Table 2).

To validate the results of the GWAS stage, 22 SNPs were selected for the replication in a set of 391 Japanese HCV patients with and without platelet reduction (Supplementary Material, Table S1). The associations of the original significant SNP (rs11697186) and the marginal SNP (rs6139030) at the GWAS stage were replicated in the second set of 391 patients in the minor-allele-dominant model [$P = 5.88 \times 10^{-10}$, odds ratio (OR) = 4.6 for rs11697186; $P = 3.83 \times 10^{-10}$, OR = 4.3 for rs6139030, Table 2]. The combined *P*-values for both stages reached 5.29×10^{-17} (OR = 4.5; 95% CI = 3.1–6.5) and 1.33×10^{-15} (OR = 3.9; 95% CI = 2.8–5.5), respectively (Table 2).

Genetic variants associated with RBV-induced anemia

We also conducted a GWAS to identify host genes associated with a quantitative change in hemoglobin (Hb) levels from baseline to week 4 of PEG-IFN/RBV treatment in the above 303 Japanese HCV patients (94 patients with an Hb reduction of ≥3 g/dl at week 4 and 209 patients without Hb reduction), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). Two SNPs (rs11697186 and rs6139030)

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Table 1. Clinical characteristics of patients in this study

	GWAS ($n = 303$)	Replication ($n = 391$)
Age	57.4 (9.7)	56.8 (9.9)
Sex (M/F)	151/152	209/182
Weight (kg)	60.6 (10.4)	61.3 (10.7)
Body mass index	23.5 (3.1)	23.7 (4.1)
Baseline Hb (g/dl)	14.1 (1.4)	14.1 (1.4)
Baseline platelet count (10^9/l)	151.3 (54.3)	159.7 (55.0)
Baseline ALT (IU/l)	83.5 (79.4)	86.8 (71.9)
Baseline creatinine (mg/dl)	0.70 (0.15)	0.72 (0.16)
Baseline liver fibrosis (F0–2/F3–4/ND)	153/77/73	175/59/43
rs8099917: TT/non-TT	165/138	296/95
rs1127354: AA/CA/CC	4/79/220	6/101/284
Week 4 Hb (g/dl)	11.8 (1.7)	11.9 (1.5)
Week 4 platelet count (10^9/l)	127.6 (48.2)	132.4 (51.0)
Hb reduction at week 4	−2.3 (1.4)	−2.2 (1.4)
Platelet reduction at week 4	−22.2 (38.4)	−24.7 (30.4)

located on *DDRGK1* gene and *ITPA* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 3.29 \times 10^{-10}$ and $P = 2.56 \times 10^{-9}$) with Hb reduction in response to PEG-IFN plus RBV treatment (Table 3).

The above 22 SNPs were selected for the replication study and fine mapping, including rs1127354, which was reported by the US group (22) to be strongly associated with Hb reduction (Supplementary Material, Table S2). All SNPs were genotyped using the DigiTag2 assay in an independent set of 391 Japanese HCV patients with quantitative change in Hb in response to PEG-IFN/RBV treatment [137 patients with Hb reduction versus 254 patients without Hb reduction (Table 3)]. The associations of the original SNPs were replicated in the second set of 391 patients in the minor-allele-dominant model ($P = 3.86 \times 10^{-16}$, OR = 0.02 for rs11697186; $P = 6.90 \times 10^{-18}$, OR = 0.03 for rs6139030, Table 3). The combined *P*-values for both stages reached 9.43×10^{-25} (OR = 0.03; 95% CI = 0.01–0.08) and 2.12×10^{-25} (OR = 0.04; 95% CI = 0.02–0.09), respectively (Table 3). The rs1127354 was also strongly associated with a quantitative change in Hb in response to PEG-IFN/RBV treatment in a set of 694 Japanese HCV patients (303 patients from the GWAS stage plus the second set of 391 patients) with and without Hb reduction ($P = 4.58 \times 10^{-26}$, OR = 0.03; 95% CI = 0.01–0.08).

Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that four significant SNPs (rs11697186, rs6139030, rs1127354 and rs13830) at the GWAS stage had a strong linkage disequilibrium (LD) ($r^2 > 0.86$) within the 22.7 kb region (Fig. 2). As the rs1127354 is known as a functional variant in the *ITPA* gene that caused ITPase deficiency and protected against RBV-induced HA (22,25), the representative SNP was applied for the following detailed studies.

ITPA/DDRGK1 variants reflect anemia and reactive increase of the platelet count

The mean quantitative reduction of blood cells from the baseline according to the *ITPA* rs1127354 genotypes is shown in Figure 3. Patients with the rs1127354 genotypes AA and CA showed lower degree of Hb reduction at weeks 2, 4, 8 and 12 during therapy compared with those with the CC genotype ($P < 0.0001$ for weeks 2, 4, 8 and 12 in Fig. 3A). The most difference of mean Hb reduction was found at week 4 (AA/CA −1.14 versus CC −2.72). These results show that the AA and CA genotypes are significantly associated with less absolute reduction in Hb levels, especially during the early weeks of therapy, and protect against the development of severe anemia. Interestingly, the CC genotype had significantly less reduction in the mean platelet count compared with the AA/CA genotype ($P < 0.0001$ for weeks 2, 4, 8; $P = 0.019$ for week 12 in Fig. 3B), due to a reactive increase of platelet count through weeks 1–4. The most difference of mean platelet reduction was found at week 4 [AA/CA −41.2 versus CC −18.0 (10^9/l)]. There was no difference in the neutrophil leukocyte count between genotypes (Fig. 3C). We then compared the percentage of patients with platelet count reduction in the *ITPA* rs1127354 genotypes at week 4 of PEG-IFN/RBV therapy (Fig. 4). The percentage of patients with a platelet count reduction of <30 (10^9/l) at week 4 was significantly higher in the rs1127354 genotypes CC ($P < 0.0001$), indicating that the degree of platelet count reduction was less in patients with the rs1127354 genotype CC. A multivariate analysis for factors associated with a platelet reduction >30 (10^9/l) at week 4 showed that lower platelet count at the baseline and the rs1127354 genotypes AA/CA were independently associated with platelet reduction (OR = 1.15; 95% CI = 1.11–1.20; $P < 0.0001$, OR = 5.92; 95% CI = 3.82–9.17; $P < 0.0001$, respectively).

Figure 5 showed reactive increase of the platelet count through weeks 1–4 of PEG-IFN/RBV therapy. Patients with anemia (Hb reduction ≥3.0 g/dl) at week 4 had a significantly higher degree of the reactive increase of the platelet count than those without anemia ($P < 0.0001$ in Fig. 5A). Within a subgroup of patients with the rs1127354 genotypes CC, patients with anemia still had a significantly higher degree of reactive increase of the platelet count than those without anemia ($P = 0.004$ in Fig. 5B). On the other hand, patients with the rs1127354 genotypes CC had a significantly higher degree of the reactive increase of the platelet count than those with genotypes AA/CA ($P < 0.0001$ in Fig. 5C), and a similar result was obtained in a subgroup of patients without anemia (Fig. 5D). To elucidate the significant factors associated with the rs1127354 genotypes by multivariate analysis, the rs1127354 genotypes AA/CA were independently associated with protection against the reduction in Hb and more reduction in platelet counts at week 4 due to a lower degree of the reactive increase of the platelet count (OR = 0.029; 95% CI = 0.009–0.092; $P < 0.0001$, OR = 4.73; 95% CI = 3.04–7.37; $P < 0.0001$, respectively). Indeed, the reactive increase of the platelet count through weeks 1–4 was positively correlated with a high platelet count at the baseline and anemia (Hb reduction ≥3.0 g/dl) at week 4, but was negatively correlated with rs1127354 genotypes AA/CA and a platelet count reduction of ≥30 (10^9/l) at week 4 (Table 4).

Relationship between *ITPA rs1127354* genotypes and treatment outcome due to dose reduction of PEG-IFN or RBV

In this population, a multivariate analysis showed that SVR was significantly associated with *IL28B* TT-genotype [OR

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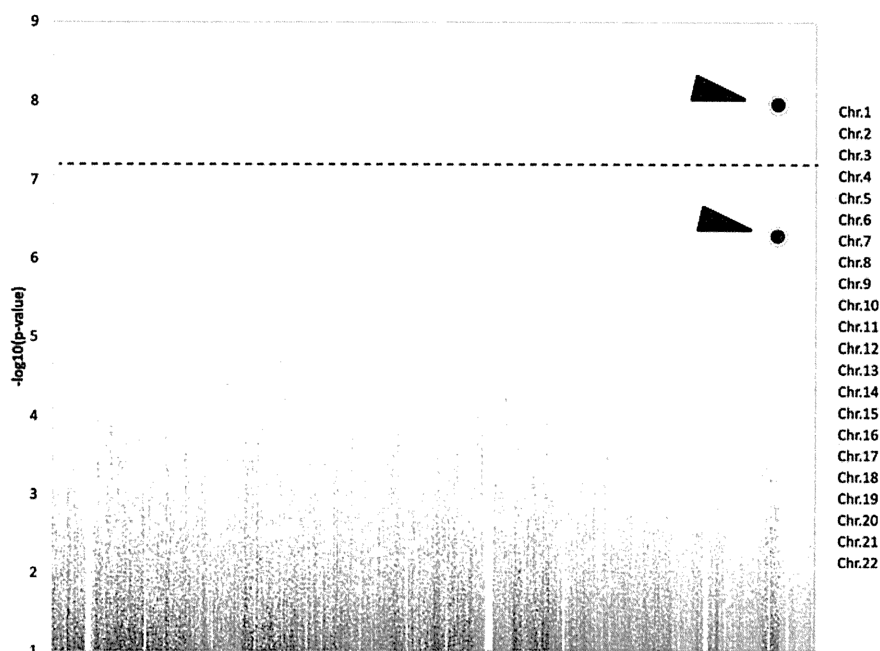

Figure 1. Genome-wide association results in 303 Japanese HCV patients with the decrease of platelets in response to PEG-IFN plus RBV treatment (107 patients with the decrease of PLT and 196 patients without the decrease of PLT). *P*-values were calculated using the χ^2 test for allele frequencies. Dots with arrow on chromosome 20 showed a significant SNP ($P = 8.17 \times 10^{-9}$ for rs11697186) and a candidate SNP with a marginal significance ($P = 4.30 \times 10^{-7}$ for rs6139030) associated with the decrease of PLT with response to PEG-IFN/RBV treatment. The dotted line indicates a genome-wide significance ($P < 8.40 \times 10^{-8}$).

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Table 2. Two SNPs (rs11697186 and rs6139030) significantly associated with the decrease of PLT in response to PEG-IFN/RBV treatment

dbSNP rsID	Nearest gene	MAF[a] (allele)	Allele (1/2)	Stage	Patients with the decrease of PLT			Patients without the decrease of PLT			OR (95% CI)[b]	*P*-value[c]
					11	12	22	11	12	22		
rs11697186	*DDRGK1*	0.15 (T)	T/A	GWAS	3 (2.8)	48 (44.9)	56 (52.3)	0 (0.0)	32 (16.6)	161 (83.4)	4.6 (2.7–7.8)	8.17×10^{-9}
				Replication	3 (1.8)	65 (39.9)	95 (58.3)	3 (1.4)	25 (12.0)	181 (86.6)	4.6 (2.8–7.7)	5.88×10^{-10}
				Combined	6 (2.2)	113 (41.9)	151 (55.9)	3 (0.7)	57 (14.2)	342 (85.1)	4.5 (3.1–6.5)	5.29×10^{-17}
rs6139030	*ITPA*	0.17 (C)	T/C	GWAS	56 (52.3)	48 (44.9)	3 (2.8)	157 (80.1)	38 (19.4)	1 (0.5)	3.7 (2.2–6.1)	4.30×10^{-7}
				Replication	96 (54.9)	74 (42.3)	5 (2.9)	181 (83.8)	32 (14.8)	3 (1.4)	4.3 (2.7–6.8)	3.83×10^{-10}
				Combined	152 (53.9)	122 (43.3)	8 (2.8)	338 (82.0)	70 (17.0)	4 (1.0)	3.9 (2.8–5.5)	1.33×10^{-15}

[a]Minor allele frequency and minor allele in 184 healthy Japanese individuals.
[b]OR for the minor allele in a dominant model.
[c]*P*-value by χ^2 test for the minor allele dominant model.

6.12 (2.78–13.46), $P < 0.0001$] as well as platelet counts [OR 1.18 (1.11–1.26), $P < 0.00001$]. We analyzed whether the rs1127354 genotype could influence the treatment outcome by PEG-IFN/RBV therapy. When analyzed in the patients available for treatment outcome (172 with *ITPA*-AA/CA and 450 with *ITPA*-CC), the percentage of patients receiving >80% of the expected PEG-IFN and RBV dose at baseline and week 4 was not significantly different among the rs1127354 genotypes. However, the rate of SVR tended to be higher in patients with *ITPA*-AA/CA genotype than those with *ITPA*-CC (48.8 versus 37.3%), because the relapse rate was lower in patients with *ITPA*-AA/CA. To investigate the influence on treatment outcome by dose reduction of PEG-IFN, in a subgroup of patients with low platelet counts (<10) at baseline (19 with *ITPA*-AA/CA and 53 with *ITPA*-CC) we analyzed the treatment outcome according to rs1127354 genotypes. The SVR rate was very low in each group (21.1% in *ITPA*-AA/CA and 17.0% in *ITPA*-CC), because many patients had the initial dose reduction of PEG-IFN (<80% of standard dose)—36.8% of patients with *ITPA*-AA/CA and 44.6% of patients with *ITPA*-CC genotype. Further prospective studies are required among the pre-cirrhotic or cirrhotic patients with low platelet counts.

DISCUSSION

Recent genome-wide association studies, including our study on HCV infection, have identified two important host genetic variants: the SNP in *IL28B* gene, which is strongly associated with response to therapy for chronic genotype 1 HCV infection (16–21), and the SNP in *ITPA* gene, which precisely predicts RBV-induced anemia in

Table 3. Two SNPs (rs11697186 and rs6139030) significantly associated with quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment

dbSNP rsID	Nearest gene	MAF[a] (allele)	Allele (1/2)	Stage	Patients with quantitative change in Hb			Patients without quantitative change in Hb			OR (95% CI)[b]	P-value[c]
					11	12	22	11	12	22		
rs11697186	*DDRGK1*	0.15 (T)	T/A	GWAS	0 (0.0)	3 (3.3)	89 (96.7)	3 (1.5)	77 (37.0)	128 (61.5)	0.06 (0.02–0.16)	3.29×10^{-10}
				Replication	0 (0.0)	2 (1.5)	134 (98.5)	6 (2.5)	88 (37.3)	142 (60.2)	0.02 (0.01–0.09)	3.86×10^{-16}
				Combined	0 (0.0)	5 (2.2)	223 (97.8)	9 (2.0)	165 (37.2)	270 (60.8)	0.03 (0.01–0.08)	9.43×10^{-25}
rs6139030	ITPA	0.17 (C)	T/C	GWAS	88 (93.6)	6 (6.4)	0 (0.0)	125 (59.8)	80 (38.3)	4 (1.9)	0.08 (0.03–0.22)	2.56×10^{-9}
				Replication	134 (97.8)	3 (2.2)	0 (0.0)	143 (56.3)	103 (40.6)	8 (3.1)	0.03 (0.01–0.08)	6.90×10^{-18}
				Combined	222 (96.1)	9 (3.9)	0 (0.0)	268 (57.9)	183 (39.5)	12 (2.6)	0.04 (0.02–0.09)	2.12×10^{-25}

[a]Minor allele frequency and minor allele in 184 healthy Japanese individuals.
[b]OR for the minor allele in a dominant model.
[c]P-value by χ^2 square test for the minor allele dominant model.

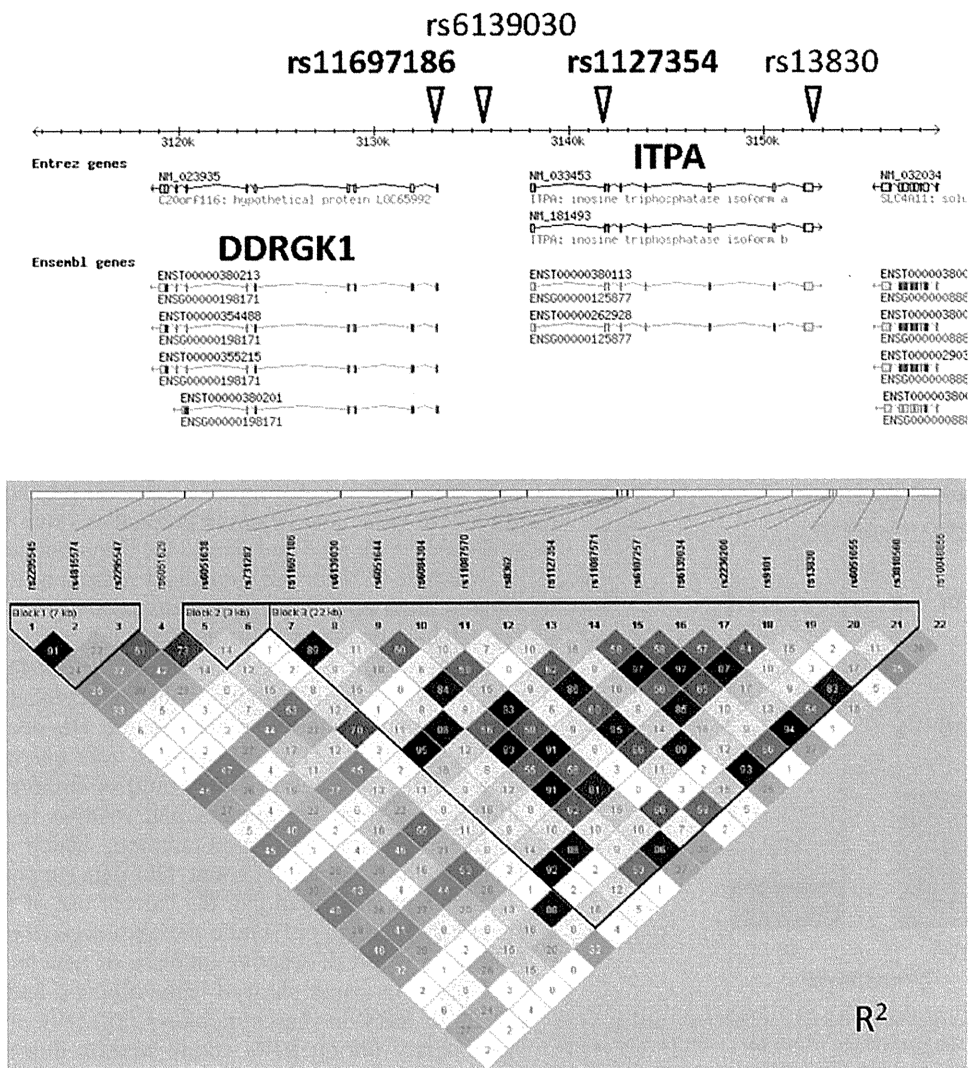

Figure 2. Pairwise LD (r^2) diagrams for *DDRGK1* and *ITPA*. Lower panel shows estimates of pairwise r^2 for 22 SNPs selected in the replication study using the second set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment.

European-American population (22) and Japanese population (26). The genetic variation of *ITPA* causing an accumulation of inosine triphosphate (ITP) has been shown to protect patients against RBV-induced anemia during treatment for CHC infection. A recent report showed the biologic mechanism that ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by RBV, in the biosynthesis of ATP (25).

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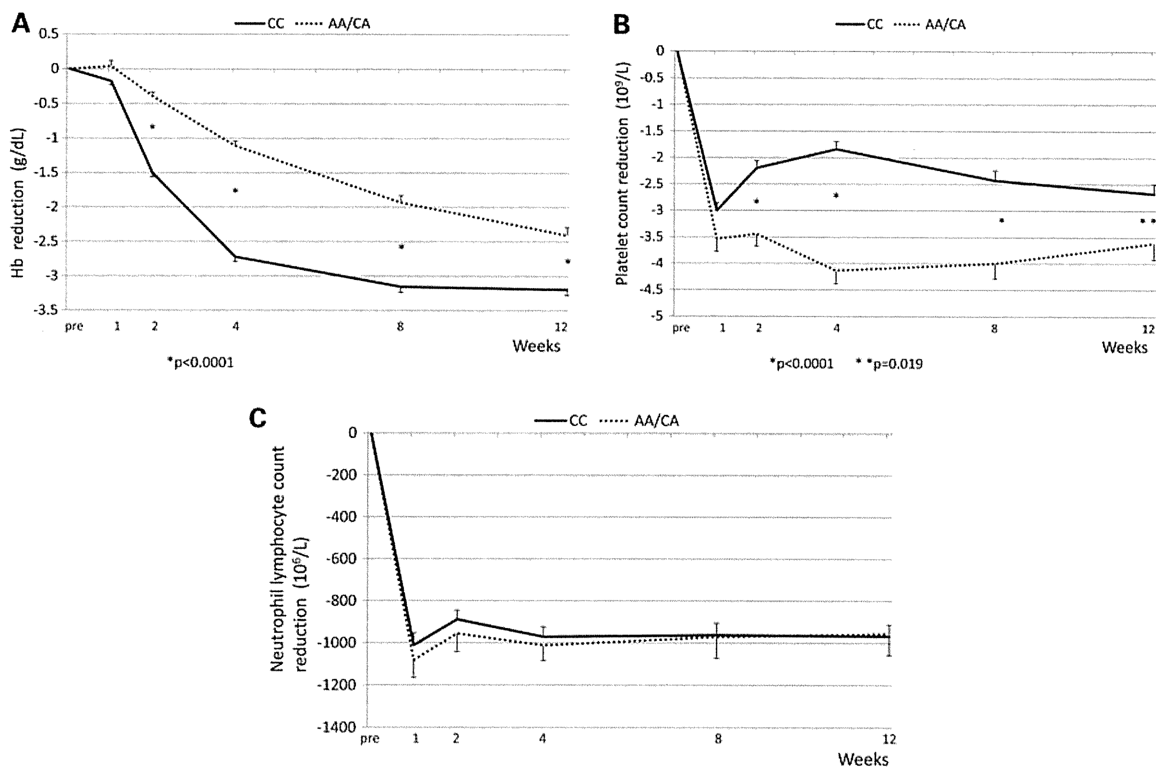

Figure 3. *ITPA* rs1127354 genotypes and the quantitative reduction of blood cells from baseline. Mean reduction of (**A**) Hb levels, (**B**) platelet counts and (**C**) neutrophil leukocyte counts during treatment according to rs1127354 genotype is shown. Solid and dotted lines indicate patients with CC and AA/CA genotypes, respectively. Error bars indicate standard error. CC genotype had more reduction in mean Hb levels during therapy compared with the AA/CA genotype (*$P < 0.0001$ for weeks 2, 4, 8, 12). CC genotype had less of a reduction in mean platelet counts (*$P < 0.0001$ for weeks 2, 4, 8, and **$P = 0.019$ for week 12), and showed a reactive increase of platelet counts through weeks 1–4.

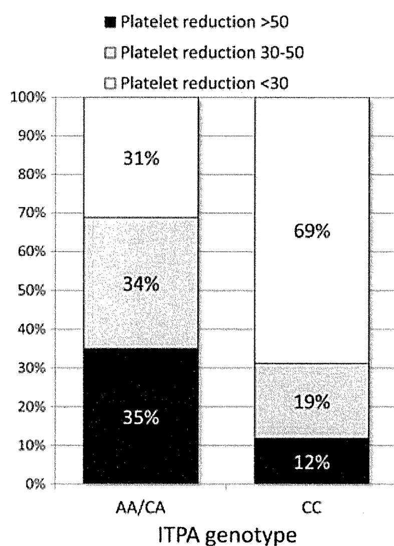

Figure 4. *ITPA* rs1127354 genotypes and reduction of platelet counts at week 4 of PEG-IFN/RBV therapy. The percentage of patients with platelet count reduction of >50 (10^9/l) (black bar), 30–50 (10^9/l) (gray bar) and <30 (10^9/l) (white bar) at week 4 is shown for rs1127354 genotypes. The incidence of platelet count reduction of >50 and <30 was significantly lower in patients with the rs1127354 genotypes CC compared with AA/CA genotypes: 12 versus 35%, $P < 0.0001$, and 69 versus 31%, $P < 0.0001$, respectively.

In this study, two SNPs, rs11697186 and rs6139030, which were within and around *DDRGK1* gene on chromosome 20, were strongly associated with thrombocytopenia as well as with Hb reduction at week 4. In clinical practice, the positive predictive value and negative predictive value by rs11697186 genotypes were 66.5 and 69.4% for thrombocytopenia, as well as 97.2 and 45% for RBV-induced anemia at week 4. As previously reported (22,26), a functional SNP (rs1127354) in the *ITPA* locus, which is in strong LD with rs11697186, was the most significant SNP associated with RBV-induced anemia and, in this study, IFN-induced thrombocytopenia in Japanese genetic populations. Note that severe Hb decline, which is mainly found in ITPA-CC patients, was inversely correlated with platelet reduction. This would contribute to an association between severe anemia and relative reactive increase of platelet count in this population, which attenuated the IFN effect on the platelet count. Our data supported a previous report which described that the current use of RBV, inducing severe anemia, might blunt the thrombocytopenic effect of IFNs as a result of reactive increase of platelet counts (27).

A previous paper showed hematological and bone marrow effects of RBV in rhesus monkeys (28). Hb values decreased significantly during RBV administration due to dose-related erythroid hypoplasia in bone marrow and returned to normal following withdrawal. On the other hand, increase of the platelet count occurred in both low- and high-dose treatment groups during RBV administration, with a fall of the platelet count to normal after drug withdrawal. The effect on platelet count was clearly dose related, with maximum counts rising to twice and three times above baseline levels in the low- and high-dose groups, respectively. This caused a significant increase of

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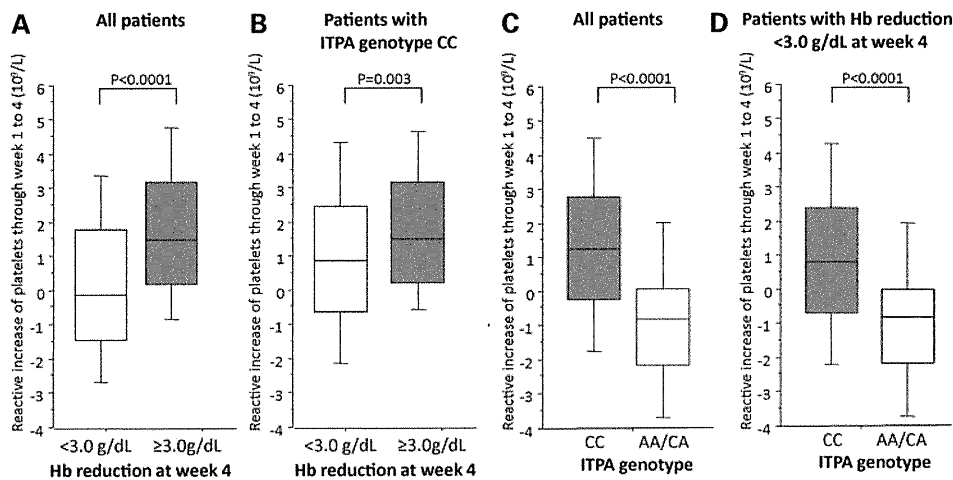

Figure 5. Reactive increase of platelet counts through weeks 1–4. Box plots of reactive increase of platelet count through weeks 1–4 according to the degree of anemia at week 4 are shown for all patients (**A**) and a subgroup of patients with the rs1127354 genotypes CC (**B**). Patients with anemia (Hb reduction ≥3.0 g/dl) at week 4 had a significantly higher degree of reactive increase of platelet count than those without anemia ($P < 0.0001$). Box plots of reactive increase of platelet counts according to the rs1127354 genotype CC are shown for all patients (**C**) and a subgroup of patients without anemia (**D**) (Hb reduction <3.0 g/dl) at week 4. Patients with the rs1127354 genotypes CC had a significantly high degree of reactive increase of platelet counts compared with those with genotypes AA/CA ($P < 0.0001$).

Table 4. Multivariate analysis of factors associated with reactive increase of platelets ≥20 (10^9/l) through weeks 1–4

	OR	95% CI	*P*-value
Baseline platelet counts	1.168	1.101–1.239	<0.0001
ITPA AA/CA	0.379	0.168–0.856	0.0196
Platelet reduction ≥30 (10^9/l) at week 4	0.051	0.021–0.120	<0.0001
Hb reduction ≥3.0 g/dl at week 4	1.602	0.914–2.809	0.0996

the platelet count associated with increased numbers of megakaryocytes. Additionally, the sequence homology of thrombopoietin (TPO) and erythropoietin (EPO) may explain the synergy of the physiologic role of TPO and EPO in platelet production. When EPO is elevated, as in iron deficiency anemia, an amino acid sequence similar to TPO may increase the platelet count (29).

Another possibility is a direct association between *ITPA* SNPs or the related SNPs with a strong LD and IFN-induced thrombocytopenia. *DDRGK1* (DDRGK domain-containing protein 1) is a novel C53/LZAP-interacting protein. C53/LZAP (also named as Cdk5rap3) is a putative tumor suppressor that plays important roles in multiple cell signaling pathways, including DNA damage response and NF-kappaB signaling (30); however, it remains largely unknown how the function of *DDRGK1* variants is regulated. Further studies are required to elucidate the possible association between *DDRGK1* variants and thrombocytopenia.

Multivariate analysis demonstrated that rs1127354 in the *ITPA* gene was independently associated with RBV-induced severe anemia and IFN-induced thrombocytopenia. This finding suggests that rs1127354 would be a useful marker to predict these hematological side effects by PEG-IFN/RBV therapy, indicating that genetic testing of *ITPA* variant might be applied to establish personalized dosages of PEG-IFN/RBV therapy. The rate of SVR tended to be higher in patients with *ITPA*-AA/CA genotype than those with *ITPA*-CC in this population. This might reflect decreased treatment efficacy (higher relapse rate) due to dose reduction of RBV in patients with *ITPA*-CC genotype. Our recent paper also demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected 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 (31) and in a subset of Japanese patients with the favorable TT genotype at rs8099917 of *IL28B* (32). Taken together, our results indicate that the *ITPA* minor variant A is not only a protective allele against PEG-IFN and RBV treatment-associated anemia in Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN. The possible mechanism of protection against RBV-induced hemolysis is that ITP deficiency or low-activity variants (*ITPA* minor variant A) in turn lead to the accumulation of ITP in red blood cells (33,34), and the ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP (25). On the other hand, half of the *ITPA*-major (CC) patients did not develop a significant Hb decline. This finding suggests other low-frequency *ITPA* variants or SNPs in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

In Japan, the older HCV-infected patients developing liver fibrosis have been prevalent (mean age 62 years) (9). Thrombocytopenia by PEG-IFN/RBV therapy could lead to poor treatment efficiency among such Japanese patients with LC due to the initial or early dose reduction of PEG-IFN. In fact, ~40% of such population in this study had the initial dose reduction of PEG-IFN, resulting in a low SVR rate. Splenectomy or embolization of the splenic artery might be one of the options to increase the SVR rate, but a sufficient treatment outcome had not been obtained at present (35). Based on the recently accumulated SNP data, if patients had favorable *IL28B* genotype and *ITPA*-CC (lower reduction of platelet counts), a standard dose of PEG-IFN might be available for

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the patients with lower platelet counts and the SVR rate might be increased due to sufficient dose of PEG-IFN.

Several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) are being tested for clinical efficacy against hepatitis C (12,13,15,16). 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 (12,13). Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN and RBV dosing to minimize drug-induced adverse events and for further optimization of clinical anti-HCV chemotherapeutics.

MATERIALS AND METHODS

Patients

From April 2007 to April 2010, samples were obtained from 303 patients with chronic HCV (genotype 1) infection who were treated at 14 multi-center hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN-α2b (1.5 μg/kg body weight, subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1000 mg daily according to body weight) for 48 weeks. Treatment duration was extended in some patients up to 72 weeks, according to the physicians' preferences. The dose of PEG-IFN or RBV was reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. EPO or other growth factors were not given. Written informed consent was obtained from each patient and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees. HBsAg-positive and/or anti-HIV-positive patients were excluded from this study.

In the following stage of replication study, SNP genotyping in an independent set of 391 Japanese HCV patients treated with PEG-IFN plus RBV treatment was completed using the DigiTag2 or TaqMan assay (ABI) following the manufacturer's protocol. The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1.

SNP genotyping and data cleaning

In the GWAS stage, we genotyped 303 Japanese HCV patients with and without the decrease of platelet counts from baseline to week 4 of PEG-IFN/RBV treatment [107 patients with a decrease of >30 (10^9/l) in platelet counts and 196 patients without a decrease of >30 (10^9/l) in platelet counts], using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to the median change. The average overall call rate of patients with and without the decrease of PLT reached 98.69 and 98.72%, respectively. We then applied the following thresholds for SNP QC in data cleaning: SNP call rate ≥95% for all samples, MAF ≥1% for all samples. A total of 595 052 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups with and without the decrease of PLT were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded.

In the following stage of the replication study and high-density association mapping, we selected 23 tag SNPs from the 44.7 kb region, including *DDRGK1* gene and *ITPA* gene by analyzing LD and haplotype structure based on the HapMap data of Japanese, using the Haploview software. Of these tag SNPs, rs1127354 within the *ITPA* gene, which was associated with RBV-induced anemia (22), was included; however, rs7270101 was excluded because recent papers studying Japanese patients showed no variants in rs7270101 (26,31,32). The SNP genotyping in an independent set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment (175 patients with quantitative change in PLT and 216 patients without quantitative change in PLT) was completed using the DigiTag2 assay (36). Twenty-two of the 23 SNPs were successfully analyzed and were used for SNP genotyping and data cleaning. All 22 SNPs in the replication study cleared HWE *P*-value >0.001.

Based on the above SNPs data obtained from 303 Japanese HCV patients, using the Affymetrix Genome-Wide Human SNP Array 6.0, we also performed GWAS between 94 patients with a quantitative change of >3 g of reduction in Hb and 209 patients without quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment. SNP genotyping in an independent set of 391 Japanese HCV patients with and without quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment (137 patients with quantitative change in Hb and 254 patients without quantitative change in Hb) was also completed using the DigiTag2 assay (36). Twenty-two of the 23 SNPs were successfully analyzed and were used for SNP genotyping and data cleaning.

An application of the Cochrane–Armitage test on all the SNPs showed the genetic inflation factor $\lambda = 1.000$ for thrombocytopenia and $\lambda = 1.006$ for anemia in the GWAS stage (Supplementary Material, Figs S1 and S2). In addition, principal component analysis was performed in 303 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (Supplementary Material, Fig. S3). These results implied that the effect of population stratification was negligible, except one sample, which was excluded from further analysis.

Laboratory and histological tests

Blood samples were obtained at baseline, 1, 2, 4, 8 and 12 weeks after the start of therapy and for hematologic tests after the start of therapy and for hematologic tests, blood chemistry and HCV-RNA. Genetic polymorphism in the *IL28B* gene (rs8099917) was determined using the ABI TaqMan assay (Applied Biosystems, Carlsbad, CA, USA). Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV-RNA level by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, CA, USA) or by Cobas Ampliprep/Cobas TaqMan assay (CAP/CTM) with a lower detection limit of

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15 IU/ml (Roche Diagnostic Systems) 24 weeks after the completion of therapy.

Statistical analysis

The observed association between an SNP and the decrease of platelets/quantitative change in Hb levels with response to PEG-IFN plus RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on chromosome X were removed because gender was not matched between groups with and without the decrease of PLT and quantitative change in Hb levels. A total of 595 052 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P = 8.40 \times 10^{-8}$ (0.05/595052) in the GWAS stage and $P = 2.27 \times 10^{-3}$ (0.05/22) in the replication stage.

The association between an SNP of the *ITPA* gene (rs1127354) and the incidence of platelet reduction at week 4 was analyzed by Fisher's exact test. The association between *ITPA* polymorphisms and the degree of reduction in platelet counts and Hb levels at each time point during therapy were analyzed by Mann–Whitney *U* test. Multivariable regression analysis was used to analyze the factors associated with *ITPA*, the rs1127354 genotype, factors associated with platelet count reductions and factors associated with the reactive increase in platelet counts. IBM-SPSS software v.15.0 (SPSS, Inc., Chicago, IL, USA) was used for these analyses.

Possible heterogeneity in allele frequencies at rs1127354 was assessed by Tarone's test. The association between the SNP and thrombocytopenia/anemia were analyzed by the Cochran–Mantel–Haenszel test. Both analyses were performed using the R (version 2.9.0) software (Supplementary Material, Table S3).

AUTHORS' CONTRIBUTIONS

Drafting of the paper, statistical analysis and approval of the final draft submitted: M.M.; drafting of the paper, statistical analysis, collecting samples and clinical data and approval of the final draft submitted: Y.T. and M.K.; statistical analysis and approval of the final draft submitted: N.N., M.S. and K.T.; collecting samples and clinical data and approval of the final draft submitted: K.M., N.S., N.E., H.Y., S.N., K.H., S.H., Y.I., E.T., S.M., M.H., Y.H., F.S., S.K. and N.I.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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

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Abstract

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

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

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

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

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Introduction

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

In genotype 2b HCV infection, 80% of patients with high viral titers can achieve a sustained virological response (SVR) to the regimen of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks [5,6]. This response is high considering that much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1 [1]. However, in other words, 20% of patients infected with genotype 2b HCV still cannot clear the virus and remain at risk of developing HCC. On the other hand, although various studies have been undertaken to clarify the factors contributing to the response to IFN-based therapy in genotype 1 infection, it remains poorly understood which patients with genotype 2b HCV infection will show unfavorable responses. Recently, the significance of IL28B single nucleotide polymorphisms (SNPs) in determining the response to PEG-IFN/RBV therapy was demonstrated in genotype 1 HCV infection [7,8]. However, the significance of IL28B SNPs was rather weak in genotype 2 HCV infection [9].

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

Table 1. Baseline Characteristics of Studied Patients.

Characteristic	SVR (n = 44)	non-SVR (n = 16)	*P* value
Gender (Male/Female)	26/18	9/7	NS†
Age (yrs)	56 (22–72)*	59 (30–80)	0.04‡
BMI	23.5 (16.6–30.3)	24.7 (18.5–31.7)	NS‡
ALT (IU/l)	51 (19–380)	41 (17–390)	NS‡
GGTP (IU/l)	36 (11–133)	40 (17–292)	NS‡
T.Chol (mg/dl)	169 (119–225)	178 (145–217)	NS‡
WBC (/μl)	4600 (2620–7200)	5080 (3270–8600)	NS‡
Hb (g/dl)	14.2 (11.5–17.3)	14.6 (11.8–16.4)	NS‡
Platelet ($\times 10^4$/mm^3)	19 (7.1–31.8)	17.8 (8–36.7)	NS‡
Fibrosis score (0–2/≥3)§	38/5	7/3	NS†
HCV RNA (KIU/ml)	2050 (100–16000)	1800 (140–6300)	NS‡
IFN dose (≥80%/60–80%)	36/8	13/3	NS†
Ribavirin dose (≥80%/60–80%)	32/12	10/6	NS†
RVR rate (%)	55.8	6.3	0.0008†
EVR rate (%)	97.7	68.8	0.004†
ETR rate (%)	100	87.5	NS†

§: SVR : n = 43, non-SVR : n = 10.
*: median (range).
†: Fisher's exact probability test.
‡: Mann-Whitney's U test.
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amino acid variation in the NS5A-ISDR [10], NS5A-IRRDR [11], NS5B [12], PKR-eIF2 phosphorylation homology domain (PePHD) of E2 [13], and Core [14] correlate with the clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy for genotype 1b HCV infection. In the meantime, these viral sequence studies have been controversial regarding their true clinical importance, because the results of different studies were not always coincident [15,16,17]. On this background, recent studies trying to analyze the correlation of complete HCV open reading frame diversity, clinical characteristics, and the response to PEG-IFN/RBV therapy for genotype 1 HCV infection, in the most comprehensive approach yet attempted, have clarified that viral amino acid variation is associated with treatment responses, with consideration of racial background [18,19]. In genotype 2 infection, however, only a few studies have investigated the association of HCV sequence variation and treatment response [20,21] and the clinical significance has been yet established. We reported recently that variation of amino acid (aa) 110 in Core and amino acids (aa) 2258–2308 in NS5A were significantly associated with treatment outcome of the PEG-IFN/RBV therapy for genotype 2a HCV infection, through the analysis of the complete HCV ORFs in Japanese patients [22].

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

Methods

Patients

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

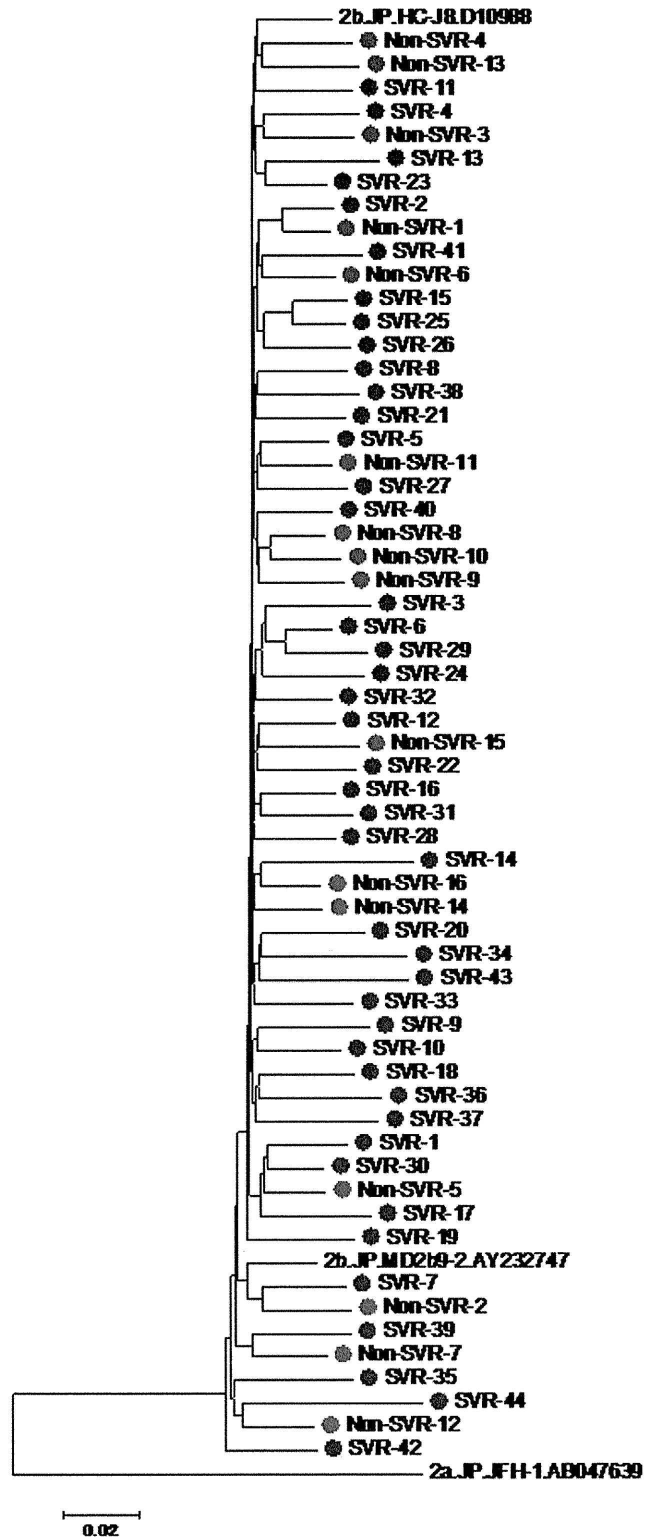

Figure 1. Phylogenetic analysis of the genotype-2b polyprotein sequences. In order to perform the phylogenetic analysis, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients along with reference sequences (2b.HC-J8.D10988, 2.JP.MD2b9-2, and 2a.JP.JFH-1.AB047639), using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

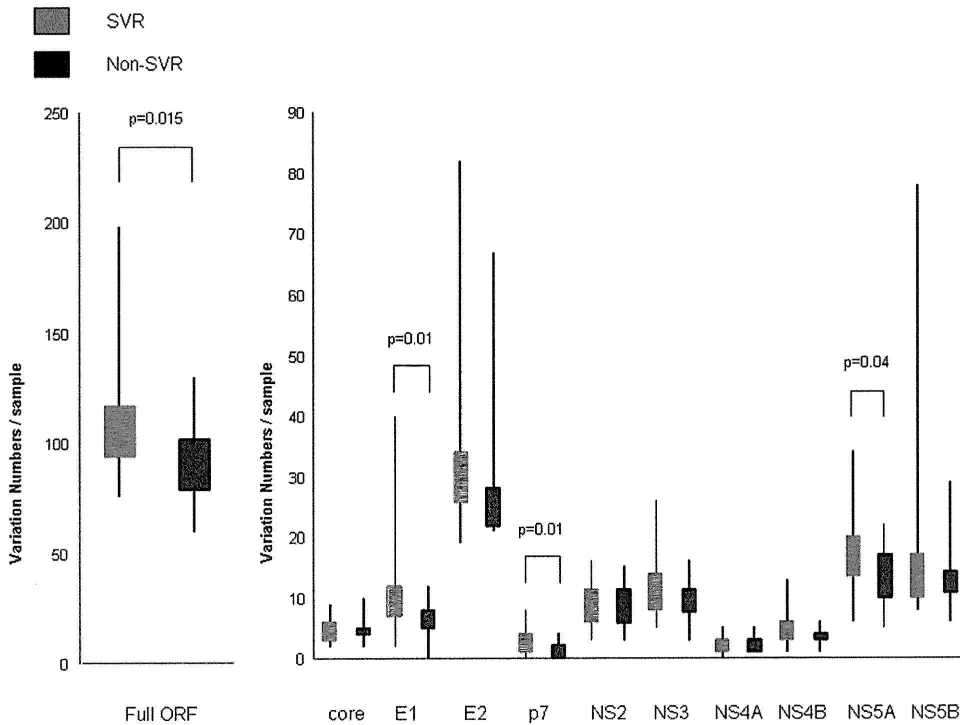

Figure 2. Number of amino acid substitutions per sample in the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) group. The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the complete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

(PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2009 at University of Yamanashi, Tokyo Medical and Dental University, and related institutions were first included in the study. They all fulfilled following criteria: (1) negative for hepatitis B surface antigen, (2) high viral load (≥100 KIU/ml), (3) absence of

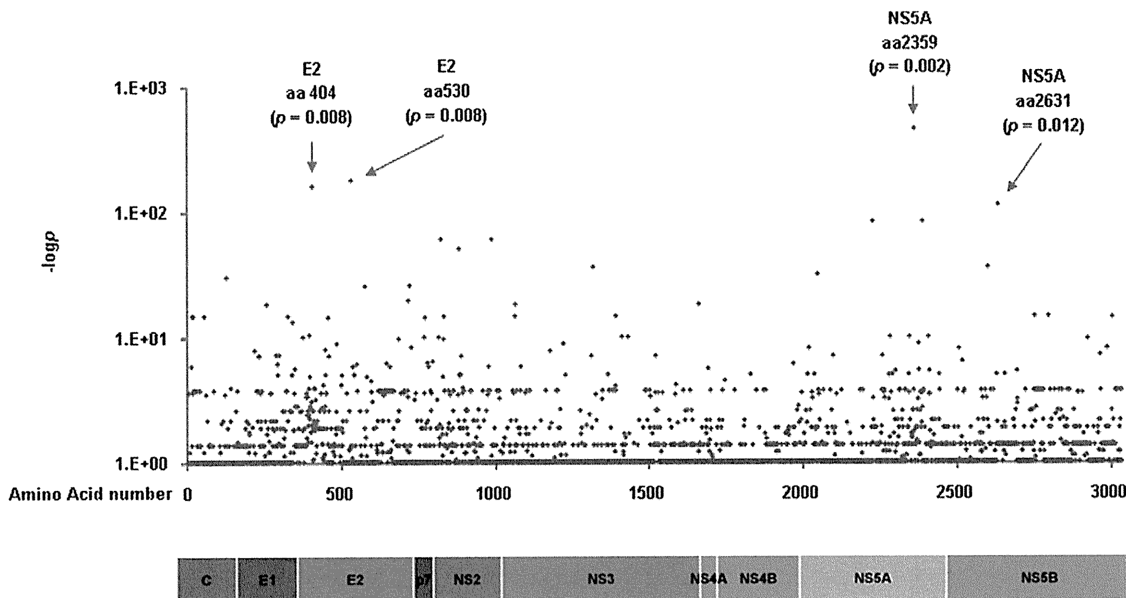

Figure 3. Different amino acid usage at each viral amino acid position between the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) patients. (a) Different amino acid usage at each viral amino acid position between the SVR and the non-SVR patients was analyzed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

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

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

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

hepatocellular carcinoma, (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease, (5) free of co-infection with human immunodeficiency virus. To clearly disclose the non-SVR viral characteristics, we have considered only those patients who achieved total drug administration of 60% or more for both PEG-IFN and RBV, with the completion of the standard treatment duration. Moreover, although we excluded patients with extended therapy to make the studied population uniform, we have included non-SVR patients with extended therapy to clarify the specific characteristics of non-SVR patients, a minor population group. As a result, 17 patients were excluded for the following reasons: 1 patient received insufficient dose, 4 patients were discontinued from the therapy within 12 weeks, and 12 SVR patients received extended therapy. Finally, 60 patients were considered as eligible for the study. During the combination therapy, blood samples were obtained at least once every month before, during and after treatment and were analyzed for blood count, ALT and HCV RNA levels. Liver biopsy specimens were obtained from most of the patients. All patients gave written informed consent to the study. The study was approved by the ethics committees of University of Yamanashi, Tokyo Medical and Dental University, and related institutions. The therapy was performed according to the standard treatment protocol of PEG-IFN/RBV therapy for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan (PEG-IFNα-2b 1.5 μg/kg body weight, once weekly subcutaneously, and RBV 600–800 mg daily per os for 24 weeks).

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

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

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

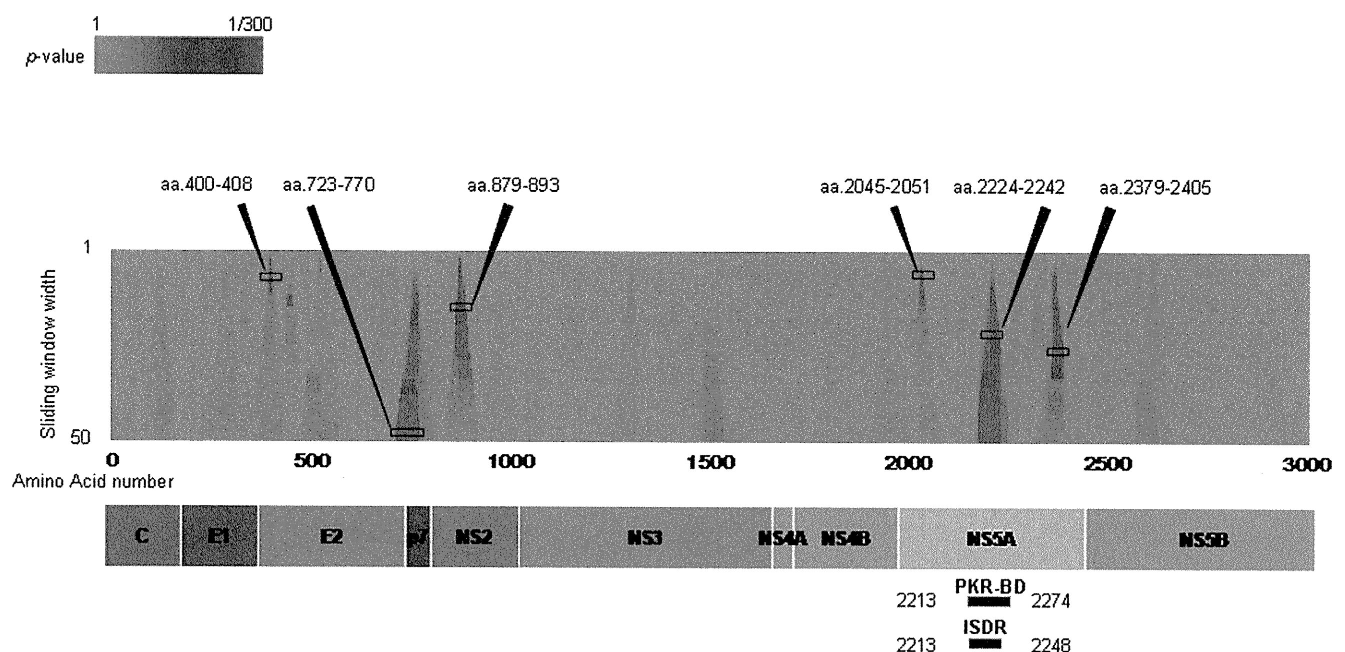

Figure 4. Sliding window analysis. (a) Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis was performed. Viral regions affecting treatment outcome are shown as red areas. There are six hot areas: amino acid 400–408 and 723–770 in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

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

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

*SVR number in patients fulfilling the criteria.
**Number of patients fulfilling the criteria.
doi:10.1371/journal.pone.0024514.t003

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

Table 4. Multivariate Logistic Regression Analysis.

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

To evaluate the optimal threshold of amino acid variations for SVR prediction in each viral region extracted, a receiver operating characteristic curve was constructed and the most optimal cut off value was determined for each region.
doi:10.1371/journal.pone.0024514.t004

Sliding Window Analysis

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

Statistical Analysis

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

Results

Characteristics of the patients studied

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

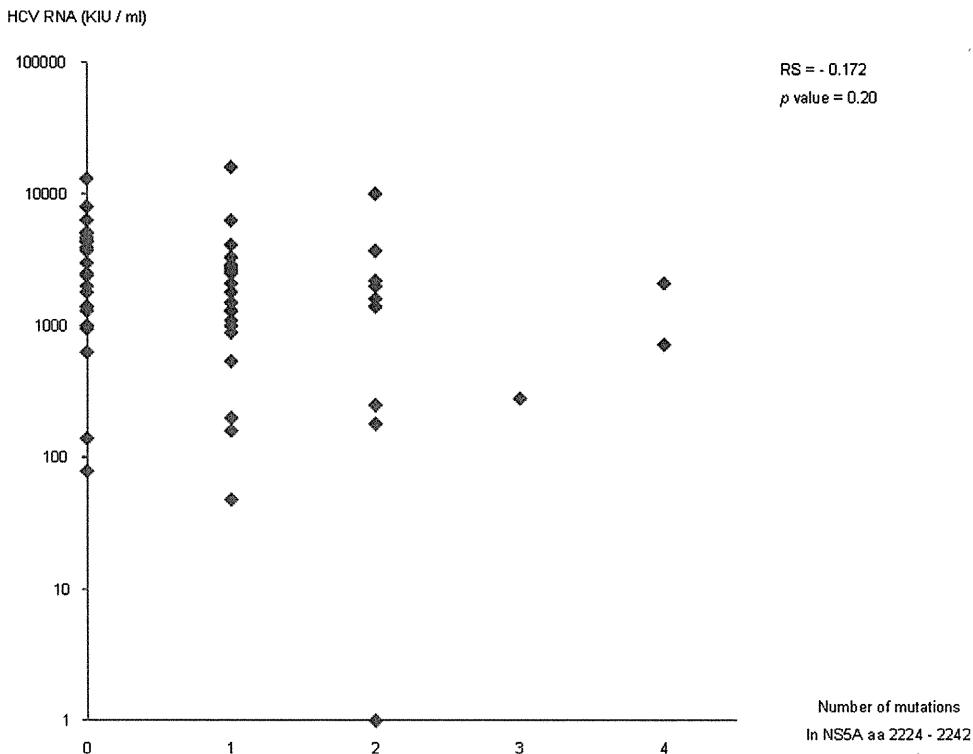

Figure 5. Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2224 to 2242. Spearman's correlation coefficient by rank test is demonstrated.
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University of Yamanashi (data not shown). The early virological response (EVR) rate was equally high in the SVR (97.7%) and non-SVR (68.8%) groups. Interestingly, most of the non-SVR patients (14/16, 87.5%) in genotype-2b HCV infection showed end-of-treatment response (ETR; undetectable serum HCV RNA at the end of therapy), demonstrating that the main cause of non-SVR was relapse (reappearance of hepatitis C viremia during the follow-up period after stopping therapy in patients with an ETR, n = 14), and not null response (detectable serum HCV RNA at the end of therapy, n = 2).

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

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

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

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

§: 1≤ : n = 31, 0 : n = 22.
*: median (range).
†: Fisher's exact probability test.
‡: Mann-Whitney's U test.
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with reference sequences (2b.HC-J8.D10988, 2.JP.MD2b9-2, and 2a.JP.JFH-1.AB047639 obtained from the Los Alamos HCV Database as representative sequences for genotype 2b and genotype 2a HCV) and constructed a phylogenetic tree (Fig. 1). As demonstrated in the tree, no evident clustering was apparent according to the difference of responses.

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

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

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

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

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

Fig. 4a and Table 3 shows the result of sliding window analysis. This approach was used to detect differing HCV amino acid "regions", rather than single amino acid positions, between the SVR and the non-SVR patients. According to the result, six regions were associated with the final outcome (p-values less than 1/20): aa 400–408 in the E2 region ($p = 0.006$), aa 723–770 in the E2 and the N-terminus of p7 region ($p = 0.001$), aa 879–893 in the NS2 region ($p = 0.01$), aa 2045–2051 in the NS5A region ($p = 0.0002$), aa 2224–2242 in the NS5A region ($p = 0.001$) and aa 2379–2405 in the NS5A region ($p = 0.03$). Interestingly, aa 2224–2242 in the NS5A was located in the interferon sensitivity determining region (ISDR). Fig. 4b shows the aligned sequences of amino acids around 2213–2274 of HCV NS5A. Among these 6 regions, aa 723–770, aa 879–893, aa 2224–2242, and aa 2379–2405 were correlated with the final outcome in an incremental manner according to the number of substitutions in those regions (Table S2, S3, S4, S5). The number of substitutions in the ISDR was also correlated to the final outcome in an incremental step-up manner (data not shown).

Multivariate analysis to detect independent predictive factors contributing to the SVR

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

Biological relevance of variation in NS5A in this study group

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

Discussion

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

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

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

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

In genotype 1 HCV, variations within the PKR-binding region of NS5A, including those within the ISDR, were reported to disrupt the NS5A-PKR interaction, possibly rendering HCV sensitive to the antiviral effects of interferon [25]. Clinically, the number of substitutions within the ISDR has been reported to correlate with the serum HCV RNA level in genotype 1 and 2a infections [13]. In addition, a recent study reported that mutations in the ISDR also show the correlation with the relapse in the PEG-IFN/RBV therapy in genotype 1b infection [26]. Because NS5A aa2224–2242, part of ISDR, was extracted as one of those regions related to the treatment response in genotype 2b infection, we undertook further analysis to investigate the correlation between amino acid variation numbers and serum HCV RNA level. Though the reason is unknown, we could not find evidence of a relationship between variation in the NS5A aa 2224–2242 and HCV RNA titer in genotype 2b infection, unlike genotypes 1 and 2a. Of note, a high SVR rate in genotype1 and genotype 2a infection is known to be closely correlated with a low HCV RNA level and multiple substitutions in ISDR. However, in genotype 2b infection in our study, there was no significant difference in the HCV RNA level between SVR and non-SVR patients, as shown in Table 1. Previously, the role of the ISDR in the contribution to SVR in genotype 1 and 2a has been discussed in detail in the context of serum HCV RNA level, and multiple substitutions in the ISDR are related to a low HCV RNA level and high SVR rate. However, it is not known which of these two factors is directly associated with viral clearance. Consideration of this three-sided relationship of ISDR, HCV RNA level and SVR rate in genotype-2b infection leads to the suggestion that amino acid variation in ISDR to be more direct contributor for SVR.

In spite of these findings, there were still limitations in our study. First, because genotype 2b infection only accounts for 10% of all HCV infection in Japan, the number of studied patients was rather small, especially non-SVR patients. In addition, because genotype 2b HCV contains as many as 3033 amino acids, it is possible that incorrect amino acids or regions were judged as significant in the complete HCV ORF comparison study as a result of type I errors. Therefore, if more patients were available for the analysis, the statistical power detecting the meaningful differences would be greater. Secondly, we could not include the IL28B SNP analysis in this study. If we could have combined the information of IL28B SNPs with the full HCV ORF information, a more comprehensive analysis would have been achieved.

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

Supporting Information

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

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

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

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

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

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

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Author Contributions

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

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