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)

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

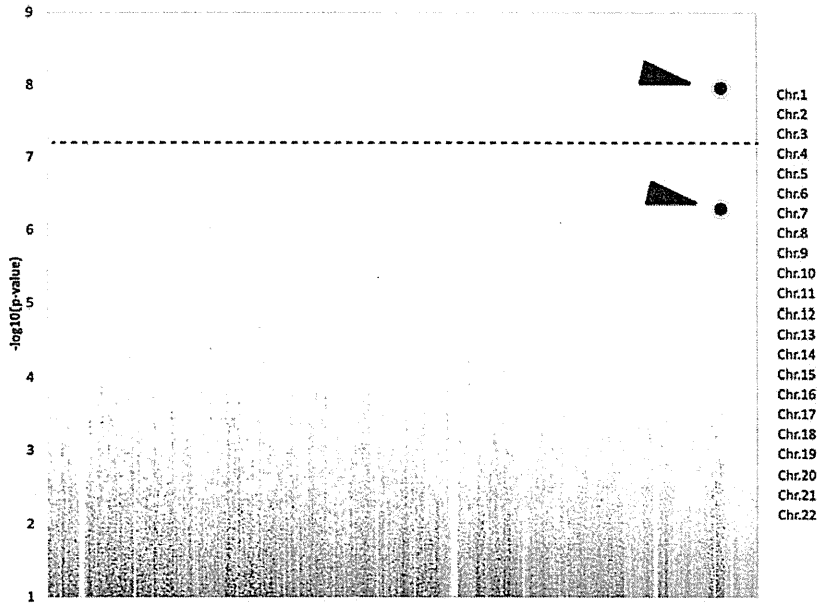

Figure 1. Genome-wide association results in 303 Japanese HCV patients with the decrease of platelets in response to PEG-IFN plus RBV treatment (107 patients with the decrease of PLT and 196 patients without the decrease of PLT). *P*-values were calculated using the χ^2 test for allele frequencies. Dots 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 ($P < 8.40 \times 10^{-8}$).

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.

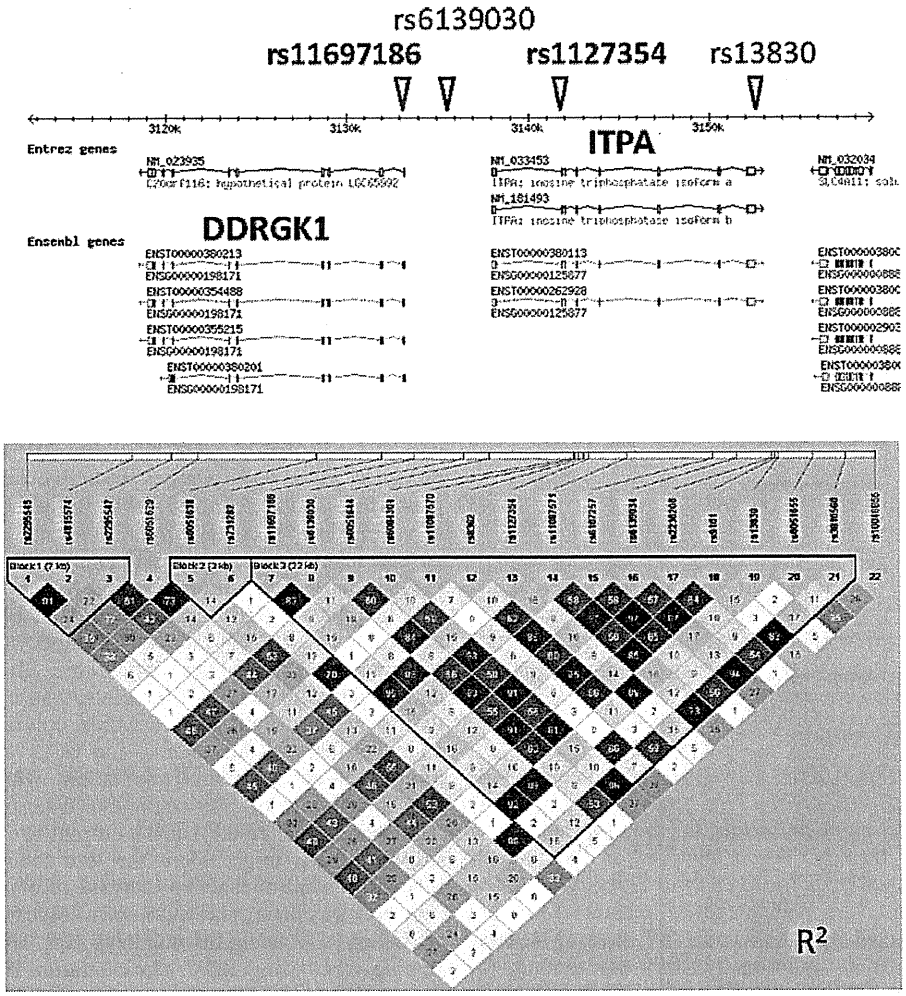

Figure 2. Pairwise LD (r^2) diagrams for *DDRGK1* and *ITPA*. Lower panel shows estimates of pairwise r^2 for 22 SNPs selected in the replication study using the second set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment.

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).

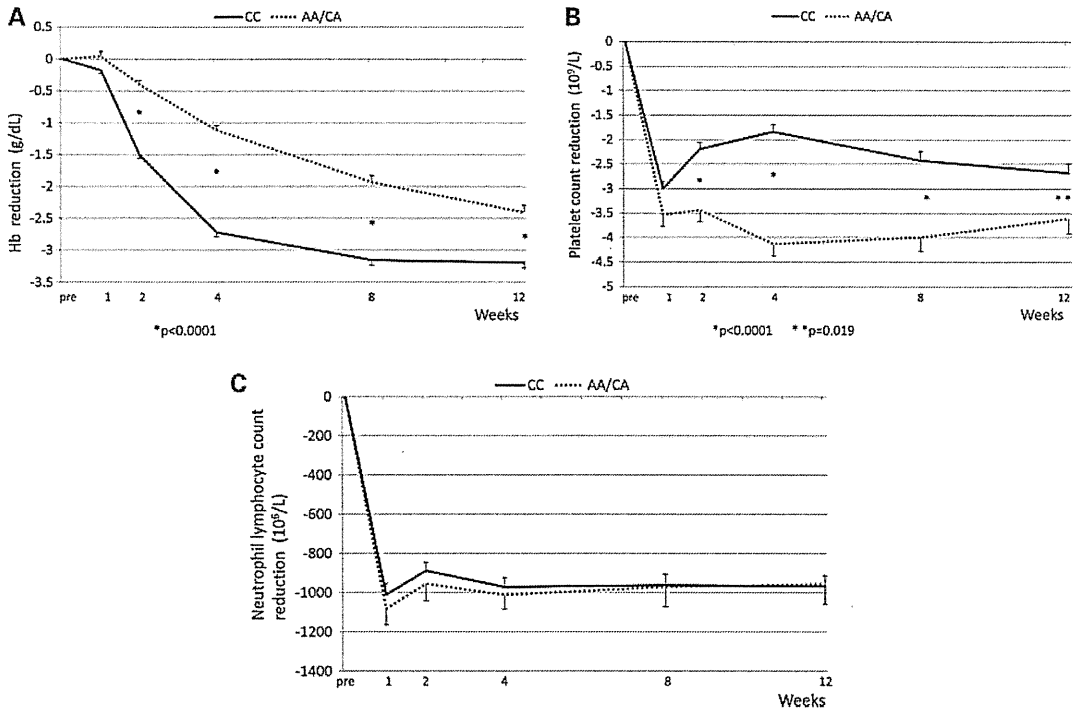

Figure 3. *ITPA* rs1127354 genotypes and the quantitative reduction of blood cells from baseline. Mean reduction of (**A**) Hb levels, (**B**) platelet counts and (**C**) neutrophil leukocyte counts during treatment according to rs1127354 genotype is shown. Solid and dotted lines indicate patients with 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.

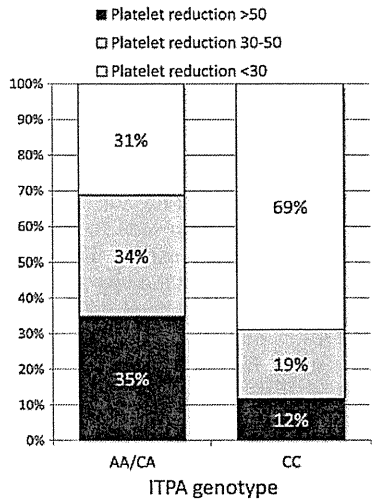

Figure 4. *ITPA* rs1127354 genotypes and reduction of platelet counts at week 4 of PEG-IFN/RBV therapy. The percentage of patients with platelet count reduction of >50 (10^9/l) (black bar), 30–50 (10^9/l) (gray bar) and <30 (10^9/l) (white bar) at week 4 is shown for rs1127354 genotypes. The incidence 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

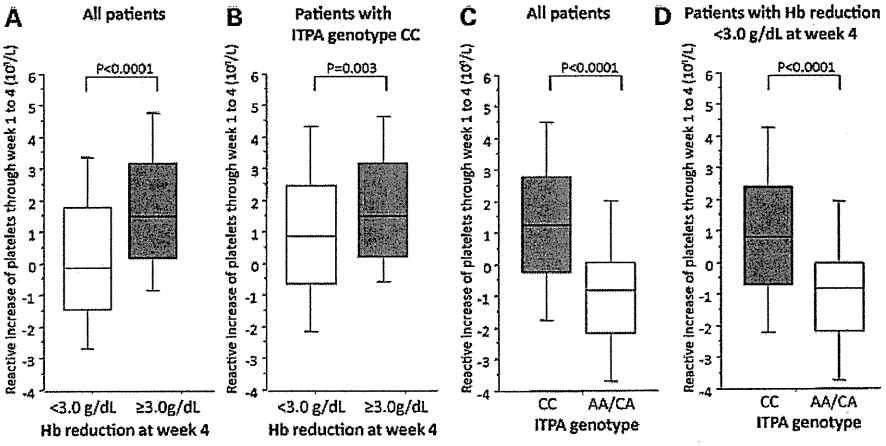

Figure 5. Reactive increase of platelet counts through weeks 1–4. Box plots of reactive increase of platelet count through weeks 1–4 according to the degree of anemia at week 4 are shown for all patients (**A**) and a subgroup of patients with the rs1127354 genotypes CC (**B**). Patients with anemia (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

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 $\geq$95% for all samples, MAF $\geq$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

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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REFERENCES

1. Global Burden of Hepatitis C Working Group (2004) Global burden of disease (GBD) for hepatitis C. *J. Clin. Phamacol.*, **44**, 20–29.
2. Shiratori, Y., Shiina, S., Imamura, M., Kato, N., Kanai, F., Okudaira, T., Teratani, T., Tohgo, G., Toda, N., Ohashi, M. *et al.* (1995) Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology*, **22**, 1027–1033.
3. Yoshida, H., Tateishi, R., Arakawa, Y., Sata, M., Fujiyama, S., Nishiguchi, S., Ishibashi, H., Yamada, G., Yokosuka, O., Shiratori, Y. *et al.* (2004) Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C. *Gut*, **53**, 425–430.
4. George, S.L., Bacon, B.R., Brunt, E.M., Mihindukulasuriya, K.L., Hoffmann, J. and Di Bisceglie, A.M. (2009) Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. *Hepatology*, **49**, 729–738.
5. Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales, F.L. Jr, Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D. *et al.* (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.*, **347**, 975–982.
6. Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M. and Albrecht, J.K. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet*, **358**, 958–965.
7. Hadziyannis, S.J., Sette, H. Jr, Morgan, T.R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer, H. Jr, Bernstein, D., Rizzetto, M. *et al.* (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann. Intern. Med.*, **140**, 346–355.
8. Hiramatsu, N., Oze, T., Tsuda, N., Kurashige, N., Koga, K., Toyama, T., Yasumaru, M., Kanto, T., Takehara, T., Kasahara, A. *et al.* (2006) Should aged patients with chronic hepatitis C be treated with interferon and ribavirin combination therapy? *Hepatol. Res.*, **35**, 185–189.
9. Iwasaki, Y., Ikeda, H., Araki, Y., Osawa, T., Kita, K., Ando, M., Shimoe, T., Takaguchi, K., Hashimoto, N., Kobatake, T. *et al.* (2006) Limitation of

combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. *Hepatology*, **43**, 54–63.
10. Sezaki, H., Suzuki, F., Akuta, N., Yatsuji, H., Hosaka, T., Kobayashi, M., Suzuki, Y., Arase, Y., Ikeda, K., Miyakawa, Y. *et al.* (2009) An open pilot study exploring the efficacy of fluvastatin, pegylated interferon and ribavirin in patients with hepatitis C virus genotype 1b in high viral loads. *Intervirology*, **52**, 43–48.
11. Bruno, R., Sacchi, P., Maiocchi, L., Patruno, S. and Filice, G. (2006) Hepatotoxicity and antiretroviral therapy with protease inhibitors: a review. *Dig. Liver Dis.*, **38**, 363–373.
12. Hezode, C., Forestier, N., Dusheiko, G., Ferenci, P., Pol, S., Goeser, T., Bronowicki, J.P., Bourliere, M., Gharakhanian, S., Bengtsson, L. *et al.* (2009) Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N. Engl. J. Med.*, **360**, 1839–1850.
13. McHutchison, J.G., Everson, G.T., Gordon, S.C., Jacobson, I.M., Sulkowski, M., Kauffman, R., McNair, L., Alam, J. and Muir, A.J. (2009) Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N. Engl. J. Med.*, **360**, 1827–1838.
14. Suzuki, F., Akuta, N., Suzuki, Y., Sezaki, H., Yatsuji, H., Kawamura, Y., Hosaka, T., Kobayashi, M., Arase, Y., Ikeda, K. *et al.* (2009) Rapid loss of hepatitis C virus genotype 1b from serum in patients receiving a triple treatment with telaprevir (MP-424), pegylated interferon and ribavirin for 12 weeks. *Hepatol. Res.*, **39**, 1056–1063.
15. Sakamoto, N. and Watanabe, M. (2009) New therapeutic approaches to hepatitis C virus. *J. Gastroenterol.*, **44**, 643–649.
16. Afdhal, N.H., McHutchison, J.G., Zeuzem, S., Mangia, A., Pawlotsky, J.M., Murray, J.S., Shianna, K.V., Tanaka, Y., Thomas, D.L., Booth, D.R. *et al.* (2010) Hepatitis C pharmacogenetics: state of the art in 2010. *Hepatology*, **53**, 336–345.
17. Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., Nakagawa, M., Korenaga, M., Hino, K., Hige, S. *et al.* (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.*, **41**, 1105–1109.
18. Ge, D., Fellay, J., Thompson, A.J., Simon, J.S., Shianna, K.V., Urban, T.J., Heinzen, E.L., Qiu, P., Bertelsen, A.H., Muir, A.J. *et al.* (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*, **461**, 399–401.
19. Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M.L., Bassendine, M., Spengler, U., Dore, G.J., Powell, E. *et al.* (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.*, **41**, 1100–1104.
20. Thomas, D.L., Thio, C.L., Martin, M.P., Qi, Y., Ge, D., O'Huigin, C., Kidd, J., Kidd, K., Khakoo, S.I., Alexander, G. *et al.* (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature*, **461**, 798–801.
21. Tanaka, Y., Nishida, N., Sugiyama, M., Tokunaga, K. and Mizokami, M. (2010) lambda-Interferons and the single nucleotide polymorphisms: a milestone to tailor-made therapy for chronic hepatitis C. *Hepatol. Res.*, **40**, 449–460.
22. Fellay, J., Thompson, A.J., Ge, D., Gumbs, C.E., Urban, T.J., Shianna, K.V., Little, L.D., Qiu, P., Bertelsen, A.H., Watson, M. *et al.* (2010) ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature*, **464**, 405–408.
23. Afdhal, N., McHutchison, J., Brown, R., Jacobson, I., Manns, M., Poordad, F., Weksler, B. and Esteban, R. (2008) Thrombocytopenia associated with chronic liver disease. *J. Hepatol.*, **48**, 1000–1007.
24. Wazny, L.D. and Ariano, R.E. (2000) Evaluation and management of drug-induced thrombocytopenia in the acutely ill patient. *Pharmacotherapy*, **20**, 292–307.
25. Hitomi, Y., Cirulli, E.T., Fellay, J., McHutchison, J.G., Thompson, A.J., Gumbs, C.E., Shianna, K.V., Urban, T.J. and Goldstein, D.B. (2011) Inosine triphosphate protects against ribavirin-induced adenosine triphosphate loss by adenylosuccinate synthase function. *Gastroenterology*, **140**, 1314–1321.
26. Ochi, H., Maekawa, T., Abe, H., Hayashida, Y., Nakano, R., Kubo, M., Tsunoda, T., Hayes, C.N., Kumada, H., Nakamura, Y. *et al.* (2010) ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy—a genome-wide study of Japanese HCV virus patients. *Gastroenterology*, **139**, 1190–1197.
27. Ong, J.P. and Younossi, Z.M. (2004) Managing the hematologic side effects of antiviral therapy for chronic hepatitis C: anemia, neutropenia, and thrombocytopenia. *Cleve. Clin. J. Med.*, **71** (Suppl. 3), S17–S21.
28. Canonico, P.G., Kastello, M.D., Cosgriff, T.M., Donovan, J.C., Ross, P.E., Spears, C.T. and Stephen, E.L. (1984) Hematological and bone marrow effects of ribavirin in rhesus monkeys. *Toxicol. Appl. Pharmacol.*, **74**, 163–172.
29. Akan, H., Guven, N., Aydogdu, I., Arat, M., Beksac, M. and Dalva, K. (2000) Thrombopoietic cytokines in patients with iron deficiency anemia with or without thrombocytosis. *Acta Haematol.*, **103**, 152–156.
30. Wu, J., Lei, G., Mei, M., Tang, Y. and Li, H. (2010) A novel C53/LZAP-interacting protein regulates stability of C53/LZAP and DDRGK domain-containing protein 1 (DDRGK1) and modulates NF-kappaB signaling. *J. Biol. Chem.*, **285**, 15126–15136.
31. Sakamoto, N., Tanaka, Y., Nakagawa, M., Yatsuhashi, H., Nishiguchi, S., Enomoto, N., Azuma, S., Nishimura-Sakurai, Y., Kakinuma, S., Nishida, N. *et al.* (2010) ITPA gene variant protects against anemia induced by pegylated interferon-alpha and ribavirin therapy for Japanese patients with chronic hepatitis C. *Hepatol. Res.*, **40**, 1063–1071.
32. Kurosaki, M., Tanaka, Y., Tanaka, K., Suzuki, Y., Hoshioka, Y., Tamaki, N., Kato, T. and Yasui, Y. (2011) Analysis of the correlations between genetic polymorphisms of the ITPA gene and hemolytic anemia or outcome after treatment with pegylated-interferon and ribavirin in genotype 1b chronic hepatitis C. *Antivir. Ther.*, in press.
33. Shipkova, M., Lorenz, K., Oellerich, M., Wieland, E. and von Ahsen, N. (2006) Measurement of erythrocyte inosine triphosphate pyrophosphohydrolase (ITPA) activity by HPLC and correlation of ITPA genotype-phenotype in a Caucasian population. *Clin. Chem.*, **52**, 240–247.
34. Fraser, J.H., Meyers, H., Henderson, J.F., Brox, L.W. and McCoy, E.E. (1975) Individual variation in inosine triphosphate accumulation in human erythrocytes. *Clin. Biochem.*, **8**, 353–364.
35. Kumada, H., Okanoue, T., Onji, M., Moriwaki, H., Izumi, N., Tanaka, E., Chayama, K., Sakisaka, S., Takehara, T., Oketani, M. *et al.* (2010) Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. *Hepatol. Res.*, **40**, 8–13.
36. Nishida, N., Tanabe, T., Takasu, M., Suyama, A. and Tokunaga, K. (2007) Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. *Anal. Biochem.*, **364**, 78–85.

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Original article

Serum interleukin (IL)-10 and IL-12 levels and *IL28B* gene polymorphisms: pretreatment prediction of treatment failure in chronic hepatitis C

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Background: Both *IL28B* gene polymorphisms and serum levels of interleukin (IL)-10, IL-12p40 and IL-18 have been reported to affect the outcome of natural and pegylated interferon and ribavirin-treated HCV infection.
Methods: To clarify their association and predictive value in treatment outcome of genotype 1 HCV-infected patients, we measured pretreatment serum IL-10, IL-12p40 and IL-18 levels using multiplex assays and determined *IL28B* gene polymorphisms (rs 8099917) in 52 cases with chronic hepatitis C.
Results: High baseline levels of IL-10 ($P<0.001$) and low levels of IL-12p40 ($P<0.001$) were significantly associated with a non-virological response (NVR) in our cohort. The *IL28B* polymorphism was tested and TT, TG or GG genotypes were found in 60%, 38% and 2% of patients, respectively, with corresponding NVR rates of 10%, 60% and 100% ($P<0.001$). Serum cytokine levels were significantly correlated with *IL28B* gene polymorphisms. When serum IL-10 levels were stratified at 5.0 pg/ml, NVR rates were 50% versus 0% ($P=0.004$) for the TT genotype and 87% versus 0% ($P=0.001$) for the TG or GG genotypes. Similarly, low IL-12p40 levels were associated with an NVR in patients with TG or GG genotypes ($P=0.006$). In multivariate analysis, high IL-10, low IL-12p40 and *IL28B* TG or GG genotypes were independently associated with an NVR.
Conclusions: Serum IL-10 and IL-12p40 levels in combination with *IL28B* genotype, especially G-allele carriage, are strong predictive markers of an NVR to HCV treatment with pegylated interferon and ribavirin.

Introduction

Chronic HCV infection often develops into chronic hepatitis leading to liver cirrhosis and/or hepatocellular carcinoma [1–3]. The successful eradication of HCV, defined as a sustained virological response (SVR), is therefore considered important. Despite recent advances, however, approximately 50% of patients with genotype 1 HCV infection do not achieve an SVR by conventional pegylated interferon (PEG-IFN) and ribavirin therapy [4,5].

It is considered beneficial to predict the response of patients with genotype 1 HCV and high viral load to PEG-IFN and ribavirin therapy before commencement of treatment because therapy can be long, costly and have many side effects. To date, many predictive factors have been reported for treatment response. Regarding viral factors, substitutions at core amino acids 70 and 91 [6] or the IFN sensitivity determining region (ISDR) have been reported [7]. Concerning host factors, Ge *et al.* [8] recently identified single nucleotide polymorphisms (SNPs) located 5′ to the *IL28B* gene that affect response to combination therapy using a genome-wide association study. Similarly, three other groups independently reported that these SNPs are associated with the effectiveness of combination treatment [9–11]. Thomas *et al.* [12] also reported that the same SNPs are associated with spontaneous clearance of HCV.

Interleukin (IL)-28A, IL-28B and IL-29 gene products belong to the IFN-λ family. These cytokines are functionally considered to be IFNs, but have been reported to be structurally related to the IL-10 family, which include IL-10, IL-22 and IL-26, and the

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

Characteristic	All (*n*=52)	VR (*n*=36)	NVR (*n*=16)	*P*-value
Age, years	58 (17–74)	57 (17–72)	60 (45–74)	0.781
Male, *n* (%)	24 (46)	18 (50)	6 (38)	0.404
Body mass index, kg/m^2	23 (18–30)	24 (18–30)	22 (19–29)	0.115
White blood cell count, cells/μl	4,470 (1,980–7,890)	4,810 (1,980–7,890)	3,700 (2,270–5,180)	0.007
Haemoglobin, g/dl	14.7 (12–18)	15.0 (13–18)	14.1 (12–16)	0.094
Platelet count, 10^4/μl	17.5 (8–30)	17.9 (8–30)	16.7 (9–27)	0.420
ALT, IU/l	75 (22–389)	68 (24–389)	91 (22–357)	0.663
AST, IU/l	58 (20–288)	49 (20–218)	78 (25–288)	0.092
HCV RNA, 10^5 IU/ml	21 (1.1–>50)	20 (1.1–>50)	18 (2.9–>50)	0.469
Core aa 70 (Arg70/Gln70/ND), *n*	30/21/1	23/12/1	7/9/0	0.139
Core aa 91 (Leu91/Met91/ND), *n*	37/14/1	26/9/1	11/5/0	0.463
ISDR of NS5A (wild/mutant), *n*	44/8	29/7	15/1	0.218
rs8099917 allele (TT/TG/GG), *n*	31/20/1	28/8/0	3/12/1	<0.001

Data are mean (range) unless indicated otherwise. aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ISDR, interferon-sensitivity determining region; NVR, non-virological response; VR, virological response.

IFN-λ family. The ligand-binding chains for IL-22, IL-26 and IFN-λ are distinct from that used by IL-10. However, all of these cytokines use a common second chain, IL-10 receptor-2, to assemble their active receptor complexes. Thus, IL-10 receptor-2 is a shared component in at least four distinct class II cytokine-receptor complexes [13]. Although IL-10 was originally described as a cytokine synthesis inhibitory factor [14,15], recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [16,17]. Furthermore, increased IL-10 levels are associated with a high risk of inefficient HCV clearance and resistance to IFN treatment [18–21]. Our recent study showed that low serum IL-10 levels as well as high IL-12p40 and IL-18 levels at baseline were independent predictive factors for a SVR to combination therapy [22]. Therefore, in the present study, we investigated the association between treatment outcome and the influence of *IL28B* genotype and serum cytokine levels in combination therapy.

Methods

Subjects

A total of 52 consecutive treatment-naive patients with genotype 1 chronic hepatitis C were included in this study. Diagnosis of chronic hepatitis C was based on the following criteria as reported previously [23]: presence of serum HCV antibodies and detectable viral RNA, absence of detectable hepatitis B surface antigen and antibody to HIV and exclusion of other causes of chronic liver disease. No patients had a history of or developed decompensated cirrhosis or hepatocellular carcinoma. The baseline characteristics of the patients are shown in Table 1.

Laboratory testing

Antibodies to HCV were measured in serum samples via third-generation enzyme-linked immunosorbent assays (EIA-3; Abbott Laboratories, North Chicago, IL, USA). Serum levels of HCV RNA were determined using the Cobas Amplicor assays (sensitivity 50 IU/ml; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II (Innogenetics, Gent, Belgium). All patients in our test cohort were infected with genotype 1b. Alanine aminotransferase, aspartate aminotransferase and other relevant biochemical tests were performed using standard methods [24].

Antiviral therapy and definition of treatment outcome

All patients received bodyweight-adjusted PEG-IFN-α2b (PegIntron; Schering–Plough KK, Tokyo, Japan; ≤45 kg, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; ≥91 kg, 150 μg/dose), and ribavirin (Rebetol; Schering–Plough KK; ≤60 kg, 600 mg/day; 61–80 kg, 800 mg/day; ≥81 kg, 1,000 mg/day) for 48 weeks.

The response to therapy categories were defined as follows: an SVR was defined as undetectable serum HCV RNA 24 weeks after completing therapy. Relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A non-virological response (NVR) was defined as a decrease in HCV RNA of <2 log copies/ml at week 12 and detectable HCV RNA during the treatment course.

Detection of amino acid substitutions in the core and NS5A regions

Core region and ISDR were determined by direct sequencing after amplification by reverse transcription and PCR as reported previously [22]. Amino acids at

positions 70 and 91 of the core region identical to the reference sequence HCV-J D90208 [25] were considered wild type [6]. The number of amino acid substitutions in the ISDR was defined as in Enomoto *et al.* [7].

Detection of serum IL-10, IL-12p40 and IL-18

Serum IL-10, IL-12p40 and IL-18 were quantified using Luminex® Multiplex Cytokine Kits (Procarta Cytokine assay kit; Panomics Inc., Fremont, CA, USA) for serum samples obtained before the start of treatment as reported previously [22]. All collected samples were immediately stored at -70°C prior to testing.

Genotyping of *IL28B*

Genomic DNA was isolated from the whole blood of patients using QuickGene-800 (Fujifilm, Tokyo, Japan). The concentration of genomic DNA was adjusted to 10–15 ng/μl for the TaqMan SNP genotyping assay. Genotyping of *IL28B* SNP (rs 8099917) was performed with a TaqMan 5′ exonuclease assay using primers supplied by Applied Biosystems (Carlsbad, CA, USA). Probe fluorescence signals were detected with a TaqMan assay for real-time PCR (7500 Real Time PCR System; Applied Biosystems) according to the manufacturer's instructions.

The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent.

Statistical analyses

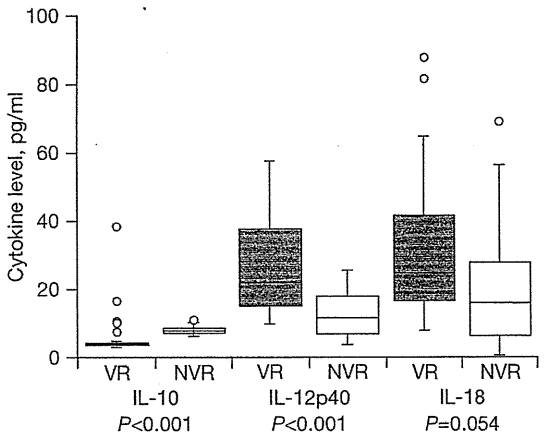
The Mann–Whitney U test was used to analyse continuous variables. The χ^2 test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was <5, Fisher's exact test was used. A *P*-value of ≤0.05 was considered statistically significant. To predict treatment outcome, we analysed receiver operating characteristic (ROC) curves for serum levels of IL-10, IL-12p40 and IL-18. Optimal cutoff values were chosen as serum cytokine levels with the highest diagnostic accuracy, that is, when the sum of the false-negative and false-positive rates was minimized. The respective overall diagnostic values were expressed using the area under the curve (AUC). Multivariate analysis was performed using a logistic regression model with stepwise method. Statistical analyses were performed using PASW Statistics 18.0J (IBM, Tokyo, Japan).

Results

Treatment outcome in patients with chronic hepatitis C

Of the 52 patients receiving PEG-IFN and ribavirin therapy, 22 (42%) achieved an SVR. Among the 30 remaining patients, 14 had a relapse and 16 had an NVR. Before treatment, the median white blood cell count in the virological response group was significantly higher than that in the NVR group (Table 1). Haemoglobin value (15.4 versus 14.1 g/dl; *P*=0.021) was significantly higher in the SVR group compared to the NVR group as well. Substitutions in the ISDR and of aa70 and aa91 in the core region were not associated with treatment outcome.

Figure 1. Detection of serum cytokines related to treatment outcome



Boxes represent the IQR of the data, lines across the boxes indicate the median values and the hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively for each group. Open circles indicate outliers. Serum interleukin (IL)-10, IL-12p40 and IL-18 levels were detected in 36 patients with a virological response (VR) and 16 patients without. NVR, non-virological response.

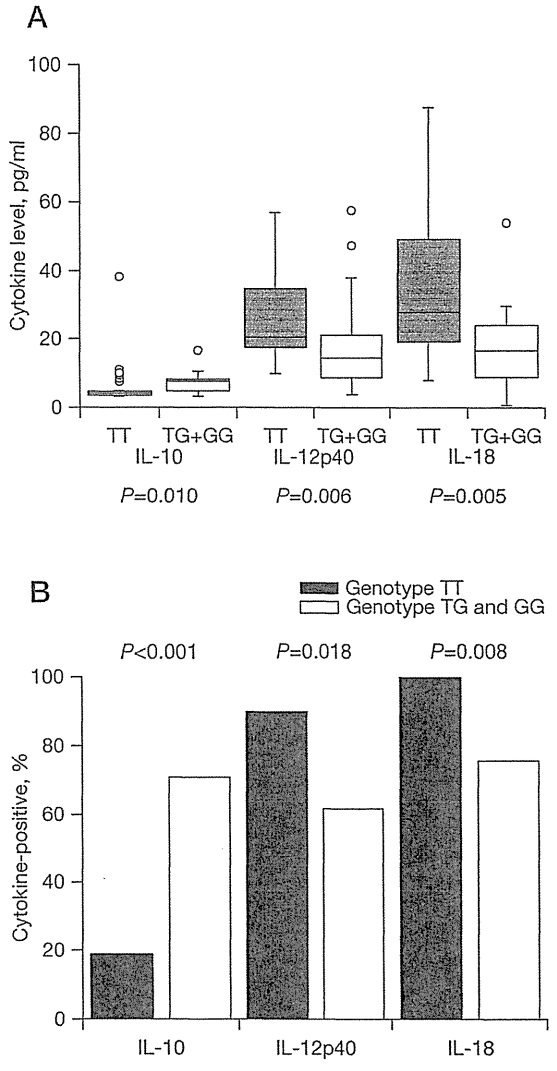
Effects of antiviral therapy on serum cytokine levels

Serum samples obtained prior to antiviral therapy were examined for the presence of IL-10, IL-12p40 and IL-18 by multiplex assays. NVR patients showed significantly higher baseline IL-10 concentrations (8.1 pg/ml) than virological responders (4.1 pg/ml; *P*<0.001; Figure 1). The median baseline serum levels of IL-12p40 (22.1 versus 11.7 pg/ml; *P*<0.001) and IL-18 (24.8 versus 16.0 pg/ml; *P*=0.054) were higher in patients who achieved a virological response than in those with an NVR (Figure 1). Furthermore, serum IL-10 level (4.0 versus 8.1 pg/ml; *P*<0.001) was significantly lower and serum IL-12p40 (25.0 versus 11.7 pg/ml; *P*<0.001) and IL-18 (31.6 versus 16.0 pg/ml; *P*=0.010) levels were significantly higher in the SVR group compared with the NVR group. We also analysed whether pretreatment serum cytokines were correlated with time to clearance of HCV RNA. Serum baseline IL-10 level was significantly lower in patients who eradicated HCV RNA 12 weeks after the start of treatment (*P*=0.002).

IL28B genotype and treatment outcome

Among the 52 patients studied for rs8099917, 31 had the TT genotype (60%), 20 had the TG genotype (38%) and 1 had the GG genotype (2%). Responses to combination therapy for rs8099917 are shown in Table 1. Overall SVR rates in patients with the TT genotype (16/31, 53%) and with the TG or GG genotypes (6/21, 29%) were not significantly different (P=0.09).

Figure 2. Serum cytokines related to *IL28B* gene polymorphisms



(A) Boxes represent the IQR of the data, lines across the boxes indicate the median values and the hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively, for each group. Open circles indicate outliers. Serum interleukin (IL)-10, IL-12p40 and IL-18 were detected in 31 patients with the TT *IL28B* genotype and 16 patients with the TG or GG genotypes. (B) The prevalence of high serum IL-10, IL-12p40 and IL-18 levels in 31 patients with the TT *IL28B* genotype and in 16 patients with the TG or GG genotypes.

However, NVR rates in patients with either TG or GG (13/21, 62%) were significantly higher than in those with TT only (3/31, 10%; P<0.001).

Association of *IL28B* genotype and serum cytokine levels

Median serum IL-10 levels were significantly higher in patients with the TG or GG genotypes (7.7 pg/ml) compared to those with TT (4.1 pg/ml; P=0.010; Figure 2A). Conversely, patients with TT had significantly higher median IL-12p40 (20.6 versus 14.5 pg/ml; P=0.006) and IL-18 (27.9 versus 16.6 pg/ml; P=0.005) levels than patients with TG or GG (Figure 2A).

ROC curve analyses were performed to determine the optimal threshold values of serum cytokines for predicting treatment outcome among the 16 NVR patients and 36 cases with a virological response in our cohort (Figure 3). The optimal threshold value of IL-10 was identical to the 5.0 pg/ml that we had reported in a prior study [22]. The cutoff values for IL-12p40 and IL-18 were 12.1 pg/ml and 6.4 pg/ml, respectively. The calculated AUC for IL-10, IL-12p40 and IL-18 was 0.89 (95% CI 0.77–0.96), 0.81 (95% CI 0.67–0.90) and 0.67 (95% CI 0.52–0.79), respectively, as shown in Figure 3.

The presence of high IL-10 levels (≥5.0 pg/ml) was significantly greater among patients with TG or GG genotypes (71%, 15 of 21) than among those with TT (19%, 6 of 31; P<0.001; Figure 2B). High IL-12p40 levels (≥12.1 pg/ml) were significantly less prevalent (P=0.018) among patients with TG or GG (62%, 13 of 21) than among those with TT (90%, 28 of 31). High IL-18 levels (≥6.5 pg/ml) were found in 100% (31 of 31) of patients with TT but only 76% (16 of 21) patients with TG or GG (P=0.008).

Predicting treatment outcome by serum cytokine levels in combination with *IL28B* genotype

The NVR prediction rate by serum IL-10 in combination with rs8099917 genotype is shown in Figure 4. In patients with TT, a significantly higher proportion of patients with high serum IL-10 levels (50%, 3 of 6) showed an NVR than patients with low IL-10 (0%, 0 of 25; P=0.004). Similarly, an NVR was significantly more likely in high versus low IL-10 levels (87%, 13 of 15 versus 0%, 0 of 6; P=0.001) in patients with TG or GG (Figure 4A).

NVR rates by serum IL-12p40 levels and IL-18 levels in combination with rs8099917 genotype are shown in Figure 4B and 4C. Among patients with the TT genotype, the NVR rate did not differ between low and high IL-12p40 levels (0% versus 11%; P=0.729) or IL-18 levels (0% versus 10%). In cases with TG or GG genotypes, the NVR rate was significantly higher for low IL-12p40 levels compared with high IL-12p40 levels (100% versus 38%; P=0.006). Patients with low serum

IL-18 had a higher NVR rate, but this difference was not statistically significant (100% versus 50%; P=0.063).

Factors associated with an NVR to PEG-IFN and ribavirin therapy

All factors found to be associated with an NVR were evaluated for independence in multivariate analysis. Genotype TG or GG (OR 10.43, 95% CI 1.73–62.96; P=0.011), serum IL-10 levels ≥5.0 pg/ml (OR 1.21, 95% CI 1.03–1.41; P=0.018) and IL-12p40 levels ≥17.4 mg/dl (OR 0.84, 95% CI 0.72–0.97; P=0.020) were all independent predictive factors of an NVR.

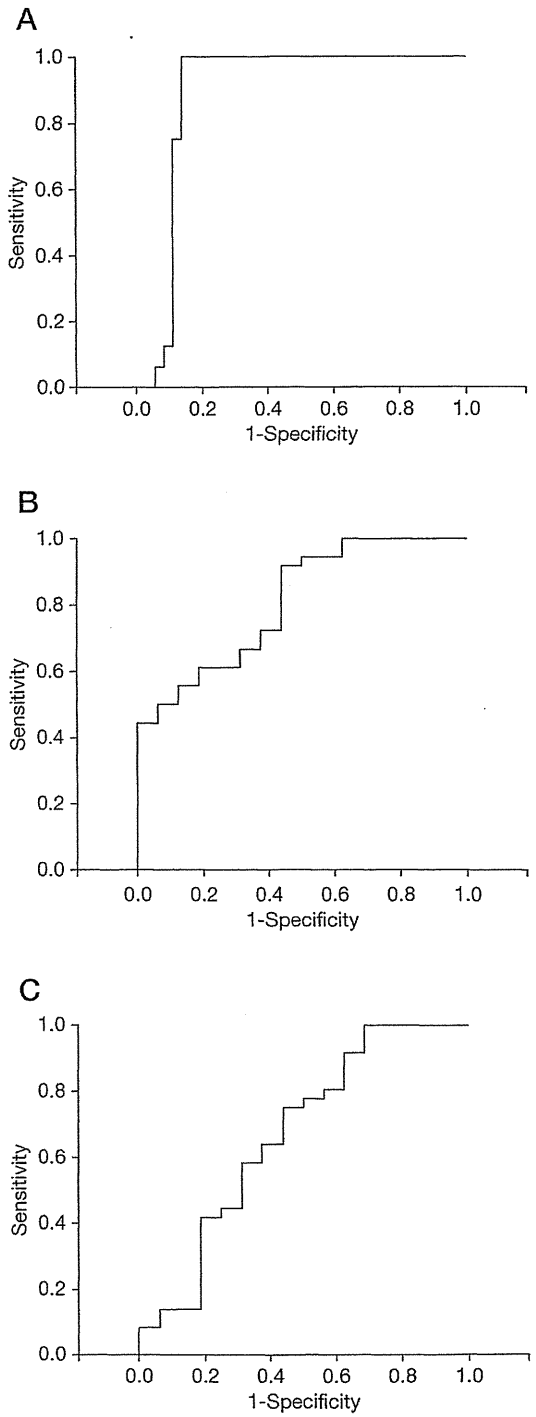
Discussion

This study examined the *IL28B* (rs8099917) genotype and serum levels of IL-10, IL-12p40 and IL-18 in patients with chronic hepatitis C to assess their predictive value in treatment outcome with PEG-IFN and ribavirin. The key findings were as follows: *IL28B* G-allele carriers were associated with an NVR to PEG-IFN and ribavirin therapy in patients infected with HCV genotype 1, consistent with recent findings; *IL28B* genotype was associated with baseline serum IL-10, IL-12p40 and IL-18 levels; in carriers of an *IL28B* G-allele, NVR rates were high (80–100%) and associated with increased IL-10 and decreased IL-12p40 and IL-18 levels, thus providing new predictive markers of an NVR in PEG-IFN and ribavirin therapy; and *IL28B* genotype, high serum IL-10 levels, and low serum IL-12p40 levels were all independent factors related to an NVR in multivariate analyses.

IL28B gene polymorphisms have recently been linked to the outcome of HCV infection during spontaneous and treatment-induced elimination of HCV [8–10,12]. In particular, carriage of a G-allele at the *IL28B* gene SNP (rs8099917) is associated with an NVR to PEG-IFN and ribavirin therapy in Japanese patients infected with HCV genotype 1 [10]. This finding was confirmed in our cohort with NVR rates of 62% with GT or GG genotypes versus 10% with TT genotypes (P<0.001). Therefore, detection of the *IL28B* genotype is a useful marker to predict the outcome of PEG-IFN and ribavirin therapy in patients with chronic hepatitis C. Data for *IL28B* SNP in healthy subjects were not available for this study.

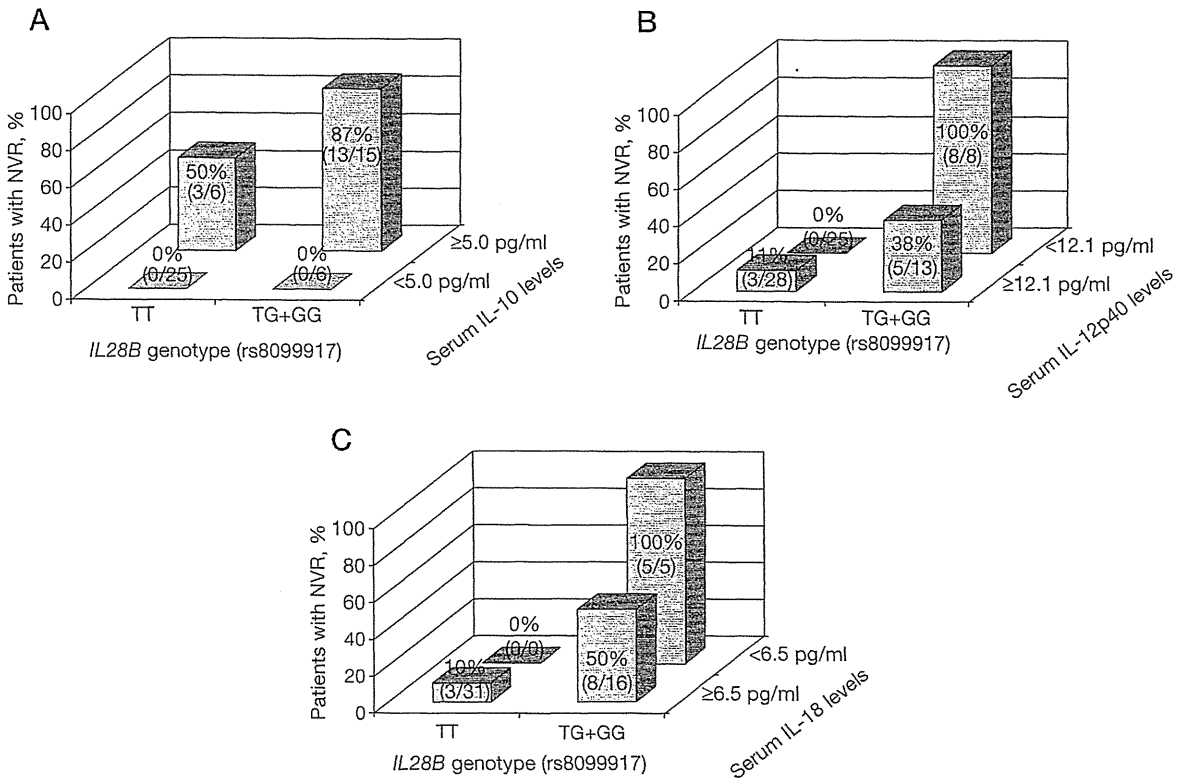
IFN-λ produces an antiviral state by triggering a cascade through the JAK-STAT pathway that up-regulates IFN-stimulated genes. *IL28B* binds to a distinct receptor that may up-regulate a different set of IFN-stimulated genes [26,27]; the precise role of IFN-λ in controlling multiple viral infections, including HCV, is currently under way. Further studies are also needed on how SNPs affect the function of *IL28B* and other cytokines.

Figure 3. Receiver-operating characteristic curves for serum cytokine levels on treatment outcome



The areas under the curve for (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 were 0.89, 0.81 and 0.67, respectively. All areas under the curve values were significantly higher than a 0.50 non-predictive value (P<0.01 for all comparisons). IL-10 was predictive of a non-response. IL-12p40 and IL-18 were predictive of a virological response.

Figure 4. Non-virological response rate determined by serum cytokine levels and *IL28B* gene genotype



The prevalence of a non-virological response (NVR) in patients with high or low serum (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 levels according to *IL28B* genotype.

A strong association between high IL-10, low IL-12p40 and low IL-18 levels and an NVR to PEG-IFN and ribavirin therapy was found in this study, which is consistent with previous studies [22,28–30]. In ROC curve analyses, AUCs were high, especially for IL-10 (AUC=0.89) and IL-12p40 (AUC=0.81), confirming that these cytokines are strong predictive markers for an NVR. This study showed a strong correlation between the *IL28B* genotype and serum IL-10, IL-12p40 and IL-18 levels at baseline. Most strikingly, all patients who had low pretreatment IL-10 levels achieved a virological response regardless of *IL28B* genotype. By contrast, among patients with high IL-10 levels (≥5.0 pg/ml), NVR rates were 87% in *IL28B* G-allele carriers and 50% for the *IL28B* TT genotype. Additionally, all *IL28B* G-allele carriers showed an NVR when pretreatment serum IL-12p40 and IL-18 levels were <12.1 pg/ml and <6.5 pg/ml, respectively. It is unclear how serum IL-10, IL-12p40 and IL-18 are associated with an NVR to antiviral therapy in patients with chronic hepatitis C. Although IL-10 was originally described as a cytokine synthesis inhibitory factor, recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [31]. Production of IL-12p40 is directed towards the elimination of intracellular pathogens and viruses because IL-12p40 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function and amplifies the cytotoxicity of cytotoxic T-lymphocytes and natural killer cells [32]. Megjugorac *et al.* [33] reported that IL-29-treated plasmacytoid dendritic cells inhibiting production of IL-13, IFN-γ and IL-10 by allogeneic T-cells were consistent with a role for this cytokine in plasmacytoid dendritic cell maturation and activation. Very recently, another report has been published demonstrating that IL-29 enhances IL-12p40 by macrophages and that IL-29 pretreatment primes the activation of macrophages induced by IFN-γ [34]. However, the association between IL-28B and such cytokines has not been studied. To explain this relationship, further studies are needed to clarify whether a direct or indirect interaction exists between pretreatment levels of these cytokines and *IL28B* genotype.

Although other predictive factors of PEG-IFN and ribavirin therapy have been reported, including core amino acid 70 and 91 and ISDR mutations [6,7], no such significant associations were found here, possibly because of our study population size, which indicates that other factors may be more significant in predicting treatment outcome. However, multivariate analysis confirmed that *IL28B* G-allele, high IL-10 and low IL-12p40 levels were significant predictors of an NVR in patients with PEG-IFN and ribavirin therapy in this study. Hence, *IL28B* G-allele carriers combined with high IL-10 and/or low IL-12 may require alteration of treatment dose, duration, or regimen with a new antiviral drug.

In conclusion, serum IL-10, IL-12p40 and IL-18 levels are associated with *IL28B* genotype in patients with genotype 1 chronic hepatitis C. Pretreatment serum IL-10 and IL-12p40 levels with *IL28B* GT or GG genotypes are particularly useful for predicting an NVR to PEG-IFN and ribavirin therapy. The clinical significance of *IL28B* genotyping combined with baseline serum IL-10 and IL-12p40 levels to predict an NVR warrants further prospective validation.

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Disclosure statement

The authors declare no competing interests.

References

1. Alter HJ, Purcell RH, Shih JW, *et al.* Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; 321:1494–1500.
2. Kiyosawa K, Sodeyama T, Tanaka E, *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; 12:671–675.
3. Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; 44 Suppl 19:102–107.
4. Manns MP, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358:958–965.
5. Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347:975–982.
6. Akuta N, Suzuki F, Sezaki H, *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; 48:372–380.
7. Enomoto N, Sakuma I, Asahina Y, *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334:77–82.
8. Ge D, Fellay J, Thompson AJ, *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; 461:399–401.
9. Suppiah V, Moldovan M, Ahlenstiel G, *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; 41:1100–1104.
10. Tanaka Y, Nishida N, Sugiyama M, *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41:1105–1109.
11. Rauch A, Kutalik Z, Descombes P, *et al.* Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010; 138:1338–1345.
12. Thomas DL, Thio CL, Martin MP, *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; 461:798–801.
13. Donnelly RP, Sheikh F, Kotenko SV, Dickensheets H. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J Leukoc Biol* 2004; 76:314–321.
14. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989; 170:2081–2095.
15. Fiorentino DF, Zlotnik A, Vieira P, *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; 146:3444–3451.
16. McGeachy MJ, Bak-Jensen KS, Chen Y, *et al.* TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 2007; 8:1390–1397.
17. Stumhofer JS, Silver JS, Laurence A, *et al.* Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 2007; 8:1363–1371.
18. Edwards-Smith CJ, Jonsson JR, Purdie DM, Bansal A, Shorthouse C, Powell EE. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa. *Hepatology* 1999; 30:526–530.
19. Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; 33:708–712.
20. Knapp S, Hennig BJ, Frodsham AJ, *et al.* Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. *Immunogenetics* 2003; 55:362–369.
21. Morgan TR, Lambrecht RW, Bonkovsky HL, *et al.* DNA polymorphisms and response to treatment in patients with chronic hepatitis C: results from the HALT-C trial. *J Hepatol* 2008; 49:548–556.
22. Yoneda S, Umemura T, Katsuyama Y, *et al.* Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. *J Infect Dis* 2011; 203:1087–1095.
23. Umemura T, Wang RY, Schechterly C, Shih JW, Kiyosawa K, Alter HJ. Quantitative analysis of anti-hepatitis C virus antibody-secreting B cells in patients with chronic hepatitis C. *Hepatology* 2006; 43:91–99.
24. Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobin G4-hepatopathy: association of immunoglobin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; 46:463–471.
25. Kato N, Hijikata M, Ootsuyama Y, *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* 1990; 87:9524–9528.
26. Honda M, Sakai A, Yamashita T, *et al.* Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010; 139:499–509.

27. Urban TJ, Thompson AJ, Bradrick SS, *et al.* IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010; 52:1888–1896.
28. Rossol S, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest* 1997; 99:3025–3033.
29. Quiroga JA, Martin J, Navas S, Carreno V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis* 1998; 178:247–251.
30. Marín-Serrano E, Rodriguez-Ramos C, Diaz F, Martin-Herrera L, Giron-Gonzalez JA. Modulation of the anti-inflammatory interleukin 10 and of proapoptotic IL-18 in patients with chronic hepatitis C treated with interferon alpha and ribavirin. *J Viral Hepat* 2006; 13:230–234.
31. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010; 10:170–181.
32. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; 13:251–276.
33. Megjugorac NJ, Gallagher GE, Gallagher G. Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). *J Leukoc Biol* 2009; 86:1359–1363.
34. Liu BS, Janssen HL, Boonstra A. IL-29 and IFNα differ in their ability to modulate IL-12 production by TLR-activated human macrophages, and exhibit differential regulation of the IFNγ receptor expression. *Blood* 2011; 117:2385–2395.

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Original Article

Serum chemokine levels are associated with the outcome of pegylated interferon and ribavirin therapy in patients with chronic hepatitis C

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Aim: Serum chemokine levels and amino acid substitutions in the interferon-sensitivity determining region (ISDR) and core region have been associated with treatment outcome of pegylated interferon and ribavirin therapy in genotype 1 hepatitis C virus (HCV)-infected patients. The present study was conducted to clarify the association between serum chemokines and treatment outcome in patients with chronic HCV-1 infection in a Japanese cohort.

Methods: A total of six serum chemokines were quantified before, during and after pegylated interferon and ribavirin treatment in 79 genotype 1 chronic HCV patients using a multiple bead array system. Viral ISDR and core region variants were determined by direct sequencing.

Results: The baseline serum levels of eotaxin, IP-10 and RANTES were significantly higher in chronic HCV patients than in controls. High levels of eotaxin and macrophage inflammatory protein (MIP)-1β before therapy and more than two mutations in the ISDR were associated with a sustained virological response, and patients with more than two mutations in the ISDR also had significantly higher MIP-1β levels. Receiver–operator curve analysis showed a 77% sensitivity and 73% specificity for predicting an SVR using MIP-1β values.

Conclusion: Serum MIP-1β levels may predict the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the ISDR.

Key words: chemokines, core, interferon sensitivity determining region, MIP-1β, pegylated interferon, ribavirin

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major cause of chronic liver disease that leads to liver cirrhosis and/or hepatocellular carcinoma (HCC).[1] HCC is ranked fourth in men and fifth in women as a cause of death from malignant neoplasms in Japan.[2,3] Interferon (IFN)-based therapy can achieve HCV eradication and decrease the risk of HCC to improve prognosis; with pegylated (PEG) IFN and ribavirin therapy, approximately 50% of patients with genotype 1 HCV infection achieve a sustained virological response (SVR).[4,5]

Chemokines and their receptors play an important role in the pathogenesis of HCV infection.[6,7] Despite the growing amount of published research supporting the complex interactions of these inflammatory biomarkers in the outcome of antiviral therapy, the majority of recent studies have nearly exclusively concentrated on only one or a few markers. Thus, it is possible that a test evaluating several biomarkers may prove to be of greater value in predicting responses to therapy.

In the present study, we sought to determine the levels of six chemokines in patients with chronic HCV-1b

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infection who underwent treatment with PEG IFN and ribavirin using a broad-spectrum bead-based multiplex immunoassay.

METHODS

Subjects

A TOTAL OF 79 treatment-naïve patients with chronic hepatitis C (40 men and 39 women; median age 60 years [range: 17–74]) were seen at Shinshu University Hospital or its affiliated hospitals in the Nagano Interferon Treatment Research Group. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γ-GTP) were tested using standard methods.[8] All patients, who were infected with genotype 1b HCV, received PEG IFN-α-2b (PegIntron; Schering-Plough, Tokyo, Japan; 1.5 μg/kg of bodyweight) and ribavirin (Rebetol; Schering-Plough; 600–1000 mg/day) adjusted to bodyweight for 48 weeks as described previously.[9] The pretreatment median value for ALT was 54 IU/L (range: 22–389), AST was 44 IU/L (range: 20–288) and HCV RNA was 1700 KIU/mL (range: 11–5100), as measured by COBAS AMPLICOR assays (Roche Diagnostic Systems, Tokyo, Japan). A group of 26 healthy individuals (13 men and 13 women; mean age 54 years [range: 28–60]) with hepatitis B and C negative serologies and normal transaminases were used as the control. All patients and controls were negative for the antibody to HIV. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent. All serum samples were immediately stored at −70°C and remained in storage until testing.

Definition of treatment outcome

An SVR was concluded in those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Post-treatment relapse was defined as the reappearance of HCV RNA in the serum after treatment in patients whose HCV RNA was undetectable during or at the completion of therapy. A non-response was defined as a decrease in HCV RNA to less than 2 log copies/mL at week 12 and detectable HCV RNA during the treatment course.

Detection of amino acid substitutions in core and interferon-sensitivity determining regions (ISDR)

The sequences of 1–191 amino acids (a.a.) in the core protein and 2209–2248 a.a. in the NS5A region of genotype 1b HCV were determined by direct sequencing using stored serum samples obtained before therapy, as reported previously. Nucleotide and a.a. sequences were compared with the nucleotide sequences of genotype 1b HCV-J.[10] Substitutions of a.a.70 arginine (Arg70) and glutamine (Gln70) or a.a.91 leucine (Leu91) and methionine (Met91)[11] and the number of a.a. substitutions in the ISDR were defined as wild-type (0), intermediate-type (1) and mutant-type (≥2).[12] Of the 79 patients, 75 were determined to have substitutions at a.a.70 and a.a.91, and 76 could be sequenced for their ISDR.

Detection of chemokines

Six chemokines (macrophage inflammatory protein [MIP]-1α, MIP-1β, eotaxin, IP-10, RANTES and interleukin [IL]-8) were quantified using Luminex Multiplex Cytokine Kits (Procarta Cytokine assay kit; Panomics, Fremont, CA, USA) from serum samples obtained before the start of treatment, 4 weeks after the start of treatment and 24 weeks after the completion of treatment, according to the manufacturer's instructions.[13]

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to analyze continuous variables as appropriate. The Wilcoxon rank sum test and the Friedman test were used to evaluate changes in serum chemokine levels over time. Spearman's rank correlation coefficients were used to evaluate the relationship between each pair of markers. The χ^2-test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was less than 5, Fisher's exact test was used. $P \leq 0.05$ was considered significant. To predict treatment outcome, each cut-off point for continuous variables was determined by receiver–operator curve (ROC) analysis. Statistical analyses were performed using SPSS ver. 18.0J.

RESULTS

OF THE 79 patients receiving PEG IFN and ribavirin therapy, 31 (39%) achieved an SVR, 23 (29%) relapsed, and 25 (32%) did not respond to treatment and were termed null viral responders (NVR). When stratified into three groups based on treatment outcome, patients with an NVR had a higher female ratio ($P = 0.030$) (Table 1). Before treatment, the median AST and γ-GTP levels in the SVR group were significantly lower than those in the relapsed and NVR groups. Substitutions of a.a.70 in the core region

Table 1 Clinical characteristics of patients with chronic hepatitis C

Characteristic	SVR ($n = 31$)	TR ($n = 23$)	NVR ($n = 25$)	P
Mean age, years (range)	55 (28–72)	57 (17–71)	59 (22–74)	0.20
Sex, male : female	23:8	9:14	8:17	0.030
Mean values (range)				
ALT (IU/L)	58 (24–172)	76 (24–389)	90 (22–357)	0.43
AST (IU/L)	41 (21–133)	57 (20–218)	78 (25–288)	0.042
γ-GTP (IU/L)	40 (13–147)	47 (12–167)	81 (17–439)	0.027
HCV RNA (10^3 IU/mL)	1962 (110–>5100)	2379 (360–>5100)	1934 (220–>5100)	0.23
Substitutions				
Core a.a. 70 (Arg70/Gln70)	22/6	14/8	11/14	0.034
Core a.a. 91 (Leu91/Met91)	20/8	17/5	17/8	0.78
ISDR of NS5A (0–1/≥ 2)	20/9	20/2	23/2	0.040

a.a., amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; ISDR, interferon-sensitivity determining region; NVR, null virological response; SVR, sustained virological response; TR, transient response; γ-GTP, γ-glutamyl transpeptidase.

($P = 0.034$) and in the ISDR ($P = 0.040$) were both significantly associated with treatment outcome. Six serum chemokines were assessed before therapy in all patients and in 26 healthy controls, revealing that the median serum levels of eotaxin, IP-10 and RANTES were significantly higher in HCV-afflicted patients. The median serum IL-8 level in cases with chronic HCV infection was significantly lower compared with the control group (Table 2).

The median serum chemokines of our cohort are shown in Table 3. Before treatment, the median serum levels of three chemokines (eotaxin, MIP-1β and RANTES) were significantly higher in patients who achieved an SVR than in those who did not. Patients with a virological response had significantly higher MIP-1α (39.0 vs 25.9 pg/mL; $P = 0.001$) and MIP-1β (192.7 vs 110.0 pg/mL; $P < 0.001$) compared with non-responders.

Table 2 Serum chemokines in patients with chronic hepatitis C and healthy controls

Chemokine	Chronic hepatitis C ($n = 79$)	Control ($n = 26$)	P
MIP-1α	36.4 (2.4–5021.3)	34.6 (10.6–92.8)	0.46
MIP-1β	160.2 (14.4–3341.6)	122.3 (21.1–1677.6)	0.18
Eotaxin	100.0 (2.4–1296.0)	19.8 (18.3–25.0)	<0.001
IP-10	1642.8 (57.7–11 487.0)	31.1 (21.3–80.6)	<0.001
RANTES	31 755.3 (17.9–83 248.0)	3460.0 (191.5–40 001.0)	<0.001
IL-8	12.9 (2.4–6324.3)	41.8 (2.4–327.6)	<0.001

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1.

Table 3 Serum chemokines in treatment outcome to antiviral therapy

Chemokine	SVR ($n = 31$)	TR ($n = 23$)	NVR ($n = 25$)
MIP-1α	36.4 (32.3–99.3)	39.0 (33.7–52.9)	25.9 (17.7–40.8)
MIP-1β	264.4 (176.3–371.6)	155.0 (112.1–300.0)	110.2 (81.0–150.2)
Eotaxin	107.0 (66.9–180.4)	44.8 (28.1–87.6)	120.1 (50.7–234.5)
IP-10	1964.4 (956.4–5485.4)	1088.2 (818.6–2006.4)	1879.8 (653.4–2969.0)
RANTES	83 248.0 (31 755.3–83 248.0)	8633.4 (3469.1–22 498.6)	30 970.8 (3638.7–83 248.0)
IL-8	12.5 (8.7–24.2)	10.6 (2.4–17.8)	13.6 (12.1–15.2)

Data are expressed as median (interquartile range) values (pg/mL).
NVR, null virological response; SVR, sustained virological response; TR, transient response.