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

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

IL28B encodes a protein also known as IFN-λ3 [O'Brien, 2009]. *IL28A* (IFN-λ2) and *IL29* (IFN-λ1) are found adjacent to *IL28B* on chromosome 19. These three IFN-λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al., 2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN-λ, and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN-λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN-α induces expression of IFN-λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN-λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN-α and that the anti-HCV activity of either IFN-α or IFN-λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN-α and IFN-λ may play a key role in the suppression of HCV [O'Brien, 2009].

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

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Detection of Hepatitis C Virus and Antibodies in Postmortem Blood and Bloodstains▽

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To evaluate the risk of accidental hepatitis C virus (HCV) infection, we examined whether anti-HCV antibodies and HCV RNA were detectable in HCV-infected blood samples from living donors, cadavers, and bloodstains. We showed that even after blood has left the body for several days, anti-HCV antibodies and HCV RNA may persist in it.

At the scenes of crimes or accidents, bloody materials are often handled without adequate precautions against infection because most first responders are not medical specialists. This study's objective was to test whether or not such samples are no longer potentially infectious. We used hepatitis C virus (HCV) as the representative infectious agent. To simulate the types of exposures first responders might encounter, we tested whether HCV RNA and antibodies are detected in blood and bloodstains kept at room temperature for up to 60 days and from blood of actual postmortem cases up to 14 days after death.

HCV-infected blood samples were obtained with informed consent from 12 patients (8 men and 4 women; mean age, 68.5 ± 10.7 years; range, 44 to 84 years) at the University Hospital, Kyoto Prefectural University of Medicine, and at Aiseikai Yamashina Hospital. Prior to our experiments, the serum titers of HCV RNA of all samples were determined, using the COBAS TaqMan HCV assay (Roche Molecular Systems, Pleasanton, CA), to range from 5.4 to 7.0 log IU/ml (average, 6.363 ± 0.42 log IU/ml). All samples were stored at −80°C until use.

Bloodstain samples were prepared by soaking cotton buds in 0.1 ml of HCV-infected whole-blood samples ($n = 8$) for 1 min and then drying them at room temperature for up to 60 days. Samples of HCV-infected whole blood ($n = 4$) were placed in sealed 2-ml test tubes and kept at room temperature for up to 60 days. The prepared blood and bloodstain samples were analyzed at 1, 3, 9, 27, and 60 days after preparation.

The postmortem whole-blood samples were obtained between December 2008 and April 2010 from 10 forensic autopsies performed on individuals (7 men and 3 women; mean age, 52 ± 13.15 years; range, 33 to 79 years) who had tested positive for anti-HCV antibodies. These blood samples were stored at −80°C for a week before use.

Anti-HCV antibodies from the bloodstain and whole-blood samples were detected using immunochromatography with Ortho Quick Chaser HCV antibody (Ortho Clinical Diagnostics, Tokyo, Japan). Before testing, the bloodstain samples were soaked in 200 μl saline; 100 μl of extracted solution was analyzed using immunochromatography.

HCV RNA was extracted from 100 μl of undiluted whole blood and 100 μl of solution extracted from blood-stained materials with a QIAamp viral RNA kit (Qiagen, Hilden, Germany). The RNA was eluted in 50 μl of RNase-free water and used for genome amplification of the partial core region using reverse transcriptase PCR (RT-PCR) with a One Step RT-PCR kit (Qiagen) in 50-μl aliquots containing 1 μl RNA, 2 μl Qiagen One Step RT-PCR enzyme mix, 400 μM deoxynucleoside triphosphate (dNTP), 0.6 μM concentrations of primers 256 (5′-CGCGCGACTAGGAAGACTTC-3′; sense) and 186 (5′-ATGTACCCCATGAGGTCGGC-3′; antisense), and Qiagen One Step RT-PCR buffer supplied by the manufacturer. The amplification was performed as described by Okamoto et al. (16). Reverse transcription was performed at 50°C for 30 min. DNA polymerase was initially activated at 95°C for 15 min for PCR. PCR amplification was performed for 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final step at 72°C for 10 min. Amplification was carried out in a PC-320 thermal cycler (ASTEC, Fukuoka, Japan). The PCR product was mixed with a 6× loading buffer double dye and subjected to electrophoresis on a 1.5% agarose gel at 100 V for 30 min. The electrophoresed agarose gel was stained with ethidium bromide (0.5 μg/ml). The image from

TABLE 1. Results of anti-HCV antibody and HCV RNA detection in blood and bloodstain samples

Test sample type	No. of positive results after the following days of storage:				
	0	3	9	27	60
Anti-HCV antibody					
Bloodstain	8	8	7	7	5
Blood	4	4	4	4	4
HCV RNA					
Bloodstain	8	8	8	8	7
Blood	4	4	4	4	4

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TABLE 2. Profiles of anti-HCV antibody-positive autopsy cases and HCV RNA detection results

Case	Age (yr)/ sex[a]	Postmortem time (day)	Cause of death	HCV RNA	Genotype
A	52/F	1	Drowning	+	1b
B	42/F	1	Unknown	+	1b
C	33/M	1	Hypothermia	+	2a
D	52/M	1	Drug intoxication	−	
E	46/M	1	Asphyxia	−	
F	45/M	2	Hemorrhage	+	1b
G	48/M	2	Drug intoxication	+	1b
H	62/M	2	Burn	−	
I	65/F	2	Strangulation	−	
J	79/M	14	Unknown	−	

[a] M, male; F, female.

the agarose gel was captured under UV transillumination on a LAS 4000 mini camera system (Fujifilm, Tokyo, Japan).

The limit of HCV detection of the RT-PCR method was 2.06 log IU/ml. This value was extrapolated from the results for five infected serum samples taken from a single serum sample (5.4 log IU/ml using the TaqMan method) that had been diluted to concentrations between ×100 and ×1,600. The genotype of the HCV strain was determined using the putative C gene of the HC-J4 isolate as described previously (16).

The analysis of anti-HCV antibodies and HCV RNA from 8 bloodstain samples and 4 whole-blood samples kept up to 60 days at room temperature is summarized in Table 1. On day 27, anti-HCV antibodies were detected in 7 of 8 bloodstain samples and in all 4 whole-blood samples. HCV RNA was detected in all samples. On day 60, anti-HCV antibodies were detected in 5 of 8 bloodstain samples and in all 4 whole-blood samples. HCV RNA was detected in 7 of 8 bloodstain samples and all 4 whole-blood samples.

Among the 10 anti-HCV antibody-positive autopsy blood samples, HCV RNA was detected in 5 samples (Table 2, cases A, B, C, F, and G). The genotype of the HCV isolated in case C was 2a, and that of the others was 1b.

The detection of HCV RNA and anti-HCV antibodies in these specimens does not prove that HCV could be transmitted to humans. Previous studies have demonstrated that the RNA of the entire HCV genome synthesized *in vitro* can infect chimpanzees and produce the progeny virus (10). However, although some models and tissue culture systems have been developed (e.g., replicon systems [11], JFH-1 cells [18], and immune-deficient mice [15]), infection and cultivation of wild-type HCV have not yet been successful in model systems. Therefore, we are unable to directly test whether the samples used in this study could infect human cells. Although these results do not prove that the samples were infectious, they highlight the need for first responders and law enforcement personnel to exercise caution when handling bloody materials, even if not fresh.

The results of our study may have an additional application. Recently, the number of unidentified cadavers has increased worldwide (3). The geographic distribution of various viruses has been used to determine the geographic origins cadavers (5–9). The worldwide distribution of HCV genotypes has also been reported (1, 2, 4, 12–14, 17, 19). In the present study, all of the samples were taken from Japanese individuals, and the viral genotypes were 1b and 2a, which are commonly detected in Japan. Therefore, it may be possible to estimate the geographic origin of a cadaver or bloodstain from the HCV viral genotype, if present. Further studies are necessary to confirm this hypothesis.

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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)

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

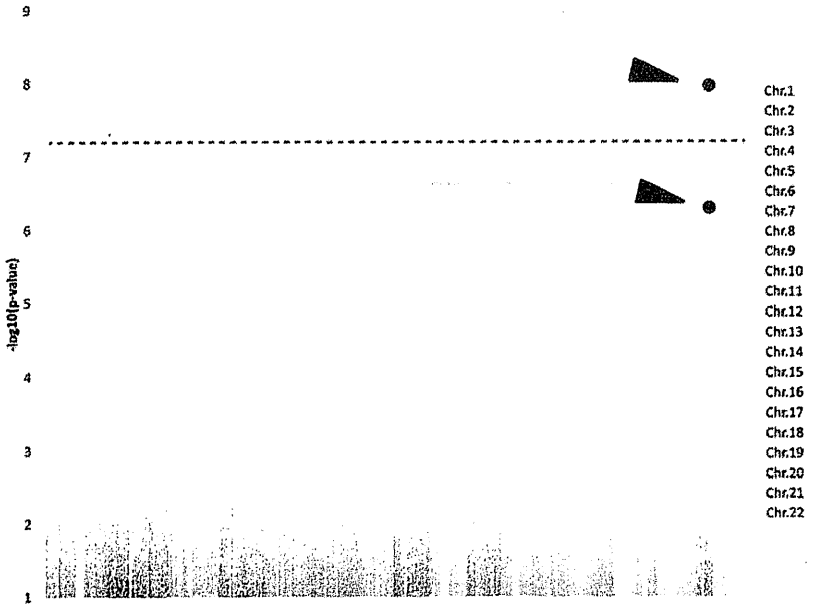

Figure 1. Genome-wide association results in 303 Japanese HCV patients with the decrease of platelets in response to PEG-IFN plus RBV treatment (107 patients with the decrease of PLT and 196 patients without the decrease of PLT). *P*-values were calculated using the χ^2 test for allele frequencies. Dots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

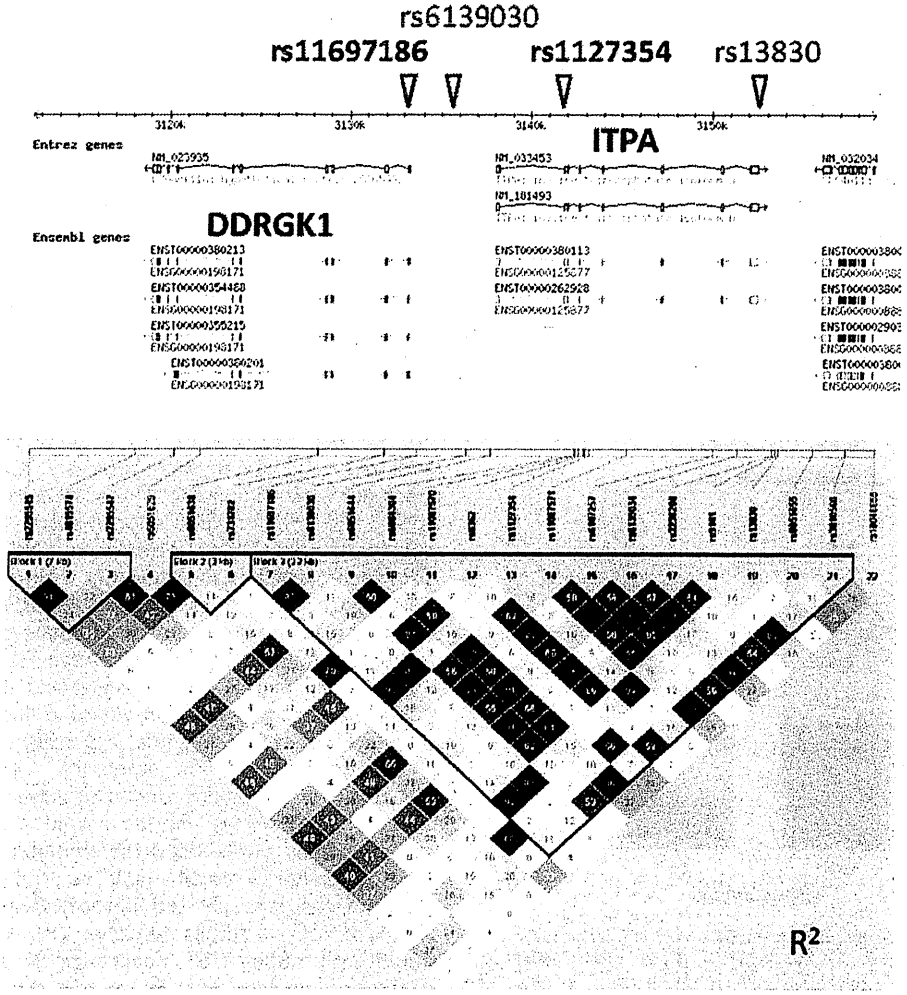

Figure 2. Pairwise LD (r^2) diagrams for *DDRGK1* and *ITPA*. Lower panel shows estimates of pairwise r^2 for 22 SNPs selected in the replication study using the second set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment.

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).

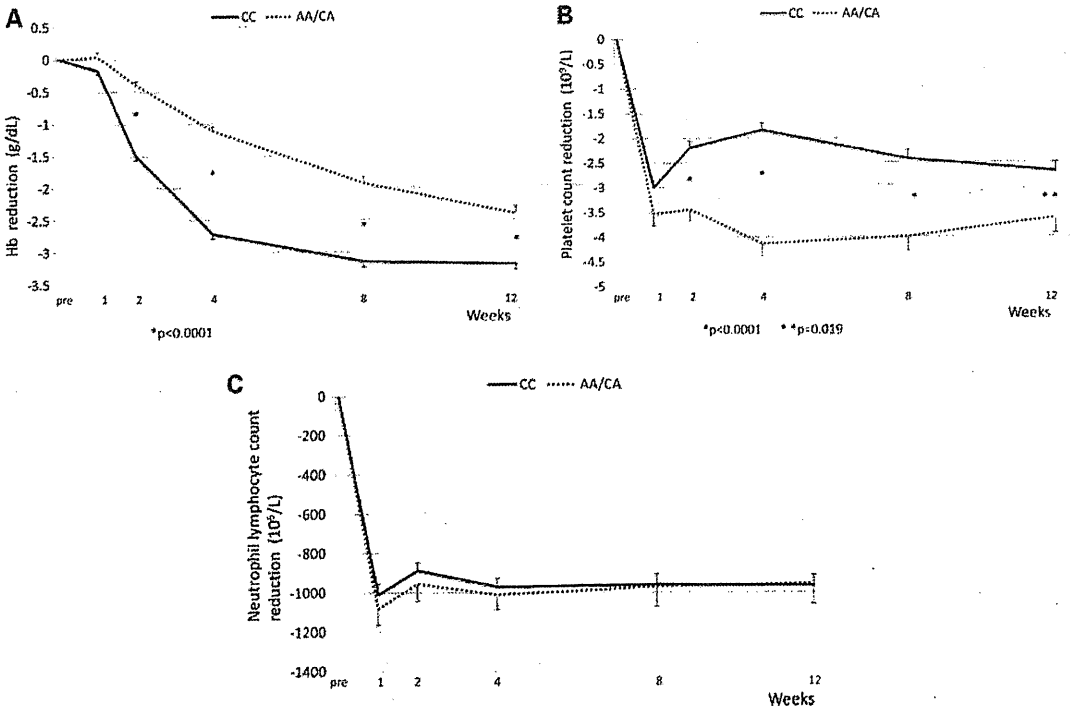

Figure 3. *ITPA* rs1127354 genotypes and the quantitative reduction of blood cells from baseline. Mean reduction of (**A**) Hb levels, (**B**) platelet counts and (**C**) neutrophil leukocyte counts during treatment according to rs1127354 genotype is shown. Solid and dotted lines indicate patients with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

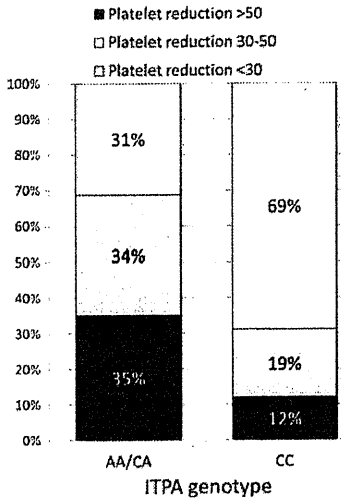

Figure 4. *ITPA* rs1127354 genotypes and reduction of platelet counts at week 4 of PEG-IFN/RBV therapy. The percentage of patients with platelet count reduction of >50 (10^9/l) (black bar), 30–50 (10^9/l) (gray bar) and <30 (10^9/l) (white bar) at week 4 is shown for rs1127354 genotypes. The incidence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

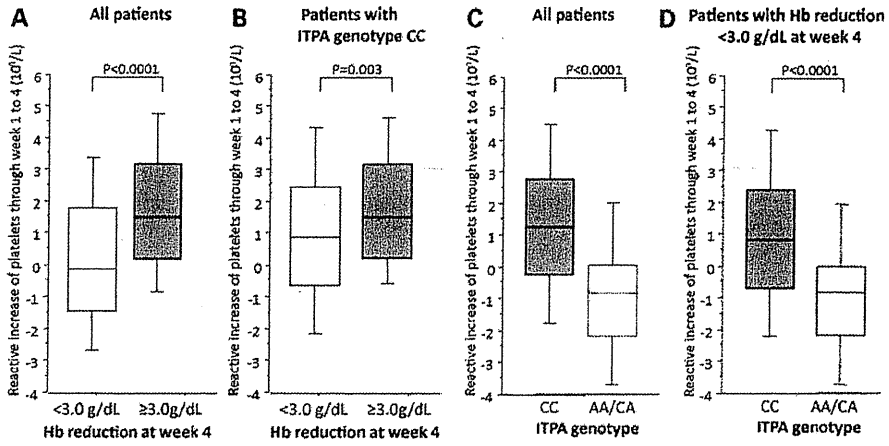

Figure 5. Reactive increase of platelet counts through weeks 1–4. Box plots of reactive increase of platelet count through weeks 1–4 according to the degree of anemia at week 4 are shown for all patients (**A**) and a subgroup of patients with the rs1127354 genotypes CC (**B**). Patients with anemia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

Hepatic steatosis in chronic hepatitis C patients infected with genotype 2 is associated with insulin resistance, hepatic fibrosis and affects cumulative positivity of serum hepatitis C virus RNA in peginterferon and ribavirin combination therapy

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Aim: Hepatic steatosis is one of the factors limiting the virological response to interferon-based antiviral therapy for chronic hepatitis C (CH-C) patients infected with genotype 1, while contradictory results have been reported for genotype 2. We aimed to clarify the effect of hepatic steatosis on therapeutic outcome and cumulative positivity of serum HCV RNA in CH-C patients infected with genotype 2 treated by peginterferon (PEG-IFN)α2b and ribavirin (RBV) combination therapy.

Methods: A total of 74 treatment-naïve non-cirrhotic CH-C patients infected with genotype 2 who received PEG-IFNα2b and RBV according to the standard regimen were divided into hepatic steatosis 0–10% and >10% groups. The clinical backgrounds, sustained virological response (SVR) rates and cumulative positivity of serum HCV RNA were compared between the two groups.

Results: Among the 74 patients, 61 (82.4%) had hepatic steatosis 0–10% and 13 (17.6%) had hepatic steatosis >10%. Scores of homeostasis model assessment-insulin resistance and hepatic fibrosis were higher in patients with hepatic steatosis >10% than hepatic steatosis 0–10% ($P = 0.040$ and 0.042, respectively). Non-SVR was more frequent in patients with hepatic steatosis >10% than hepatic steatosis 0–10% ($P = 0.003$). Cumulative positivity of serum HCV RNA was significantly higher in patients with hepatic steatosis >10% than hepatic steatosis 0–10% ($P = 0.004$).

Conclusions: In CH-C patients infected with genotype 2 treated by PEG-IFNα2b and RBV combination therapy, hepatic steatosis >10% was associated with increased insulin resistance, advanced hepatic fibrosis and higher cumulative positivity of serum HCV RNA, which lead to a higher risk of non-SVR.

Key words: chronic hepatitis C, genotype 2, hepatic fibrosis, hepatic steatosis, homeostasis model assessment-insulin resistance

INTRODUCTION

WITH ADVANCES IN the practice of clinical hepatology, around 50% of chronic hepatitis C (CH-C) patients infected with genotype 1, and more than 80% of genotype 2, can be cured by peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy.[1,2] However, although PEG-IFN and RBV combination therapy is a powerful tool for the treatment of CH-C patients infected with genotype 2 worldwide, a significant number remain viremic.

Because of the high rate of sustained virological response (SVR) to PEG-IFN and RBV combination therapy in CH-C patients infected with genotype 2, the clinical backgrounds associated with resistance to therapy have not been evaluated in detail. Previously, CH-C patients infected with genotype 2 who had

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hepatic steatosis were reported to be resistant to interferon (IFN) monotherapy[3,4] and PEG-IFN and RBV combination therapy,[5] but several recent publications have contradicted these earlier reports.[6,7]

A recent trend in PEG-IFN and RBV combination therapy for CH-C is response-guided therapy. Several trials have evaluated short courses of treatment for CH-C patients infected with genotype 2 or genotype 3 with a rapid virological response (RVR) but controversial results also have been reported.[8–13] Recent papers on genotype 2/3 patients reported that low platelet counts and a body mass index (BMI) of 30 or higher are associated with relapse of RVR after a short regimen of 12 weeks,[14] and a body weight greater than 75 kg also affected the probability of relapse,[15] whereas the impact of hepatic steatosis on the anti-viral effect was not fully studied. In the present study, we investigated the effects of hepatic steatosis on clinical backgrounds of CH-C patients infected with genotype 2. Specifically, we investigated the impact of hepatic steatosis on cumulative positivity of serum HCV RNA during PEG-IFN and RBV combination therapy and therapeutic outcome.

METHODS

Patients

THIS STUDY WAS conducted at the university hospital of Kyoto Prefectural University of Medicine, Kyoto, Japan and related hospitals in Kinki Area (Kyoto, Osaka, Nara, Shiga Prefecture). The study protocol was approved by the ethical committee of each institution in 2005. Written informed consent was obtained from all patients before treatment. Enrollment of the patients began in January 2006 and ended in December 2008 and the follow up study was completed in August 2009. Patients with liver cirrhosis, co-infection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV), autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Patients with uncontrollable hypertension or diabetes mellitus, BMI ≥30 kg/m^2, and those with a history of alcohol abuse also were excluded. The criteria for enrollment were platelet count (PLT) greater than 10×10^4/mm^3, neutrophil count greater than 1100/mm^3, and hemoglobin concentration greater than 11.0 g/dL.

Among the 136 patients enrolled, 10 dropped out because of severe adverse effects and eight were lost to follow up. Forty-four patients without liver biopsy also were excluded. Finally, 74 eligible and previously untreated Japanese CH-C patients infected with genotype 2, aged 27 to 73 years and who had high viral loads (≥100 KIU/mL by the Amplicor HCV RNA kit, version 2.0, Roche Diagnostics, Tokyo, Japan), were studied.

All 74 patients had provided a liver biopsy within a year prior to treatment. Liver biopsy was performed using a Sure-cut needle guided by ultrasound. Liver biopsy specimens were fixed in 10% formalin and stained with hematoxylin and eosin and Masson's trichrome. Histopathological diagnosis was based on the scoring of New Inuyama classification.[16] The fibrosis scores were F0: no fibrosis, F1: portal fibrous widening, F2: portal fibrous widening with bridging fibrosis, F3: bridging fibrosis plus lobular distortion. The inflammation scores were A0: none to minimal, A1: mild, A2: moderate, A3: severe. Evaluation of the percentage of hepatic steatosis was carried out by two expert hepatologists who were blinded to the treatment outcome of each patient and classified as ≥0% and ≤1%, >1% and ≤5%, >5% and ≤10%, >10% and ≤15%, >15% and ≤20%, >20% and ≤25%, >25% and ≤30%.

Study design

All patients received weekly injections of PEG-IFNα2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ, USA) of 1.5 μg/kg.bw and oral administration of RBV (Rebetol; Shering-Plough) of 600 to 1000 mg/day according to the 24 week standard regimen. The amount of RBV was adjusted based on body weight (bw): 600 mg for <60 kg.bw, 800 mg for ≥60 kg.bw and <80 kg.bw, 1000 mg for ≥80 kg.bw. The dose of PEG-IFNα2b was decreased by 50% when the PLT count fell below 8×10^4/mm^3 or the neutrophil count fell below 750/mm^3. The dose of RBV was lowered by 200 mg/day when the hemoglobin concentration fell below 10 g/dL. The full dose was reinstated when the adverse events improved.

Hepatitis C virus RNA negativity at treatment week 4, based on a qualitative polymerase chain reaction (PCR) assay, was defined as RVR. HCV RNA negativity at 24 weeks after the cessation of combination therapy was defined as SVR. Those who failed to attain SVR were defined as non-SVR patients. All patients were examined serially (at 2, 4, 8, 12, 24 weeks) by qualitative HCV RNA assays and again 24 weeks after termination of the therapy.

Statistical analysis

All data analyses were carried out using the SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL, USA). Individual characteristics were compared between the groups using the Mann-Whitney *U*-test or Fisher's exact test. For some variables, receiver operating characteristic analysis was performed followed by proper cat-

egorization of the data. Cumulative positivity of serum HCV RNA was calculated by the Kaplan–Meier method and analyzed by the log-rank test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Evaluation of hepatic steatosis in chronic hepatitis C patients infected with genotype 2

RECEIVER OPERATING CHARACTERISTIC curve analysis was performed to examine the relationship between hepatic steatosis and SVR (Fig. 1). Because the sum of sensitivity and specificity was maximum when the hepatic steatosis was 11.50% (data not shown), we classified the patients into hepatic steatosis 0–10% and hepatic steatosis >10% groups. Based on these results, the degree of hepatic steatosis was classified into seven categories from ≥0% and ≤1% to >25% and ≤30% (Fig. 2) and presented separately for the SVR and non-SVR groups (Table 1).

Correlation between hepatic steatosis and clinical and other histological features

The baseline characteristics of the 74 patients (31 male and 43 female) with chronic hepatitis C infected with genotype 2 are shown in Table 2. There were no significant differences between the patients with hepatic steatosis 0–10% and hepatic steatosis >10% in clinical backgrounds such as gender, age, baseline HCV RNA load and other laboratory data, except that homeostasis model assessment-insulin resistance (HOMA-IR) was significantly ($P = 0.040$) higher in patients with hepatic steatosis >10%. BMI and γ-glutamyl transferase (γ-GTP) also tended to be higher in patients with hepatic steatosis >10%; however, statistical significances were not demonstrated ($P = 0.052$ and $P = 0.050$, respectively). The scores of hepatic fibrosis, but not of hepatic inflammation, were significantly ($P = 0.042$) higher in patients with hepatic steatosis >10% (Table 3). Although we investigated the factors associated with hepatic steatosis >10% by multivariate regression analysis, neither HOMA-IR nor hepatic fibrosis were independently associated with it.

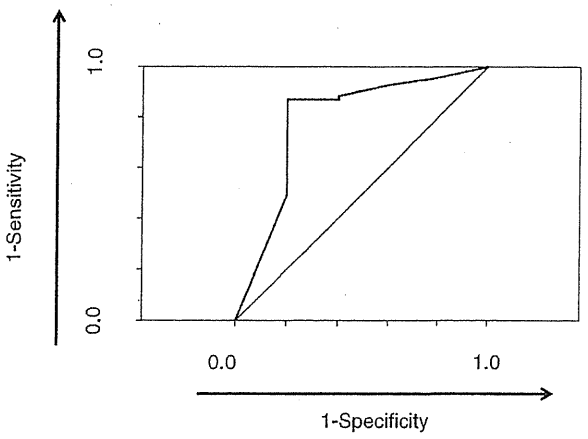

Figure 1 Receiver operating characteristic curve analysis of hepatic steatosis and sustained virological response (SVR). Receiver operating characteristic curve analysis was performed to examine the relationship between hepatic steatosis and SVR in all patients. Because the sum of sensitivity and specificity was maximum when the hepatic steatosis was 11.50% (data not shown), we classified the patients into hepatic steatosis 0–10% and hepatic steatosis >10% groups.

Table 1 Hepatic steatosis in chronic hepatitis C patients infected with genotype 2

Hepatic steatosis (%)	No. patients		
	SVR	non-SVR	Total
–1%	34	1	35
–5%	17	0	17
–10%	9	0	9
–15%	1	1	2
–20%	3	1	4
–25%	2	1	3
–30%	3	1	4
	69	5	74
IL28B major homo/ hetero (total)	10/0 (10)	3/2 (5)	

The degree of hepatic steatosis was classified as ≥0% and ≤1%, >1% and ≤5%, >5% and ≤10%, >10% and ≤15%, >15% and ≤20%, >20% and ≤25%, >25% and ≤30% and presented for the SVR and non-SVR groups.
SVR, sustained virological response.

Impact of hepatic steatosis on therapeutic response

The relationship between hepatic steatosis and SVR or RVR ratio is shown in Table 3. The SVR ratio of patients with hepatic steatosis 0–10% was significantly ($P = 0.003$) higher than that of patients with hepatic steatosis >10% and the RVR ratio of patients with hepatic steatosis 0–10% also tended to be higher; however, statistical significance was not demonstrated ($P = 0.055$). This result tempted us to investigate the impact of hepatic steatosis on the cumulative positivity of serum HCV RNA in the early phase of PEG-IFNα2b and RBV combination therapy.

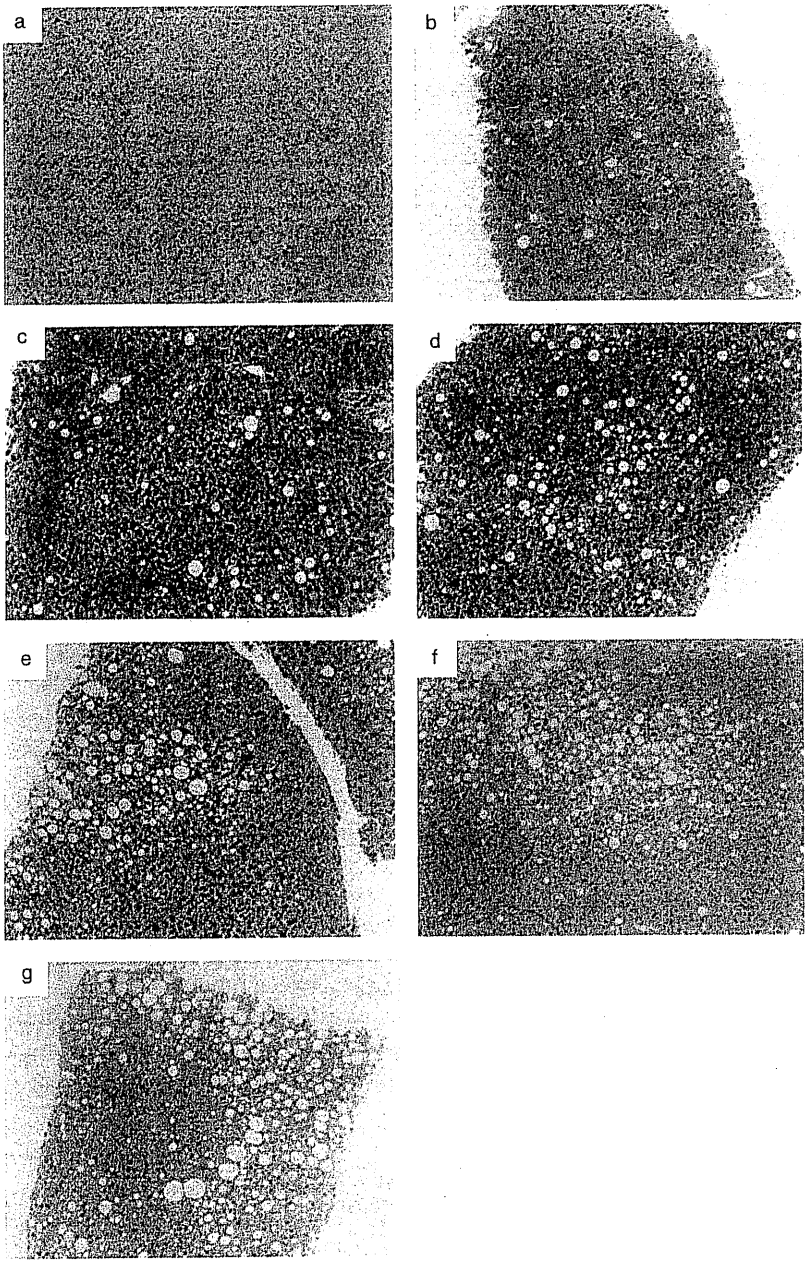

Figure 2 Histological classification of hepatic steatosis. Typical histological classification was presented. The degree of hepatic steatosis was classified as ≥0% and ≤1% (a), >1% and ≤5% (b), >5% and ≤10% (c), >10% and ≤15% (d), >15% and ≤20% (e), >20% and ≤25% (f), >25% and ≤30% (g) by two hepatologists who were blinded to the treatment outcome of each patient.

Impact of hepatic steatosis on cumulative positivity of serum HCV RNA

Here, we divided the patients based on receiver operating characteristic curve analysis and demonstrated that hepatic steatosis >10% was associated with resistance to PEG-IFNα2b and RBV combination therapy for CH-C patients infected with genotype 2. To support this finding, we compared the cumulative positivity of serum HCV RNA between the patients with hepatic steatosis 0–10% and those with hepatic steatosis >10% in the early phase of PEG-IFNα2b and RBV combination therapy using the Kaplan–Meier method.

The patients with hepatic steatosis >10% had a significantly ($P = 0.004$) higher cumulative positivity of HCV RNA than the patients with hepatic steatosis

Table 2 Relationship between hepatic steatosis (0–10% or >10%) and the clinical backgrounds of the patients

Hepatic steatosis	>10% (13) Median [range]	0–10% (61) Median [range]	*P*-value
Genotype 2a/2b/N.D.	5/6/2	28/13/10	0.148
Gender (M/F)	5/8	26/35	0.782
Age (years)	56 [33.0–70.0]	54 [27.0–73.0]	0.430
BMI (kg/m^2)	23.9 [19.9–28.8]	22.0 [17.5–29.6]*	0.052
HCVRNA (KIU/mL)	1850 [120–5000]†	1300 [130–5000]†	0.439
Hb (g/dL)	14.5 [11.8–15.1]	13.9 [11.3–17.4]	0.691
Plt (×10^4/mm^3)	19.2 [10.6–24.0]	18.9 [10.4–37.9]	0.551
WBC (×10^3/mm^3)	5.20 [3.33–7.40]	4.90 [2.30–9.40]	0.612
ALT (IU/L)	78 [16.0–248]	47 [15.0–377]	0.125
rGTP (IU/L)	56 [19–285]	23 [8–158]*	0.050
T-chol (mg/dL)	187 [130–250]	178 [108–274]	0.227
Feritin (ng/dL)	150 [22–1107]	103 [20–664]‡	0.330
HOMA-IR	4.6 [1.5–6.5]	2.0 [0.3–11.3]*	0.040
Creatinine (mg/dL)	0.6 [0.5–0.9]	0.7 [0.3–1.1]	0.172

The clinical backgrounds of the 74 patients were compared by Mann–Whitney *U*-test or Fisher's exact test. Continuous variables are presented as medians (ranges). Individual characteristics between the groups were evaluated using the Mann–Whitney *U*-test. All patients with >10% hepatic steatosis were evaluated for each parameter. In patients with 0–10% hepatic steatosis *60 patients were evaluated for body mass index (BMI), 56 patients for γ-glutamyl transferase (γ-GTP) and homeostasis model assessment-insulin resistance (HOMA-IR). †The upper limit of measurement is 5000 KIU/mL. ‡The lower limit of measurement is 20 ng/dL.
ALT, alanine aminotransferase; F, female; Hb, hemoglobin; HCV, hepatitis C virus; M, male; N.D., not determined; PLT platelet count; T-chol, total cholesterol; WBC, white blood cell count.

0–10% (Fig. 3). To exclude the possibility that adherence to PEG-IFNα2b or RBV influenced the changes in serum HCV RNA in this study, we examined the cumulative positivity of serum HCV RNA in patients who achieved ≥80% adherence (as a percentage of the expected total dose) to both PEG-IFNα2b and RBV. Patients with hepatic steatosis >10% also had significantly ($P = 0.045$) higher cumulative positivity of serum HCV RNA than those with hepatic steatosis 0–10% (data not shown). The SVR ratio of patients with ≥80% adherence to both PEG-IFNα2b and RBV did not differ significantly from those without (data not shown).

DISCUSSION

PREVIOUS PAPERS[3–5] REPORTED that hepatic steatosis affected the efficacy of IFN-based therapy for CH-C patients infected with genotype 2, whereas recent papers by Poustchi *et al.*[6] and Rodriguez-Torres *et al.*[7] denied it. Because steatosis was roughly graded (for example, less than 5%, 5–33%, 34–66%, and more than 67%) in these studies, we considered that tight grading of hepatic steatosis may lead to a different result. As we have expected, in the present study, tight grading of hepatic steatosis revealed a close relation between

Table 3 Relationship between hepatic steatosis (0–10% or >10%) and other histological features and therapeutic response

Hepatic steatosis	>10%	0–10%	*P*-value
Hepatic inflammation (0,1/2,3)	5/8	37/24	0.143
Hepatic fibrosis (0,1/2,3)	5/8	42/19	0.042
Hepatic iron deposit Yes/No/N.D.	3/5/5	16/39/6	0.455
Rapid virological response Yes/No	6/7	45/16	0.055
Sustained virological response Yes/No	9/4	60/1	0.003

The histological findings of the 74 patients were evaluated according to the scoring system of New Inuyama classification.[16] Histological features such as inflammation, fibrosis and iron deposition, were compared between the hepatic steatosis 0–10% and >10% groups by Fisher's exact test. The ratio of rapid virological response and sustained virological response were compared between the hepatic steatosis 0–10% and >10% groups by Fisher's exact test.
N.D., not determined.

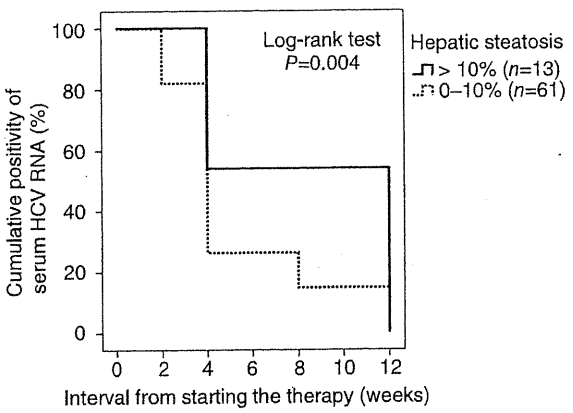

Figure 3 Cumulative positivity of serum hepatitis C virus (HCV) RNA during the early phase of pegylated interferon (PEG-IFN)α2b and ribavirin (RBV) combination therapy. Cumulative positivity of serum HCV RNA as determined by qualitative HCV RNA assay was compared between the hepatic steatosis >10% and hepatic steatosis 0–10% groups using the Kaplan–Meier method, and then analyzed by log-rank test. A *P*-value of <0.05 was considered to be statistically significant. (⌐¬): >10% ($n = 13$); (···): 0–10% ($n = 61$).

hepatic steatosis and HOMA-IR, hepatic fibrosis, and especially, cumulative positivity of serum HCV RNA during the PEG-IFN/RBV combination therapy, which was not studied as far as we investigated.

In response-guided therapy for hepatitis C, early disappearance of HCV RNA from the serum is the best predictor of SVR.[17] Therefore, our finding that >10% hepatic steatosis affected the cumulative positivity of serum HCV RNA during the PEG-IFN/RBV combination therapy for genotype 2 infected patients is useful to establish more accurate prediction of therapeutic outcome. So, we think that evaluation of hepatic steatosis before PEG-IFN/RBV combination therapy is very important.

The percentage of patients with hepatic steatosis >10% varies according to the papers; however, our result that patients with hepatic steatosis >10% was 17.6% (13/74) do not seem to be extraordinary considering a report by Rodriguez-Torres *et al.*[7] or a recent paper by Kurosaki *et al.*[18] Our results showing that hepatic steatosis >10% was associated with higher HOMA-IR and advanced hepatic fibrosis (Tables 2,3) are reasonable, judging from previous reports.[19,20] While the number of non-SVR patients was small, hepatic steatosis >10% was associated with resistance to PEG-IFN and RBV combination therapy (Table 3). To support this finding, we compared the cumulative positivity of serum HCV RNA between the patients with hepatic steatosis >10% and ≤10% and found that it was significantly ($P = 0.004$) higher in patients with hepatic steatosis >10%, compared to those without.

Patton *et al.*[20] demonstrated first that hepatic steatosis reduced the likelihood of achieving SVR in CH-C patients infected with genotype 1. This finding was confirmed by Lok *et al.*[21] in non-diabetic patients. Our result that hepatic steatosis >10% was associated with resistance to combination therapy in CH-C patients infected with genotype 2 is supported by the report by Poynard *et al.*[5] showing that absence of hepatic steatosis was associated with a higher SVR ratio to PEG-IFN and RBV combination therapy, except for genotype 3. In our study, in addition, increased cumulative positivity of HCV RNA was demonstrated in patients with hepatic steatosis >10% (Fig. 3). We speculate that there is a close relationship between hepatic steatosis >10% and impaired IFN-mediated anti-HCV activity in CH-C patients infected with genotype 2.

It is well known that metabolic diseases such as diabetes mellitus can trigger hepatic steatosis and HCV infection also causes hepatic steatosis by way of increased expression of genes including sterol regulatory element-binding protein 1c.[22] Recently, Vanni *et al.*[23] demonstrated that HCV infected patients with more hepatic steatosis revealed higher intra-hepatic lipid oxidation, which in turn stimulated gluconeogenesis and induced higher suppressor of cytokine signaling-3 (SOCS-3) expression resulting in increased insulin resistance. Because IFN unresponsiveness is, in part, linked to upregulated hepatic expression of SOCS-3 and insulin resistance as shown by increased HOMA-IR,[24] we hypothesize that enhanced hepatic staetosis in CH-C patients infected with genotype 2 may be associated with increased insulin resistance and increased hepatic expression of SOCS-3, which might have interfered with IFN signal transduction pathway.[25] It should be clarified whether or not hepatic steatosis in CH-C patients infected with genotype 2 really links to IFN unresponsiveness through this molecular pathway in the next study.

To exclude the possibility that adherence to PEG-IFNα2b or RBV may have influenced the response to the therapy, we compared the cumulative positivity of serum HCV RNA among the patients with ≥80% adherence to both drugs and demonstrated that patients with hepatic steatosis >10% showed a higher cumulative positive serum HCV RNA (data not shown).

Recently, an interleukin 28B (IL28B) gene polymorphism has been reported to be strongly associated with