

The rollover study ETV-060 was designed to provide open-label entecavir for patients who had completed therapy in the Japanese Phase II program. Patients who completed 24 weeks of treatment in ETV-047 enrolled in ETV-060 and received 0.5 mg entecavir once daily. After 96 weeks of treatment in study ETV-060, patients could complete the study and were eligible to receive commercially available entecavir, which was approved by Japanese health authorities while study ETV-060 was ongoing.

The current analysis describes results for a subset of 33 patients who received lamivudine for 24 weeks in ETV-047 and entecavir 0.5 mg once daily for up to 96 weeks in ETV-060.

#### Efficacy analyses

Efficacy assessments evaluated the proportions of patients who had available samples (non-completer = missing) every 24 weeks through 120 weeks' treatment. Efficacy end points assessed included HBV DNA <400 copies/mL by PCR assay, ALT normalization ( $\leq 1.0 \times \text{ULN}$ ), HBeAg seroconversion among patients who were HBeAg-positive at baseline, and hepatitis B surface antigen (HBsAg) loss. Serum HBV DNA was determined by Roche Amplicor<sup>®</sup> PCR assay (Roche Diagnostics K.K., Tokyo, Japan; limit of quantification = 400 copies/mL) in a central laboratory. Clinical laboratory tests, PCR assays for HBV DNA, and serologic tests for HBV were performed at SRL, Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. On-treatment testing for resistance was carried out using a direct-sequencing PCR method.

#### Safety analyses

Safety analyses include the incidence of adverse events, serious adverse events, laboratory abnormalities, and discontinuations due to adverse events on-treatment throughout treatment in study ETV-060. On-treatment ALT flares were defined as ALT  $>2 \times$  baseline and  $>10 \times$  ULN.

#### Resistance analysis

Resistance testing was performed using a direct-sequencing PCR method. Paired samples from all patients with HBV DNA  $\geq 400$  copies/mL were analyzed for substitutions associated with entecavir or lamivudine resistance at week 96 (72 weeks of entecavir therapy) or week 120 (96 weeks of entecavir therapy). Patients who discontinued therapy prior to week 120 had their last on-treatment sample analyzed. All patients with virologic breakthrough ( $\geq 1 \log_{10}$  increase from nadir on two consecutive measurements) were also tested for resistance.

## Results

### Study population

Of the 34 patients in ETV-047 who received treatment with lamivudine 100 mg once daily for 24 weeks, 33 entered ETV-060 and received treatment with entecavir 0.5 mg once daily. Two patients discontinued treatment during ETV-060: one due to an adverse event (depression) and the other due to insufficient effect. In addition, one patient completed treatment at week 76 (52 weeks of entecavir therapy) after meeting the criteria for protocol-defined complete response (undetectable HBV DNA by PCR assay, undetectable HBeAg and normal serum ALT).

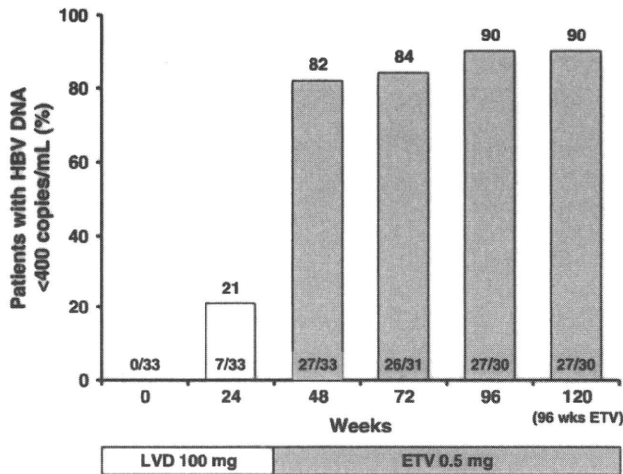
Baseline demographic and disease characteristics for the switch cohort are presented in Table 1. The majority of patients (82%) in the cohort were male with a mean age of 43 years. The mean duration of entecavir therapy was 105.9 weeks (range 25–141 weeks). Baseline mean HBV DNA and ALT levels were 7.9  $\log_{10}$  copies/mL and 184 IU/L, respectively. Ninety-one percent of patients were HBeAg-positive and 88% had HBV genotype C infection.

### Virologic end points

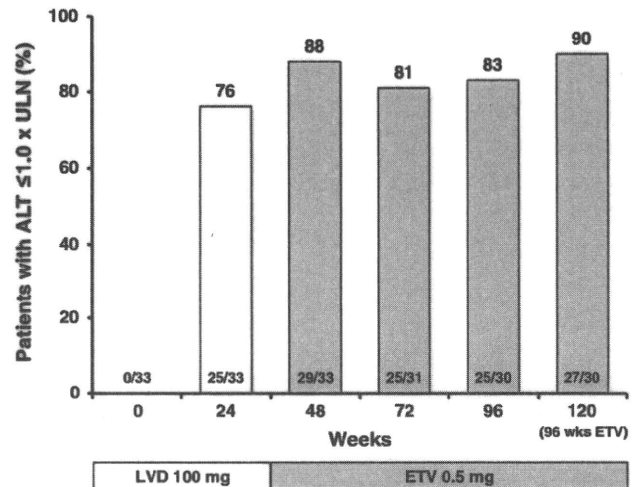
After completion of 24 weeks of lamivudine treatment in ETV-047, 21% (7/33) of patients in the switch cohort had achieved HBV DNA <400 copies/mL (Fig. 1). Following the switch to entecavir, the proportion of patients achieving HBV DNA <400 copies/mL increased to 82% (27/33) by week 48 (24 weeks of entecavir therapy). Viral suppression

**Table 1** Baseline (pretreatment) demographics and disease characteristics: switch cohort

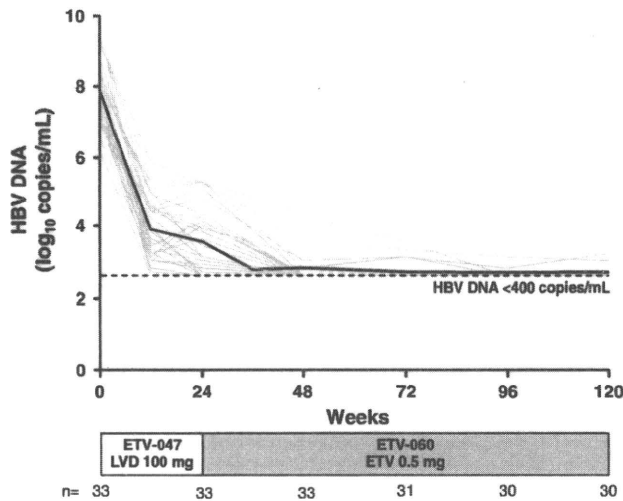
Characteristic	ETV-047/60 lamivudine to entecavir switch cohort (n = 33)
Age, mean (years)	42.7
Male, n (%)	27 (82)
Ethnicity Japanese, n (%)	33 (100)
Entecavir treatment periods, mean (range) (weeks)	105.9 (25–141)
HBeAg-positive, n (%)	30 (91)
HBV DNA by PCR, mean $\log_{10}$ copies/mL (SD)	7.9 (0.80)
ALT (IU/L), mean (SD)	184.8 (132.9)
HBV genotype, n (%)	
A	2 (6)
B	2 (6)
C	29 (88)
Others	0



**Fig. 1** Proportion of patients with HBV DNA <400 copies/mL through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples. *ETV* entecavir, *HBV* hepatitis B virus, *LVD* lamivudine



**Fig. 3** Proportion of patients with ALT normalization ( $\leq 1.0 \times$  ULN) through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples. *ALT* alanine aminotransferase, *ETV* entecavir, *LVD* lamivudine, *ULN* upper limit of normal

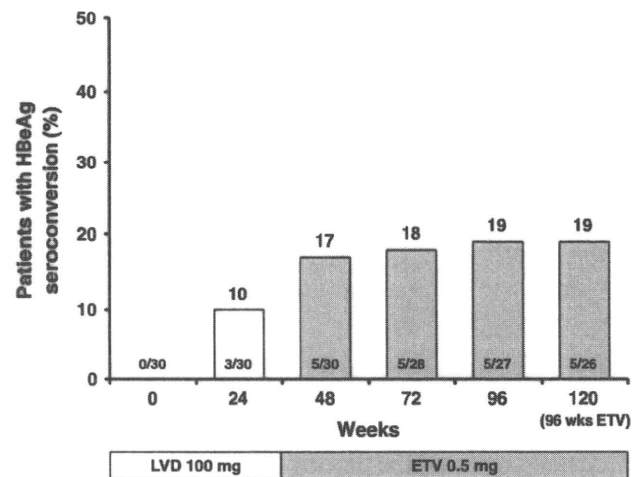


**Fig. 2** HBV DNA suppression through week 120 (96 weeks of entecavir therapy). Individual patient HBV DNA profiles are plotted in gray. Mean HBV DNA levels are represented by the solid black line. *ETV* entecavir, *HBV* hepatitis B virus, *LVD* lamivudine

was maintained with longer entecavir treatment, with 84% (26/31) and 90% (27/30) achieving HBV DNA <400 copies/mL at weeks 72 and 120, respectively (48 and 96 weeks of entecavir therapy). Mean HBV DNA levels decreased from a baseline of 7.90 to 3.52 log<sub>10</sub> copies/mL after 24 weeks of lamivudine therapy in ETV-047, and reached 2.69 log<sub>10</sub> copies/mL after 96 weeks of entecavir therapy in ETV-060 (week 120; Fig. 2). No viral breakthrough was observed during entecavir therapy.

**Biochemical end points**

ALT normalization ( $\leq 1.0 \times$  ULN) was demonstrated in 76% (25/33) of patients after 24 weeks of lamivudine therapy in



**Fig. 4** Proportion of patients with HBeAg seroconversion through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples among the 30 patients HBeAg-positive at baseline. *ETV* entecavir, *HBeAg* hepatitis B e antigen, *LVD* lamivudine

ETV-047 (Fig. 3). Following treatment with entecavir in ETV-060, ALT normalization was maintained in 90% (27/30) of patients achieving this end point by week 120. Minor fluctuations in the proportion of patients achieving ALT normalization were attributed to patients discontinuing entecavir therapy during the course of study ETV-060.

**Serologic end points**

HBeAg seroconversion was assessed among the 30 patients in the switch cohort who were HBeAg-positive at baseline in ETV-047 (Table 1; Fig. 4). Three patients (10%)

achieved HBeAg seroconversion during the initial 24-week lamivudine treatment period in ETV-047 (Fig. 4). Following switch to entecavir in ETV-060, two additional patients developed HBeAg seroconversion by week 120 (96 weeks of entecavir therapy). None of the patients in the switch cohort experienced HBsAg loss during treatment in ETV-047 or ETV-060.

### Resistance

Four of the 33 patients who received entecavir therapy in ETV-060 had HBV DNA  $\geq 400$  copies/mL either at treatment discontinuation or at week 120. One patient discontinued therapy at week 68 (44 weeks of entecavir therapy) due to insufficient effect. HBV DNA prior to treatment discontinuation was  $3.1 \log_{10}$  copies/mL, however, resistance testing revealed no substitutions associated with entecavir resistance. The remaining three patients had HBV DNA  $\geq 400$  copies/mL at weeks 96 and 120; however, only two patients had samples available for testing. Neither patient's samples had substitutions associated with entecavir or lamivudine resistance either at weeks 96 or 120.

### Safety

Entecavir was well tolerated during long-term treatment and the safety profile of patients in the switch cohort was consistent with that previously reported for patients who received continuous entecavir therapy in studies ETV-047 and ETV-060 (Table 2). Serious adverse events (Meniere's disease, subcutaneous abscess and ALT flare) were reported in three patients (9.1%). The most frequently reported adverse events during treatment in ETV-060, occurring in  $\geq 10\%$  of patients, were nasopharyngitis (76%), diarrhea (21%), back pain (18%), influenza (18%), and allergic rhinitis (15%). One patient discontinued entecavir therapy due to depression, which the investigator considered was

possibly related to entecavir therapy. An ALT flare (ALT  $>2 \times$  baseline and  $>10 \times$  ULN) occurred in one patient at week 18, and was judged a serious adverse event by the investigator, but was not associated with a change in HBV DNA. No deaths were reported during the study.

### Discussion

Profound long-term suppression of HBV DNA is required for patients to meet the goals of CHB therapy, which are to prevent cirrhosis, hepatic failure, HCC and liver-related death [14–16]. A major concern with long-term therapy is the increasing risk of selecting resistance mutations, especially for therapies with a low-genetic barrier to resistance, such as lamivudine. The current analysis presents results for a cohort of Japanese patients who were switched directly from lamivudine to long-term entecavir therapy. The results show that this switch cohort achieved additional HBV DNA suppression after the switch to entecavir. The proportion of patients with HBV DNA  $<400$  copies/mL increased from 21% after 24 weeks of lamivudine treatment to 82% following an additional 24 weeks of entecavir treatment. Mean HBV DNA decreased from  $3.52 \log_{10}$  copies/mL at week 24 to  $2.80 \log_{10}$  copies/mL at week 48. Rates of HBV DNA suppression were maintained in this cohort, with 90% of patients achieving HBV DNA  $<400$  copies/mL through 96 weeks of entecavir therapy (week 120). These results are comparable to those achieved by the cohort of patients who received entecavir 0.5 mg once daily in the Japanese Phase II studies and the rollover study ETV-060 [17]. At baseline in ETV-060, 56% of this cohort had achieved HBV DNA  $<400$  copies/mL, increasing to 83% through 96 weeks of entecavir therapy. Among patients with abnormal ALT levels at ETV-060 baseline, 88% of patients in the entecavir 0.5 mg cohort achieved normalized ALT levels at week 96 as compared to 90% of patients in the switch cohort. Rates of HBeAg seroconversion at week 96 in ETV-060 were also similar (20 vs. 19%, respectively). These rates of viral suppression also show comparison favorably to those reported for the global nucleoside-naïve cohorts treated for a similar period of time [18, 19]. The potent antiviral activity of entecavir and its high genetic barrier to resistance is expected to minimize the potential for resistance in the switch cohort, allowing long-term therapy for patients. Liver biopsies were not obtained from patients in the switch cohort; however, the histologic benefits of long-term entecavir therapy have been recently reported for a cohort of naïve Japanese patients in the ETV-060 rollover study [20]. Following treatment with entecavir 0.5 mg daily for 3 years, all patients experienced histologic improvement and 57% experienced improvement in fibrosis score. In

**Table 2** Summary of safety in ETV-060: switch cohort

On-treatment in ETV-060	Patients, n (%)
Any adverse events	33 (100)
Clinical adverse events	33 (100)
Laboratory adverse events	33 (100)
Grade 3/4 clinical adverse event	1 (3)
Grade 3/4 laboratory adverse event	5 (15)
Clinical serious adverse event <sup>a</sup>	3 (9)
Discontinuations due to adverse events	1 (3)
Deaths	0
ALT flares <sup>b</sup>	1 (3)

<sup>a</sup> Including ALT flares

<sup>b</sup> ALT  $>2 \times$  baseline and  $>10 \times$  ULN

addition, the results from a separate global study have confirmed the histologic benefits of long-term entecavir treatment [21].

Previous Japanese (ETV-052/060) and global (ETV-026) studies have examined the efficacy of entecavir in lamivudine-refractory patients. In these studies, entecavir demonstrated efficacy, with 54% of Japanese patients achieving HBV DNA <400 copies/mL through 3 years' treatment [10, 22]. However, as a result of the lower genetic barrier in these patients, a major drawback of entecavir therapy in this population is the development of resistance. The cumulative probabilities of genotypic entecavir resistance among lamivudine-refractory patients were 33% through 3 years' treatment in Japanese patients and 51% through 5 years' treatment among patients in the global cohort [12, 22]. In the current study, no entecavir- or lamivudine-associated resistance substitutions were detected after 96 weeks of entecavir treatment. However, in contrast to the previous studies where the majority of patients had high baseline HBV DNA and documented lamivudine resistance [10, 23], patients in the switch cohort received entecavir after achieving variable degrees of HBV DNA suppression with 24 weeks of lamivudine therapy. Therefore, the fact that no resistance has been observed in this cohort to date is not unexpected. This observation is consistent with an analysis of lamivudine-refractory patients enrolled in the worldwide lamivudine-refractory study ETV-026. Patients with baseline HBV DNA <7 log<sub>10</sub> copies/mL had a higher probability of achieving HBV DNA <300 copies/mL as compared to those who had baseline HBV DNA ≥7 log<sub>10</sub> copies/mL (73 vs. 16%) [24]. Furthermore, among the 42 entecavir-treated patients in ETV-026 who achieved HBV DNA <300 copies/mL through 96 weeks of therapy, only one patient subsequently developed entecavir resistance.

Current recommendations on the treatment of patients with documented lamivudine resistance suggest that patients should receive a second drug without cross resistance. The combination of lamivudine and adefovir has been shown to be superior to adefovir monotherapy for the treatment of lamivudine resistance, especially in preventing the selection of adefovir resistance [25–27]. Although only short-term clinical data are available for tenofovir, rates of viral suppression among lamivudine-experienced or -resistant patients who received tenofovir monotherapy do not differ significantly from those of treatment-naïve patients [28, 29]. Small studies have also shown pegylated interferon alpha-2a to be a safe and beneficial treatment option for lamivudine-experienced patients [30]. However, the treatment options for Japanese lamivudine-resistant patients are more limited, since neither tenofovir nor pegylated interferon alpha-2a are currently approved in Japan.

The Japanese guidelines recommend that patients with detectable YMDD mutations should receive treatment with a combination of lamivudine and adefovir [13]. However, the guidelines also allow patients who have received <3 years of lamivudine therapy, have HBV DNA <400 copies/mL, and no breakthrough hepatitis or YMDD mutations to switch directly to entecavir. The results presented in this analysis suggest that the strategy of switching to entecavir is an effective one that may avoid the additional cost and potential toxicity of combination treatment with lamivudine and adefovir. Among patients in the switch cohort, 96 weeks of entecavir treatment was well tolerated and the safety profile was comparable with previous experience in Japanese patients. One patient experienced an ALT flare (ALT >2 × baseline and >10 × ULN) 18 weeks after initiating entecavir, which was not associated with a change in HBV DNA. This low rate of ALT flares is consistent with previous findings and demonstrates that lamivudine-treated patients can be switched safely to entecavir with a minimal risk of such flares [10, 22].

In summary, the data from the switch cohort presented in this analysis demonstrate that CHB patients can be switched from lamivudine to long-term entecavir. The treatment with entecavir resulted in increased rates of virologic suppression with no evidence of resistance through 2 years of therapy. These findings support recommendations in the current Japanese treatment guidelines that patients on stable lamivudine therapy with no YMDD mutations should be switched to entecavir.

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## Original Article

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

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

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

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

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

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

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

## INTRODUCTION

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

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

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

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

## METHODS

### Patients

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

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

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

### Patient evaluation

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

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

### SNP genotyping

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

### Outcomes

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

### Statistical analyses

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

## RESULTS

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

**Table 1** Baseline characteristics of participating patients

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

†Data are expressed as mean ± standard deviation.

‡Data are shown as median (range) values.

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

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

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

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

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

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

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

†Data are show as median (range) values.

‡Data are expressed as mean ± standard deviation.

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

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

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

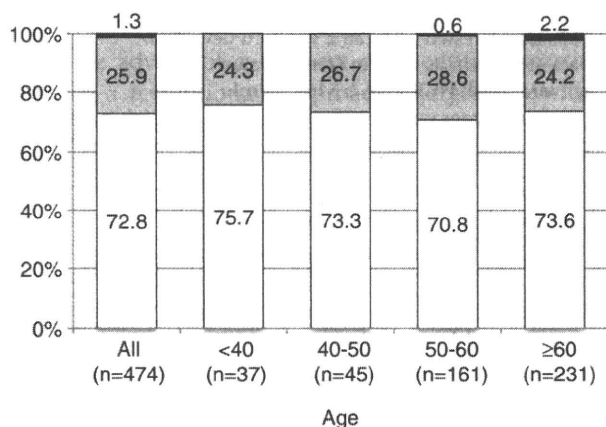


Figure 1 *ITPA* variant, rs1127354, known to be responsible for inosine triphosphatase deficiency and its age-related differences. *ITPA*, inosine triphosphatase. The numbers in parentheses denote numbers of patients.

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

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

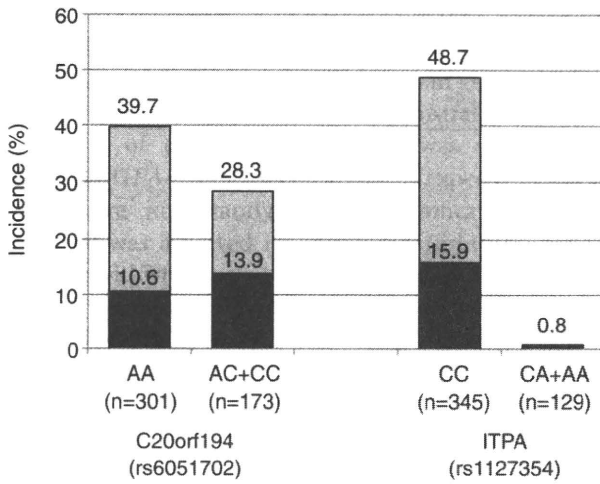
Table 3 Association of *C20orf194* and *ITPA* gene variants with treatment-induced Hb decline

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

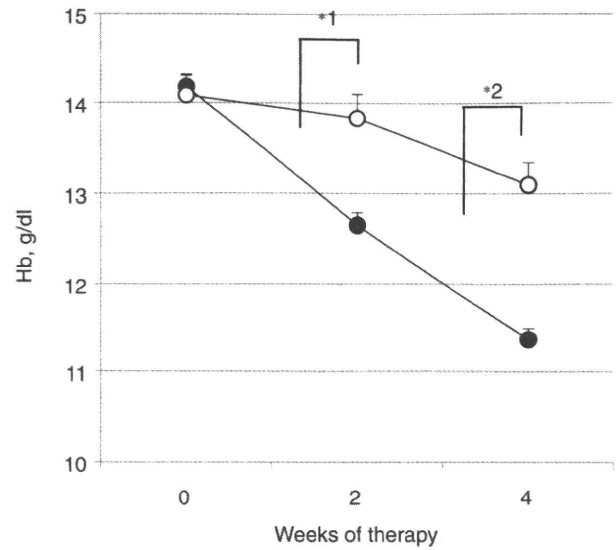
\*The SNP-phenotype associations were analyzed using a basic allelic test.

P-values were calculated by  $\chi^2$  analysis.

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



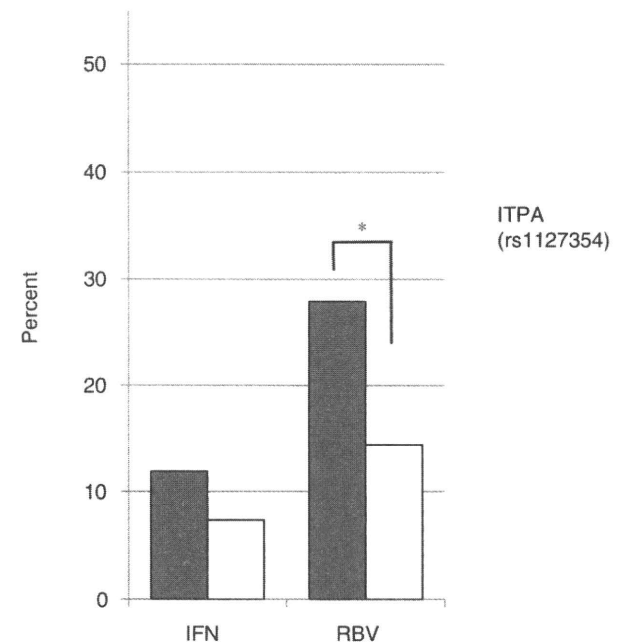
**Figure 2** Effects of *c20orf194* and *ITPA* single nucleotide polymorphisms (SNP) on clinically significant anemia induced by pegylated interferon plus ribavirin treatment. Percentages of patients with hemoglobin (Hb) decline of >3 g/dL or Hb levels of >10 g/dL at week 4 of treatment are shown for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



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

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

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



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



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

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

High viral load; serum HCV RNA  $\geq 5$  logIU/mL.

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

*P*-values were calculated by  $\chi^2$ -test analyses of SVR versus TVR plus NR.

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

The response to PEG IFN plus RBV treatment is affected by several viral and host factors such as age, sex,<sup>36,37</sup> NS5A-ISDR<sup>38</sup> and core region.<sup>32,33</sup> To maintain good adherence to drugs, especially RBV, it is important to achieve good treatment responses. Increased RBV exposure during the treatment phase was associated with an increased likelihood of SVR in the US<sup>39</sup> and Japanese studies.<sup>40</sup> Because patients with ITPA minor variant A are

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

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

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

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

clinical efficacy against hepatitis C.<sup>21,22</sup> 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.<sup>18,19,54</sup> Our present results may give a valuable pharmacogenetic diagnostic tool for the tailoring of RBV dosage to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV chemotherapeutics.

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## Pretreatment prediction of response to peginterferon plus ribavirin therapy in genotype 1 chronic hepatitis C using data mining analysis

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### Abstract

**Background** This study aimed to develop a model for the pre-treatment prediction of sustained virological response (SVR) to peg-interferon plus ribavirin therapy in chronic hepatitis C.

**Methods** Data from 800 genotype 1b chronic hepatitis C patients with high viral load ( $>100,000$  IU/ml) treated by peg-interferon plus ribavirin at 6 hospitals in Japan were randomly assigned to a model building ( $n = 506$ ) or an internal validation ( $n = 294$ ). Data from 524 patients treated at 29 hospitals in Japan were used for an external validation. Factors predictive of SVR were explored using data mining analysis.

**Results** Age ( $<50$  years), alpha-fetoprotein (AFP) ( $<8$  ng/mL), platelet count ( $\geq 120 \times 10^9/l$ ), gamma-glutamyl-transferase (GGT) ( $<40$  IU/l), and male gender were used to build the decision tree model, which divided patients into 7 subgroups with variable rates of SVR ranging from 22 to 77%. The reproducibility of the model was confirmed by the internal and external validation ( $r^2 = 0.92$  and  $0.93$ , respectively). When reconstructed into 3 groups, the rate of SVR was 75% for the high probability group, 44% for the intermediate probability group and 23% for the low probability group. Poor adherence to drugs lowered the rate of SVR in the low probability group, but not in the high probability group.

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**Conclusions** A decision tree model that includes age, gender, AFP, platelet counts, and GGT is useful for predicting the probability of response to therapy with peg-interferon plus ribavirin and has the potential to support clinical decisions regarding the selection of patients for therapy.

**Keywords** Data mining · Decision tree · Alpha-fetoprotein · HCV · Peg-interferon

## Introduction

The current standard therapy for genotype 1 chronic hepatitis C is 48 weeks of pegylated interferon (PEG-IFN) plus ribavirin (RBV) [1]. Sustained virological response (SVR), defined as undetectable HCVRNA post-treatment is regarded as a cure of chronic hepatitis C. However, the rate of SVR to this regimen is only 50% in patients with HCV genotype 1b and a high HCVRNA titer [2, 3]. Since PEG-IFN and RBV combination therapy is costly and accompanied by potential adverse effects, the ability to predict the possibility of SVR before therapy may significantly influence the selection of patients for therapy. A recent report revealed that single nucleotide polymorphisms located in the *IL28B* are strongly associated with a response to PEG-IFN plus RBV therapy [4–6]. Besides, the amino acid substitutions in the NS5A [7–9] or core region of HCV were also associated with response to therapy [10, 11]. Unfortunately, these host genetic and viral factors are not yet readily available for general application in actual clinical practice. Fibrosis of the liver is also an important predictor of response, but resources may be limited in some countries. Clinical and non-invasive parameters may be better suited for general practice, but there is no established means by which the likelihood of a response can be predicted prior to therapy.

Data mining is a method of predictive analysis that explores data, without setting the hypothesis, to discover hidden patterns and relationships in highly complex datasets and enables the development of predictive models. Decision tree analysis is a core component of data mining and predictive modeling [12], and it is utilized by decision makers in various fields of business. Recent publications on decision tree analysis indicate its usefulness for defining prognostic factors in various diseases such as prostate cancer [13], diabetes [14], melanoma [15, 16], colorectal carcinoma [17, 18], and liver failure [19]. The results of the analysis are presented as a tree structure, which is intuitive and facilitates the allocation of patients into subgroups by following the flow chart form [20]. We have recently reported the usefulness of decision tree analysis for the prediction of early virological response (undetectable

HCVRNA within 12 weeks of therapy) to PEG-IFN and RBV combination therapy in chronic hepatitis C [21].

In the present study, we used decision tree analysis to explore baseline predictors of response to PEG-IFN/RBV therapy so that a pre-treatment algorithm could be created to discriminate chronic hepatitis C patients who are likely to respond to PEG-IFN/RBV therapy from those who are not. For the purpose of use in general practice, only clinical and non-invasive parameters were included in the analysis.

## Materials and methods

### Patients

This was a multicenter retrospective cohort study supported by the Japanese Ministry of Health, Labor and Welfare. Data were collected from a total of 800 chronic hepatitis C patients who received therapy for 48 weeks with PEG-IFN alpha-2b and RBV at Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Osaka University, Nagoya City University Graduate School of Medical Sciences, Yamanashi University, and their related hospitals. The inclusion criteria to be enrolled in this study were as follows (1) infection by genotype 1b, (2) HCVRNA higher than 100,000 IU/ml by quantitative PCR (Cobas Amplicor HCV Monitor v 2.0, Roche Diagnostic systems, CA), which is typically used for the definition of high viral load in Japan, (3) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (4) lack of other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis and (5) completion of at least 12 weeks of therapy. Patients received PEG-IFN alpha-2b (1.5 µg/kg) subcutaneously every week and were administered a weight-adjusted dose of RBV (600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg for >80 kg), which is the recommended dosage in Japan. Patients who were treated for more than 49 weeks were not included in the study. For the analysis, patients were randomly assigned to either the model building ( $n = 506$ ) or the internal validation ( $n = 295$ ) group. Consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review committee. The baseline characteristics and representative laboratory test results are listed in Table 1. The overall rate of SVR was 47% in the model building set and 49% in the validation set. There were no significant differences in the clinical backgrounds between these 2 groups.

For external validation of the model, we collaborated with another study group supported by the Japanese Ministry of Health, Labor and Welfare. This multicenter study group consisted of 29 medical centers and hospitals



**Table 1** Comparison of pre-treatment factors between model building and internal validation patients

	Model (n = 506)	Validation (n = 295)
Age (years)	56 (14–75)	55 (18–74)
Male gender <sup>a</sup>	261/506 (52%)	160/295 (54%)
Body mass index (kg/m <sup>2</sup> )	22.9 (14.3–34.0)	23.2 (16.1–33.8)
Albumin (g/dl)	4 (2.7–5.0)	4 (2.8–4.9)
Creatinine (mg/dl)	0.7 (0.4–1.5)	0.7 (0.4–1.1)
AST (IU/l)	60 (11–370)	62 (11–240)
ALT (IU/l)	73 (11–413)	73 (14–390)
GGT (IU/l)	56 (10–328)	55 (7–409)
Total cholesterol (mg/dl)	173 (73–297)	171 (29–273)
Triglyceride (mg/dl)	105 (33–474)	109 (32–372)
White blood cell count (/μl)	4,745 (1,800–10,900)	4,823 (1,200–9,700)
Neutrophil count (/μl)	2,563 (667–7,870)	2,484 (508–7,579)
Red blood cell count (/μl)	448 (313–577)	451 (313–574)
Hemoglobin (g/dl)	14.1 (9.4–18.3)	14.1 (10.0–18.0)
Hematocrit (%)	41.7 (13.3–53.7)	41.9 (15.5–52.7)
Platelets (10 <sup>9</sup> /l)	164 (52–380)	158 (43–312)
AFP (ng/ml)	14.7 (0.9–680)	13 (0.8–323)
HCVRNA (10 <sup>3</sup> IU/ml)	1,852 (100–5,100)	1,870 (100–5,100)
Fibrosis stage: F3–4	73/417 (18%)	48/247 (19%)

Data expressed as median (range) unless otherwise indicated

AST aspartate aminotransferase, ALT alanine aminotransferase, GGT gamma-glutamyltransferase, AFP alpha-fetoprotein

<sup>a</sup> Data expressed as number/available data (percentage)

belonging to the National Hospital Organization. A dataset collected from 524 patients who were treated with PEG-IFN alpha-2b/RBV was used as an external validation dataset, i.e., completely independent from the dataset that was used for model building.

#### Laboratory tests

Blood samples were obtained before therapy and at least once every month during therapy, and were used for hematologic tests, blood chemistry analysis and determination of HCV RNA. Pretreatment levels of HCV RNA were quantified by Cobas Amplicor (Roche Diagnostic Systems, Pleasanton, CA). SVR was defined as undetectable HCV RNA at week 24 after completion of therapy, as determined by qualitative PCR with a lower end detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems). Liver biopsy was available in 664 patients. Fibrosis and activity

were scored according to the METAVIR scoring system [22]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of 0–3: A0 (no activity), A1 (mild activity), A2 (moderate activity) and A3 (severe activity).

#### Statistical analysis

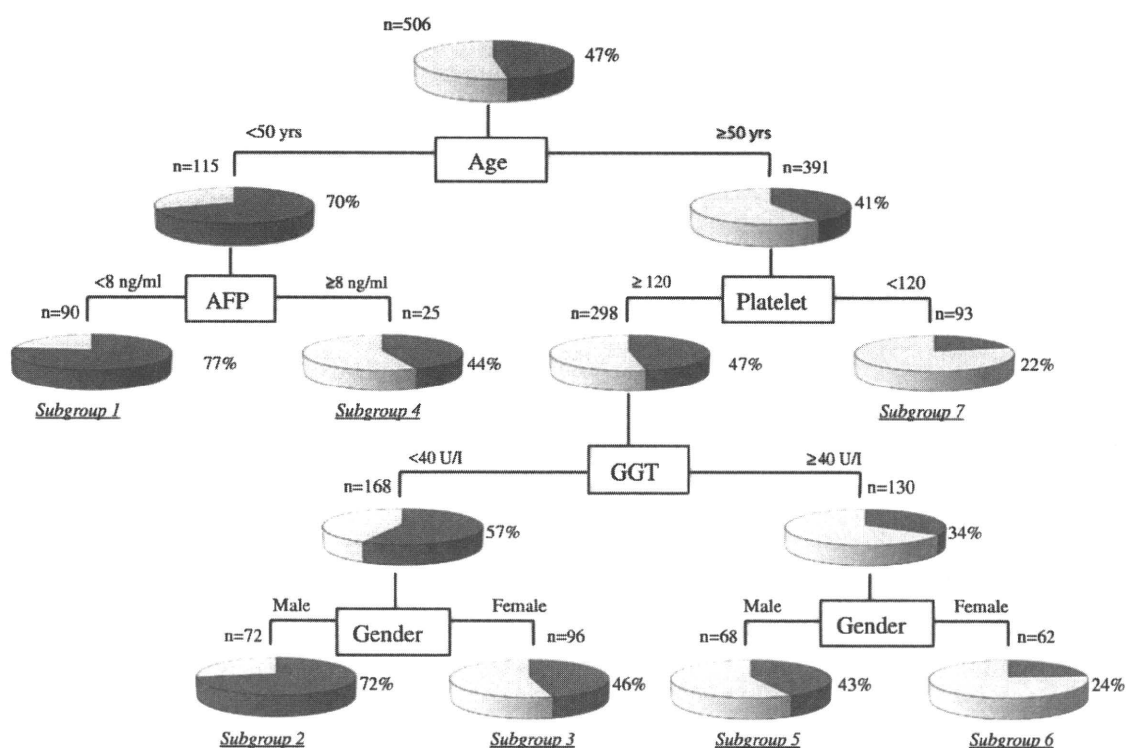
A database of pretreatment variables was created containing 6 variables from hematological tests (red blood cells, hemoglobin, hematocrit, white blood cells, neutrocytes and platelets), 8 variables from the blood chemistry test [creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase (GGT), total cholesterol, triglyceride and alpha-fetoprotein (AFP)], serum level of HCV RNA and 3 variables for patient characteristics (age, gender and body mass index). Based on this database, the recursive partitioning analysis algorithm referred to as decision tree analysis was implemented to define meaningful subgroups of patients with respect to the possibility of achieving SVR.

Decision tree analysis is a family of nonparametric regression methods. Software is used to automatically explore the data to search for optimal split variables and to build a decision tree structure [23]. For the analysis, the entire study population was evaluated to determine which variables and cutoff points yielded the most significant division into 2 prognostic subgroups that were as homogeneous as possible for the probability of SVR. Thereafter, the same analytic process was applied to all newly defined subgroups. A restriction was imposed on the tree construction such that the procedure stopped when either no additional significant variable was detected or when the sample size was below 20. For this analysis, the data mining software IBM SPSS Modeler 13 (IBM SPSS Inc., Chicago, IL) was utilized. SPSS software v.15.0 (SPSS Inc., Chicago, IL) was used for multivariate logistic regression analysis.

## Results

### Decision tree analysis

Decision tree analysis was carried out on the model building dataset from 506 patients using 18 variables. Figure 1 shows the results. The analysis automatically selected 5 predictive variables to produce a total of 7 subgroups of patients. Age was selected as the variable of initial split with an optimal cutoff of 50 years. The possibility of achieving SVR was 41% for patients older than 50 compared to 70% for patients



**Fig. 1** Decision tree analysis. Boxes indicate the factors for splitting and the cutoff value for the split. Pie charts indicate the rate of SVR for each group. Terminal subgroups of patients discriminated by the

analysis are numbered from 1 to 7. AFP alpha-fetoprotein, GGT gamma-glutamyltransferase

younger than 50. Among patients younger than 50, the level of serum AFP, with an optimal cutoff of 8 ng/ml, was selected as the variable of second split. Patients with lower AFP levels had a higher probability of SVR (77 vs. 44%). Among older patients, platelet count was selected as the second variable of split, with an optimal cutoff of  $120 \times 10^9/l$ . Patients with higher platelet counts had a higher probability of SVR (47 vs. 22%). Among patients with platelet counts higher than  $120 \times 10^9/l$ , GGT was selected as the third variable of split with an optimal cutoff of 40 IU/l. Patients with a lower GGT level had a higher probability of SVR (57 vs. 34%). Gender was selected as the fourth variable of split, with male gender being a predictor of a higher SVR probability (72 vs. 46% in patients with GGT levels <40 IU/l and 43 vs. 24% in those with GGT  $\geq 40$  IU/l). HCVRNA load was included in the analysis but was not selected as a significant variable.

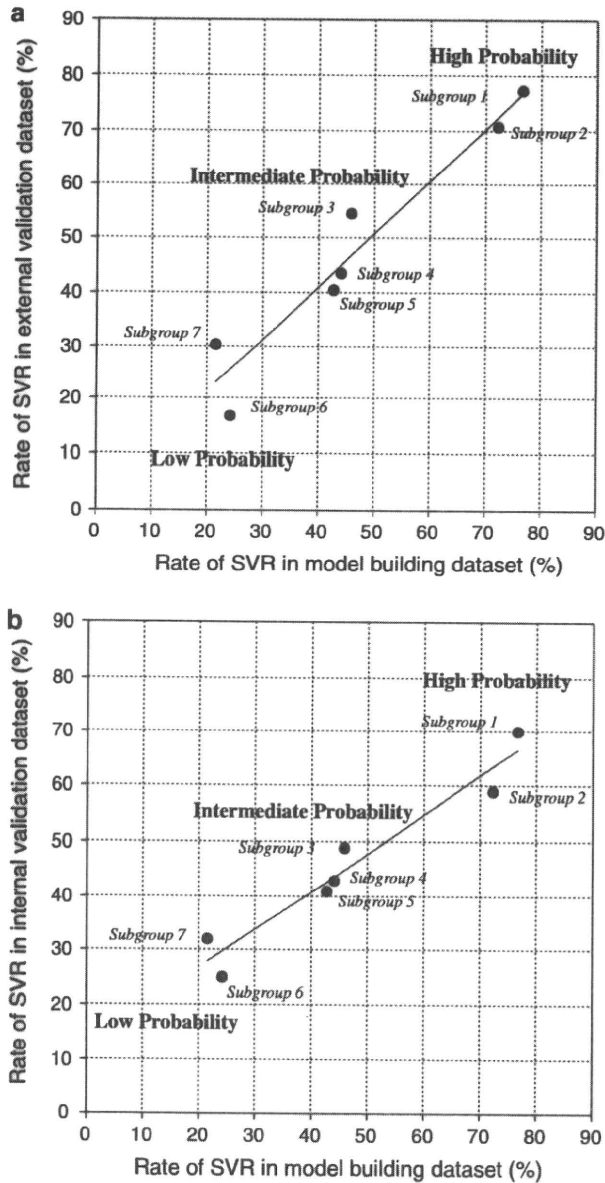
The probabilities of SVR for the 7 subgroups derived by this process were highly variable. The subgroup of young patients (<50 years) with low serum AFP (<8 ng/ml) (subgroup 1) or the subgroup of older ( $\geq 50$  years) male patients with high platelet counts ( $\geq 120 \times 10^9/l$ ) and low serum GGT (<40 IU/l) (subgroup 2) showed the highest

probability of SVR (72 and 77%), while the subgroup of older ( $\geq 50$  years) patients with low platelet counts (< $120 \times 10^9/l$ ) (subgroup 7) and older ( $\geq 50$  years) female patients with high serum GGT (subgroup 6) showed the lowest probability of SVR (22 and 24%).

#### Validation of the decision tree

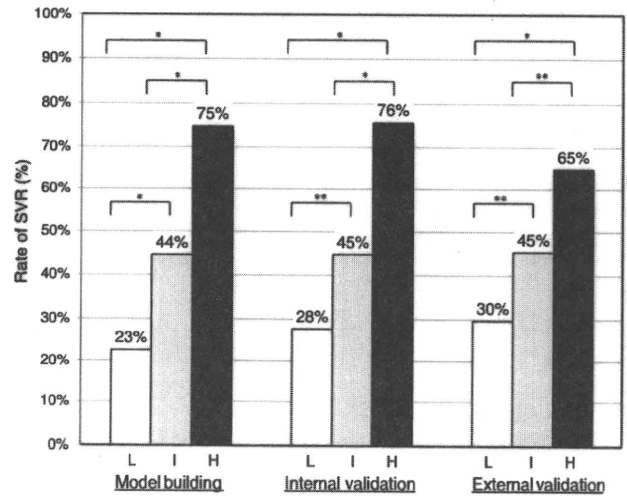
The results of the decision tree analysis were validated with an internal validation dataset of 295 cases, which was independent of the model building dataset. Each patient in the validation set was allocated to subgroups 1–7 using the flow-chart form of the decision tree. The rates of SVR were 77% for subgroup 1, 71% for subgroup 2, 55% for subgroup 3, 44% for subgroup 4, 41% for subgroup 5, 17% for subgroup 6, and 30% for subgroup 7. The rates of SVR for each subgroup of patients were closely correlated between the model building dataset and the internal validation dataset ( $r^2 = 0.925$ ) (Fig. 2a).

To further confirm the universality of the results, data collected from 524 patients by a collaborating study group were used for external validation. Thus, the dataset used for external validation was completely independent of the



**Fig. 2** Validation of the decision tree analysis by an internal and external validation dataset: subgroup-stratified comparison of the SVR rate. The rate of SVR in each subgroup was plotted. The X axis represents the model building, and the Y axis represents the validation datasets. **a** Internal validation and **b** external validation. There was a close correlation between the model building and the internal validation dataset (correlation coefficient  $r^2 = 0.925$ ) and between the model building and the external validation dataset (correlation coefficient  $r^2 = 0.936$ )

original dataset used for model building. Each patient in the external validation set was allocated to subgroups 1–7 using the flow-chart form of the tree. The rates of SVR were 70% for subgroup 1, 59% for subgroup 2, 49% for subgroup 3, 43% for subgroup 4, 41% for subgroup 5, 25% for subgroup 6, and 32% for subgroup 7. The rates of SVR for each subgroup of patients were closely correlated



**Fig. 3** Comparison of SVR rates between groups divided by the decision tree. The rate of SVR was compared among the 3 groups of patients divided by the decision tree analysis (white, gray and black boxes, indicating a low (L), intermediate (I) and high (H) probability group, respectively). The rate of SVR was significantly different among the 3 groups. \* $p < 0.0001$ , \*\* $p < 0.001$

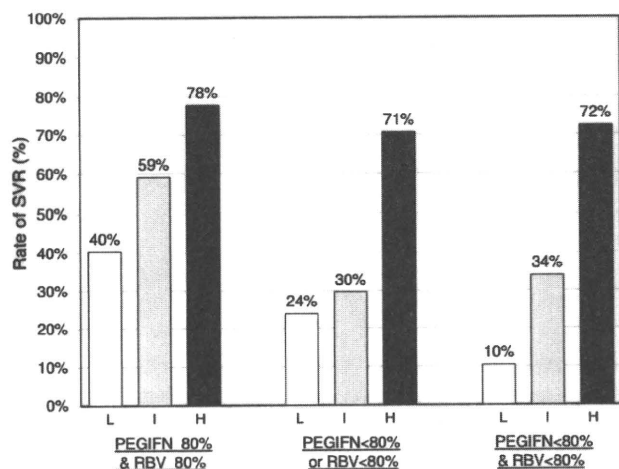
between the model-building dataset and the validation dataset ( $r^2 = 0.936$ ) (Fig. 2b).

#### Construction of 3 groups according to the probability of SVR

Seven subgroups were reconstructed into 3 groups according to their predicted rates of SVR: the high probability group consisted of subgroups 1 and 2, the intermediate probability group consisted of subgroups 3, 4 and 5, and the low probability group consisted of subgroups 6 and 7. The rate of SVR was significantly different among the 3 groups (Fig. 3). The rate of SVR in the high probability group was consistently high: 75% for model building patients, 76% for internal validation patients and 65% for external validation patients. Conversely, the rate of SVR in the low probability group was consistently low: 23% for model building patients, 28% for internal validation patients and 30% for external validation patients. The rate of SVR in the intermediate probability group was 44% for model building patients, 45% for internal validation patients and 45% for external validation patients. Since 28–32% of patients were classified as high probability and 30–32% were classified as low probability, roughly 60% of patients were classified as having either a high or low probability of achieving SVR.

#### Effect of dose reductions of PEG-IFN and RBV on SVR

The cumulative dose of PEG-IFN and RBV was not included as a variable of analysis since the present study



**Fig. 4** Comparison of SVR rates among groups stratified by drug adherence. The 3 groups of patients divided by the decision tree analysis (white, gray and black boxes indicating a low (L), intermediate (I) and high (H) probability group, respectively) were further stratified according to the cumulative drug exposure of PEG-IFN and RBV. The good adherence group ( $\geq 80\%$  planned dose of both PEG-IFN and RBV) had a higher rate of SVR compared with the poor adherence group ( $< 80\%$  planned dose of both PEG-IFN and RBV) in the low ( $p = 0.0003$ ) and intermediate ( $p = 0.007$ ) but not in the high probability group ( $p = 0.53$ )

aimed to develop a pre-treatment model for the prediction of response. To analyze the possible effect of drug reductions on the result of the decision tree analysis, 3 groups of patients divided by the decision tree analysis (low, intermediate and high probability group) were further stratified according to the cumulative drug exposure of PEG-IFN and RBV (Fig. 4). Even after adjustment for adherence, 3 groups of patients still had low, intermediate and high probability of achieving SVR, respectively. Of note, the good adherence group ( $\geq 80\%$  planned dose of both PEG-IFN and RBV) had higher rates of SVR compared with the poor adherence group ( $< 80\%$  planned dose of both PEG-IFN and RBV) in the low ( $p = 0.0003$ ) and intermediate ( $p = 0.007$ ) probability group, but not in the high probability group ( $p = 0.53$ ).

#### Factors associated with SVR by multivariate logistic regression analysis

We also explored the factors associated with SVR using a standard statistical analysis. By univariate analysis, age, gender, serum albumin, creatinine, alanine aminotransferase, GGT, red blood cell count, hemoglobin, hematocrit, platelet count and AFP were found to be associated with SVR (Table 2). HCVRNA load was not associated with SVR. By multivariate analysis, age, gender, GGT and platelet count were found to be independently associated with SVR (Table 3). Of note, AFP, which was selected as a

significant predictor of response in the decision tree analysis, was not found to be an independent response predictor in the standard multivariate analysis. This indicates a unique feature of the decision tree analysis; i.e., it could identify significant predictors that specifically apply to selected patients, in this case patients younger than 50 years old.

#### Relationships between decision tree model and stage of fibrosis or HCV RNA load

Liver biopsy was performed in 664 patients. The distribution of fibrosis in three probability groups differed significantly. Advanced fibrosis (F3 or F4) was higher in the low probability group (39%) compared to the intermediate probability group (13%) ( $p < 0.0001$ ) and to the high probability group (6%) ( $p < 0.0001$ ). Advanced fibrosis was also higher in the intermediate group compared to the high probability group ( $p = 0.01$ ). AFP was significantly associated with liver fibrosis stage: medians of AFP levels were 4.9, 5.9, 13.0 and 18.6 for F1, F2, F3 and F4, respectively ( $p < 0.0001$ , Spearman's rank correlations). Lower platelet counts correlated with advanced fibrosis stages (data not shown). The SVR rate was higher in the high probability group compared to the intermediate or low probability group after stratification by HCV RNA load. Among patients with low HCVRNA load ( $< 400,000$  IU/ml), the rate of SVR was 93, 59 and 50% for the high, intermediate and low probability group, respectively ( $p = 0.002$  for high vs. intermediate and  $p < 0.001$  for high vs. low probability groups). Among patients with a high HCVRNA load ( $\geq 400,000$  IU/ml), the rate of SVR was 73, 42 and 21% for the high, intermediate and low probability group, respectively ( $p < 0.001$  for high vs. low, high vs. intermediate and intermediate vs. low probability groups).

#### Discussion

Currently, the combination of PEG-IFN and RBV is the recommended therapy for chronic HCV infection. The rate of SVR with 48 weeks of therapy is around 50% in patients with HCV genotype 1b and a high HCV RNA titer [2, 3]. To date, the virological response during therapy is the most reliable means for predicting the likelihood of SVR [2, 24, 25]. More potent therapy, such as a triple combination of protease inhibitor, PEG-IFN and RBV, is being evaluated in clinical trials but is not readily available [26, 27]. Under the circumstances, pre-treatment prediction of the likelihood of SVR may be useful for both patients and physicians to support clinical decisions as to whether to start PEG-IFN/RBV therapy or delay treatment until a new more effective therapy becomes available.