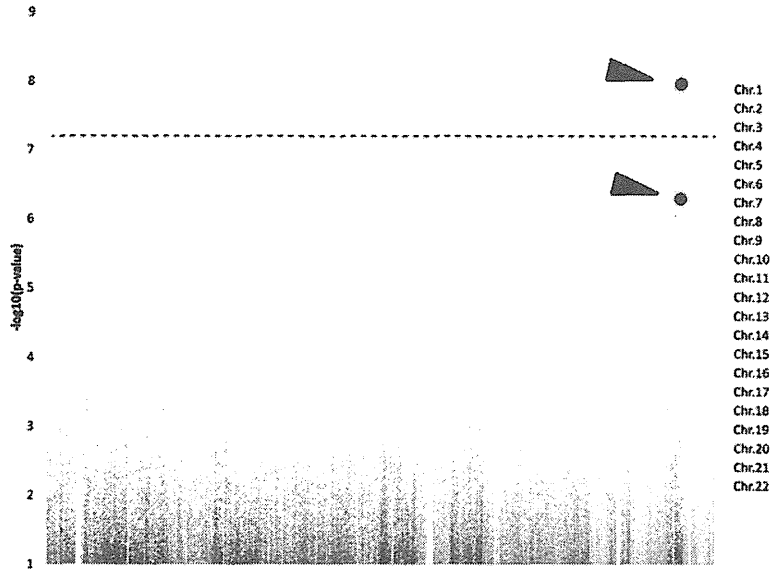

Figure 1. Genome-wide association results in 303 Japanese HCV patients with the decrease of platelets in response to PEG-IFN plus RBV treatment (107 patients with the decrease of PLT and 196 patients without the decrease of PLT). *P*-values were calculated using the χ^2 test for allele frequencies. Dots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

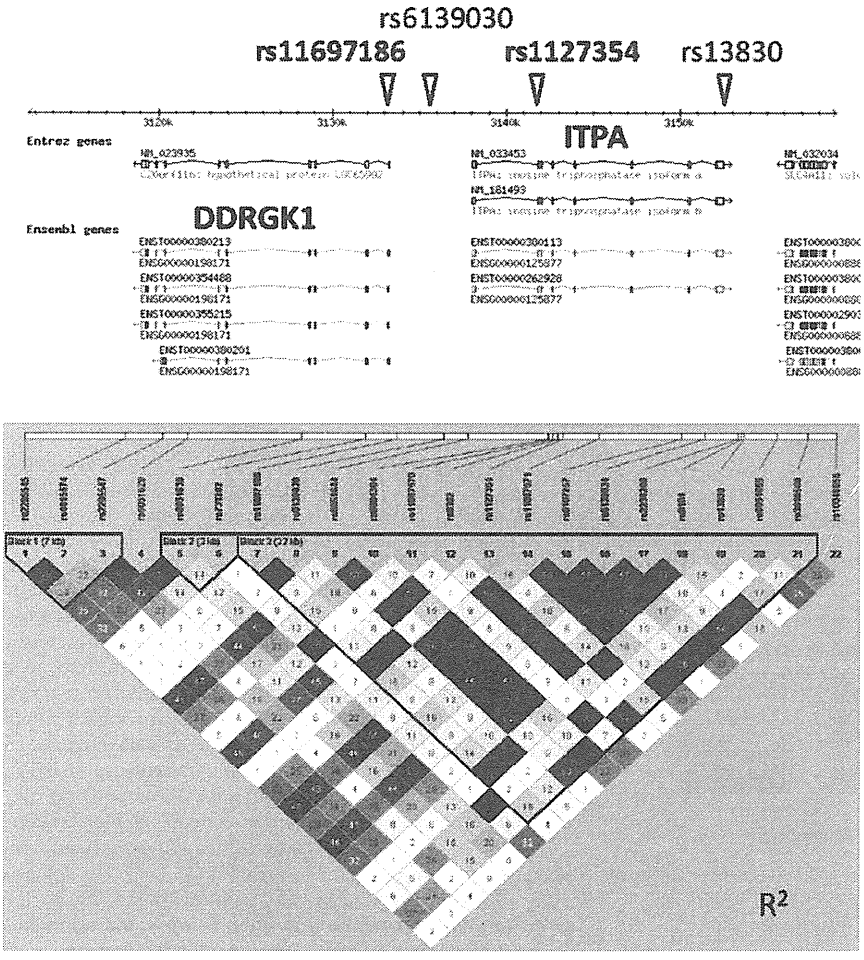

Figure 2. Pairwise LD (r^2) diagrams for *DDRGK1* and *ITPA*. Lower panel shows estimates of pairwise r^2 for 22 SNPs selected in the replication study using the second set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment.

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).

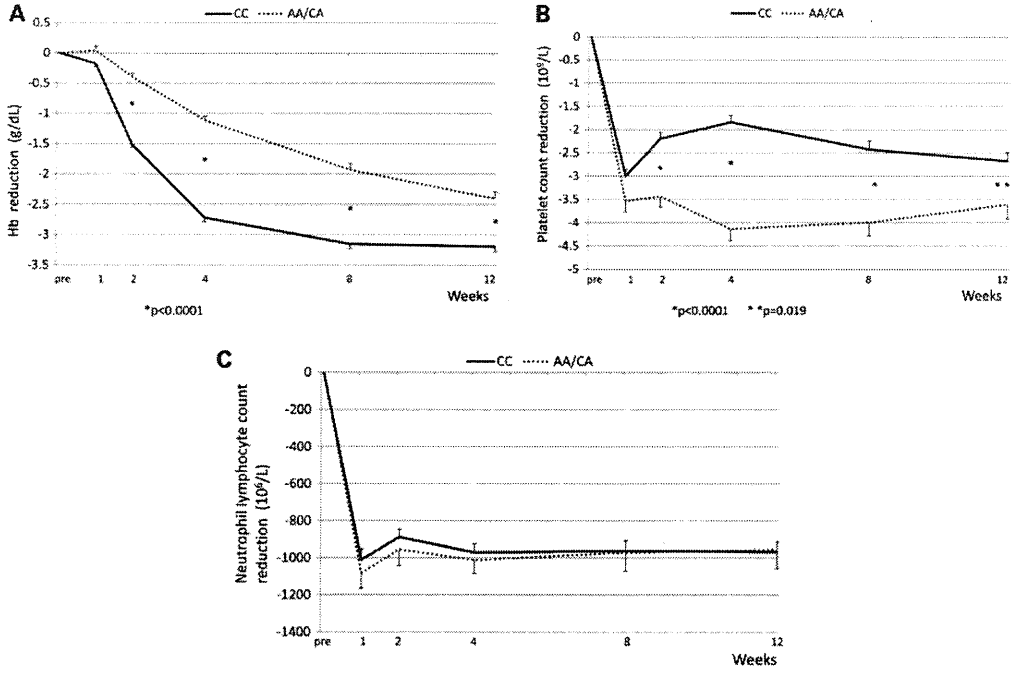

Figure 3. *ITPA* rs1127354 genotypes and the quantitative reduction of blood cells from baseline. Mean reduction of (**A**) Hb levels, (**B**) platelet counts and (**C**) neutrophil leukocyte counts during treatment according to rs1127354 genotype is shown. Solid and dotted lines indicate patients with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

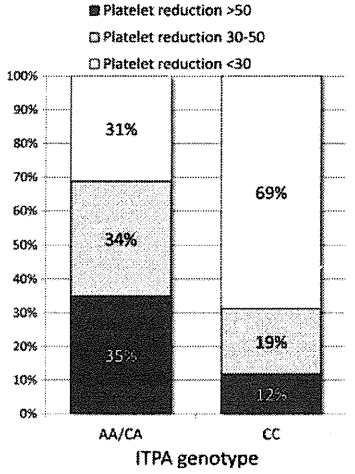

Figure 4. *ITPA* rs1127354 genotypes and reduction of platelet counts at week 4 of PEG-IFN/RBV therapy. The percentage of patients with platelet count reduction of >50 (10^9/l) (black bar), 30–50 (10^9/l) (gray bar) and <30 (10^9/l) (white bar) at week 4 is shown for rs1127354 genotypes. The incidence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

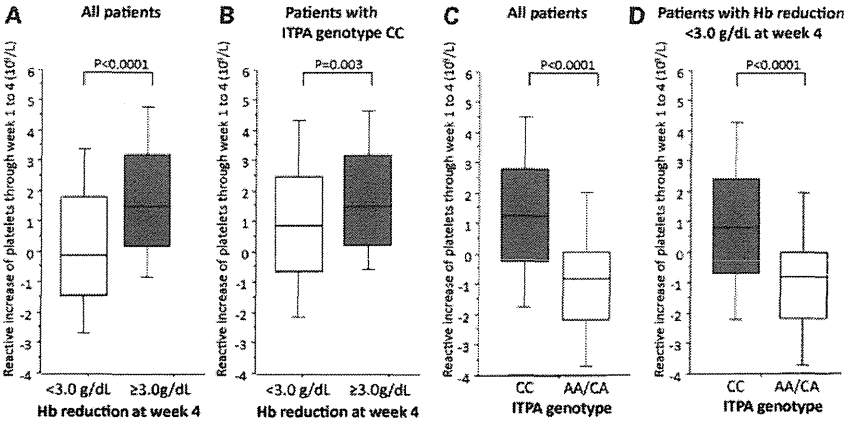

Figure 5. Reactive increase of platelet counts through weeks 1–4. Box plots of reactive increase of platelet count through weeks 1–4 according to the degree of anemia at week 4 are shown for all patients (**A**) and a subgroup of patients with the rs1127354 genotypes CC (**B**). Patients with anemia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

ACKNOWLEDGEMENTS

This study is based on 14 multi-center hospitals throughout Japan: Hokkaido area (Hokkaido University Hospital), Kanto area (Saitama University Hospital; Konodai Hospital; Musashino Red Cross Hospital; Tokyo Medical and Dental University Hospital; Yamanashi University Hospital), Koshin area (Shinshu University Hospital; Kanazawa University Hospital), Tokai area (Nagoya City University Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital; Hyogo College of Medicine Hospital), Chugoku/Shikoku area (Ehime University Hospital; Kawasaki Medical College Hospital) and Kyushu area (National Nagasaki Medical Center). We thank Ms Yasuka Uehara-Shibata, Yuko Ogasawara-Hirano, Yoshimi Ishibashi, Natsumi Baba and Megumi Yamaoka-Sageshima (Tokyo University) for technical assistance. We also thank Dr Masaaki Korenaga (Kawasaki), Dr Akihiro Matsumoto (Shinshu), Dr Kayoko Naiki (Saitama), Dr Takeshi Nishimura (Kyoto), Dr Hirayuki Enomoto (Hyogo), Dr Minako Nakagawa (Tokyo Medical and Dental University) and Ochanomizu Liver Conference Study Group for collecting samples, and Dr Mamoru Watanabe (Tokyo Medical and Dental University) and Dr Moriichi Onji (Ehime University) for their advice throughout the study.

Conflict of Interest statement. Y.T., E.T. and S.K. are currently conducting research sponsored by Merck Sharp & Dohme, Corp. and Chugai Pharmaceutical Co. Ltd. The other co-authors have no conflict of interest.

FUNDING

This study was supported by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H22-kannen-005), and the Ministry of Education, Culture, Sports, Science, and Technology.

REFERENCES

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

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

Hepatology Research

Hepatology Research 2011; 41: 217–224 doi: 10.1111/j.1872-034X.2010.00768.x

Original Article

Changes in hepatitis C viral load during first 14 days can predict the undetectable time point of serum viral load by pegylated interferon and ribavirin therapy

Jun Itakura,[1] Yasuhiro Asahina,[1] Nobuharu Tamaki,[1] Itsuko Hirayama,[1] Yutaka Yasui,[1] Tomohiro Tanaka,[1] Mitsuaki Sato,[1] Ken Ueda,[1] Teiji Kuzuya,[1] Kaoru Tsuchiya,[1] Hiroyuki Nakanishi,[1] Masayuki Kurosaki,[1] Gretchen S. Gabriel,[2] George J. Schneider[2] and Namiki Izumi[1]

[1]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; and* [2]*Abbott Molecular, Des Plaines, Illinois, USA*

Aim: In the treatment of chronic hepatitis C, pegylated interferon (PEG-IFN) and ribavirin combination therapy must be continued for an adequate duration to improve the rate of sustained virological response. We attempted to predict the time point at which serum hepatitis C virus (HCV) RNA are undetectable during combination therapy.

Methods: Patients with HCV genotype 1b were enrolled in a model preparation ($n = 35$) and a validation group ($n = 70$). All patients received PEG-IFN-α-2b/ribavirin combination therapy for at least 48 weeks, and serological samples were screened a minimum of 17 times during the therapy. Serum HCV RNA were measured by the Abbott RealTime HCV assay. Using the HCV dynamics model described by Neumann *et al.*, we used multiple linear regression analysis to select factors that affected the undetectable time point.

Results: Difference in viral load between weeks 1 and 2 was the only predictive factor for the undetectable time point of serum HCV RNA ($r^2 = 0.67$, $P < 0.0005$), and we derived the following prediction equation: undetectable time point (week) = 13.495 × (viral load at day 14 [log IU/mL] – viral load at day 7 [log IU/mL]) + 25.456. The equation was applicable to the validation group.

Conclusion: We created a formula for predicting the undetectable time point from viral load measurements early in PEG-IFN-α-2b/ribavirin combination therapy. An early response reflects sensitivity to therapy, and the estimation of an undetectable time point would be useful for determining the optimal duration of treatment for chronic hepatitis C patients.

Key words: hepatitis C, interferon, kinetics, real-time polymerase chain reaction, undetectable time point

INTRODUCTION

INTERFERON (IFN)-BASED therapy is the main form of therapy for chronic hepatitis C, but it requires a long-term period to complete, typically lasting at least 48 weeks for hepatitis C virus (HCV) genotypes 1 and 4. The final therapeutic effect is eradication of HCV, which is referred to as a sustained virological response (SVR). Although combination therapy with pegylated (PEG)-IFN-α and ribavirin is now established as the standard treatment for chronic HCV infection genotype 1b, the SVR rate in these patients is still approximately 50%.[1–3] Moreover, it is difficult to know the treatment outcomes during treatment and follow-up period.

Various factors have been investigated to predict the treatment efficacy before initiation of therapy, including pretreatment viral load,[4] viral genotype,[5] and gene sequences, such as IFN sensitivity determining region,[6] and host factors, including sex, age, fibrosis stage and race.[7,8] These factors cannot be modified by therapy and are unfortunately not completely reliable for predicting therapeutic response. However, other studies have documented the importance of the period when HCV is cleared from the serum (we define this as the

Correspondence: Dr Namiki Izumi, Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. Email: nizumi@musashino.jrc.or.jp

There are no conflicts of interests regarding this study.

Received 13 November 2009; revised 18 November 2010; accepted 18 December 2010.

"undetectable time point").[9–13] When an undetectable time point is achieved within 4 weeks of therapy initiation, the SVR rate is high. In contrast, the later the undetectable time point, the lower the SVR rate. One disadvantage with this prediction method during therapy is that SVR cannot be predicted until serum viral clearance. If one can predict the undetectable time point early during the treatment, physicians can modify and optimize the ongoing treatment.

There are various patterns of patient response to IFN therapy. In clinical settings, the following three response patterns are observed: (i) SVR; (ii) non-virological response (NVR), in which viral loads continue to be detected during therapy; and (iii) relapse, in which viral loads transiently drop below the detection limit but become detectable again after the end of therapy.[8] Mathematical models have been developed for analyzing therapy-induced changes in HCV viral load. Neumann *et al.*[14] introduced a model for IFN monotherapy in 1998, and a pharmacokinetic model for PEG-IFN has been developed by Powers *et al.*[15] These models are very useful for understanding the therapeutic effects of IFN on HCV.

In recent years, techniques to quantify serum viral RNA levels have advanced. The detection limit and the dynamic range of the quantitative real-time polymerase chain reaction (PCR) assay are lower and wider than those of Amplicor PCR assay.[16,17] As a result, the real-time PCR assay can show us the more accurate viral dynamics. In the present study, we used the model of Powers *et al.*[15] and real-time PCR to measure serum viral loads. Our aim was to ascertain whether it is possible to predict the undetectable time point during the early stage of PEG-IFN-α-2b/ribavirin combination therapy for genotype 1b patients with a high viral load, which is the most difficult-to-treat phenotype of HCV.

METHODS

Patients

THE MODEL PREPARATION group comprised 35 patients with biopsy-proven chronic hepatitis C who were treated at the Musashino Red Cross Hospital from 2000–2001. All patients had HCV genotype 1b and a high viral load (>100 000 IU/mL) as determined by the Amplicor-HCV Monitor Assay (Roche Diagnostics, Tokyo, Japan). Patients with other liver disease, such as liver cirrhosis, autoimmune hepatitis or alcoholic liver injury, were excluded. None of the patients had hepatitis B virus-related antigens, antibodies or anti-HIV antibodies. At the time of enrollment, it was confirmed that none of the patients were taking drugs that could affect their immune system. The dosage of ursodeoxycholic acid and glycyrrhizin was not changed during therapy.

The model validation group comprised 70 patients with biopsy-proven chronic hepatitis C who were treated at the Musashino Red Cross Hospital from 2004–2006. As with the model preparation group, all patients had HCV genotype 1b and a high viral load, and patients with liver cirrhosis or alcoholic liver injury were excluded. None of the patients had hepatitis B virus-related antigens, antibodies or anti-HIV antibodies.

Informed consent was obtained from all patients in writing. The present study was approved by the Ethics Review Board of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment protocol

All patients received at least 48 weeks of PEG-IFN-α-2b (PegIntron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) combination therapy. In the model validation group, if viral clearance was not achieved by week 12, combination therapy was prolonged to 72 weeks. PEG-IFN-α-2b (1.5 μg/kg per week) was administrated s.c. Ribavirin was administrated p.o. at 600 mg/day twice daily to patients weighing less than 60 kg, and 800 mg/day was given to patients weighing between 60 and 80 kg. The dosage of PEG-IFN-α-2b was reduced to 0.75 μg/kg per week when white blood cells, neutrophils or platelets dropped below 1500, 750 or $80 \times 10^3/mm^3$, respectively. When hemoglobin concentration dropped below 10 g/dL, the dosage of ribavirin was reduced from 600 to 400 mg/day for patients weighing less than 60 kg, and from 800 to 600 mg/day for patients weighing between 60 and 80 kg. Both drugs were discontinued when white blood cells, neutrophils, platelets or hemoglobin levels dropped below $1000/mm^3$, $500/mm^3$, $50 \times 10^3/mm^3$ or 8.5 g/dL, respectively.

HCV dynamics in serum

To analyze viral dynamics, serum samples were collected from each patient according to the following schedule with respect to the start of PEG-IFN-α2b/ribavirin combination therapy: immediately before and at 4, 8 h, and 1, 2, 4, 7, 8, 14 and 28 days after the therapy was started; and then at 4-week intervals until completion of the therapy. HCV viral loads were measured in all serum samples using the Abbott RealTi*me* HCV assay (Abbott Molecular, Des Plaines, IL, USA) at an Abbott laboratory in the USA.[16] The dynamic range

was 1.08–8 $\log_{10}$ IU/mL. The assay is standardized to the 2nd World Health Organization (WHO) International Standard for HCV RNA (National Institute for Biological Standards and Control code 96/798). Nucleic acid extraction was performed on 0.5-mL samples using an Abbott *m2000sp* (Abbott Molecular). The Abbott *m2000rt* (Abbott Molecular) was used for reverse transcription, PCR amplification and detection/quantification. A single-stranded linear probe was used as the HCV probe.

Definitions of response to therapy

The undetectable time point was defined as the first time the viral load dropped below the detection limit (1.08 $\log_{10}$ IU/mL) during therapy. Patients with SVR had no detectable viral load 6 months after the end of PEG-IFN-α-2b/ribavirin combination therapy. Patients in relapse had no detectable viral load at the end of therapy but had a detectable viral load 6 months after the end of therapy. Patients with NVR had a detectable viral load throughout the treatment period.

Calculation of the HCV dynamic parameters

Hepatitis C virus dynamic parameters (c, δ, ε, T_0 and V_0) were calculated from viral loads with equations for HCV dynamics.[15] The parameter c is the constant viral death rate, δ is the death rate of infected cells, ε is the effect of PEG-IFN on blocking production of virus from infected cells, and T_0 and V_0 are the numbers of uninfected cells and virus at the start of therapy, respectively.

Statistical analysis

SAS ver. 9.13 was used for the statistical analysis. *P*-values of less than 0.05 were considered significant.

RESULTS

Baseline patient characteristics

TABLE 1 SHOWS the baseline characteristics of the patients. The SVR rate was 60% and 27 patients accomplished undetectable serum HCV until 24 weeks after the therapy was started. The therapy was discontinued in three of the 35 patients because of a reduction in

Table 1 Patient characteristics at baseline

	Model preparation group ($n = 35$)	Model verification group ($n = 70$)
Age (years)	52.1 ± 9.9	57.8 ± 11
Sex (male/female)	24/11	36/34
BMI	23.7 ± 2.9	23.9 ± 3.7
Hemoglobin (g/dL)	14.7 ± 1.2	14.2 ± 1.6
Platelet count ($\times 10^3/\mu L$)	17.9 ± 4.8	15.5 ± 5.2
Albumin (g/dL)	4.2 ± 0.33	3.92 ± 0.048
ALT (U/L)	91.7 ± 64	80.0 ± 7.4
Liver histology (Metavir score)		
A (0/1/2/3/4/not measured)	0/17/13/5/0/0	0/40/26/2/0/2
F (0/1/2/3/4/not measured)	0/17/15/3/0/0	2/23/25/18/0/2
Viral load (log IU/mL)		
At pretreatment	5.49 ± 0.52	5.54 ± 0.92
At 7th day of treatment	4.05 ± 0.98	4.75 ± 1.05
at 14th day of treatment	3.23 ± 1.41	4.23 ± 1.29
Durations of therapy		
(48 weeks/72 weeks/dropout)	32/0/3	45/7/18
Drug adherence†		
(PEG-IFN/ribavirin/both/non-)	7/5/2/21	6/21/30/13
Outcome (SVR/relapse/NVR)	21/6/8	20/26/24
Actual undetectable time point‡		
(14/28 days/8/12/16/20/24/28/32 weeks/therapy end)	3/7/8/4/1/2/2/0/0	2/2/12/14/4/4/2/2/4

†Patients numbers with dose reduction during the therapy.
‡NVR cases were excluded.
BMI, body mass index; ALT, alanine aminotransferase; PEG-IFN, pegylated interferon; SVR, sustained virological response; NVR, non-virological response.

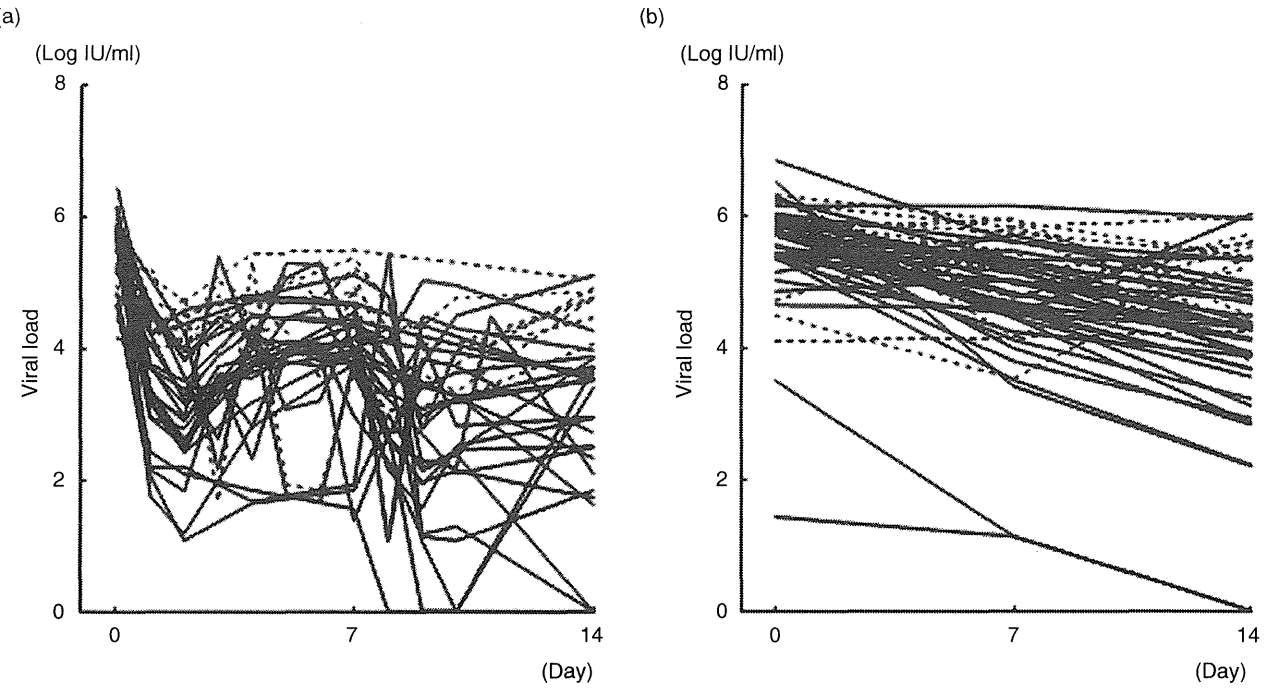

Figure 1 Early hepatitis C virus (HCV) dynamics of model preparation group (a) and of model validation group (b). The patients with incomplete blood collection were excluded from the figure of the model validation group. Solid line, dynamics of those who accomplished undetectable serum HCV until the therapy ended; dotted line, of those in whom serum HCV was detected through the whole therapy.

the hemoglobin concentration, a reduction in the neutrophil count and a worsening of depressive symptoms. In comparison to the model preparation group, there were more NVR patients, and the SVR rate was 29% in the model validation group. There were six patients who accomplished undetectable serum HCV after 24 weeks, and the latest patients achieved it 40 weeks after the therapy started. More patients had advanced hepatic fibrosis in the model validation group than in the model preparation group. Eighteen patients discontinued the combination therapy for various reasons, for example, decreased neutrophil count. The early HCV dynamics of both group are shown in Figure 1.

Undetectable time point prediction

From the model preparation group, 29 patients were analyzed and six patients were excluded for the following reasons: therapy was discontinued before viral clearance in one patient, PEG-IFN dosage was decreased before viral clearance in three patients, viral load increased during therapy in one patient, and an incomplete series of samples were obtained from one patient.

First, we hypothesized that the HCV dynamic parameters have a possibility to predict the undetectable time point. HCV dynamic parameters were calculated with three dataset patterns of viral loads, as follows: (i) immediately before and at 4, 8 h, and 1, 2, 4, 7 and 8 days; (ii) before and at 8 h, and 1, 2, 4 and 7 days; and (iii) before and at 4, 8 h, and 1, 2, 4 and 7 days after the therapy was started. Unfortunately, no significant factors for prediction of the undetectable time points were detected in these HCV dynamic parameters (Table 2), even when adding parameters of age and sex.

Next, we investigated the possibility using early-stage treatment dynamics. Multiple linear regression analysis was conducted for viral load, and changes in viral load up to day 14 as the explanatory variables and undetectable time points as the objective variables. Among various factors which became significant alone, the decrease in viral load from day 7 to 14 was found to be the best predictor for the undetectable time points by multiple linear regression analysis ($r^2 = 0.67$, Table 3). Then, whole datasets were analyzed again including HCV dynamic parameters, sex, age, viral loads and viral

Table 2 Calculated HCV-dynamic parameters of model preparation group

Dataset	Dataset 1† median (range)	*P*	Dataset 2‡ median (range)	*P*	Dataset 3§ median (range)	*P*
c	0.77 (0.032–5.21)	0.73	1.54 (0.0515–7.58)	0.37	2.75 (0.040–6.19)	0.85
δ	0.0033 (0–0.69)	0.76	0.013 (0–0.99)	0.094	0.053 (0–0.70)	0.91
ε	0.28 (0.023–0.84)	0.30	0.067 (0.0083–0.72)	0.038	0.28 (0.023–0.71)	0.18
T_0	0.36 (0.0001–0.95)	0.63	0.415 (0.0049–0.98)	0.23	0.36 (0.007–0.90)	0.21
V_0	5.49 (4.40–6.69)	0.53	4.99 (4.10–6.48)	0.090	5.29 (4.30–6.69)	0.29
R^2	0.012		0.090		0.056	

†Dataset 1: serum hepatitis C virus (HCV) load immediately before and at 4, 8 h, and 1, 2, 4, 7, 8 days after the therapy was started.
‡Dataset 2: serum HCV load before and at 8 h, and 1, 2, 4, 7 days after the therapy was started.
§Dataset 3: serum HCV load before and at 4, 8 h, and 1, 2, 4, 7 days after the therapy was started.

load changes. The results showed that only the change in viral load from day 7 to 14 was associated with the prediction of the undetectable time point ($r^2 = 0.67$). Finally, prediction in each patient was valid (Cook's D = 0.046, mean, data not shown), and we derived the following prediction formula:

Undetectable time point (week) = 13.495 × (viral load at day 14 [log IU/mL] − viral load at day 7 [log IU/mL]) + 25.456.

The degree of decrease in viral load from day 7 to 14 for the model preparation group and the actual

Table 3 Early viral dynamics of model preparation group, correlation to undetectable time point and the result of multiple linear regression analysis

	Viral load (log IU/mL)	Spearman's rank correlation test coefficient (*P*-value)	Multiple linear regression analysis r^2 (*P*-value)
Pretreatment (0 days)	5.48 ± 0.30	0.27 (0.28)	Excluded
4 h	5.66 ± 0.22	0.045 (0.82)	Excluded
8 h	5.55 ± 0.19	0.026 (0.89)	Excluded
1 day	3.74 ± 0.75	0.68 (<0.001)	Excluded
2 days	3.20 ± 0.76	0.66 (<0.001)	Excluded
4 days	4.01 ± 0.74	0.56 (0.002)	Excluded
7 days	4.05 ± 0.75	0.77 (<0.001)	Excluded
8 days	3.34 ± 0.80	0.67 (<0.001)	Excluded
14 days	3.52 ± 0.95	0.87 (<0.001)	Excluded
Subtracted values of viral load (log scale)			
1 day – 0 days	−1.78 ± 0.88	0.59 (0.001)	Excluded
2 days – 0 days	−2.18 ± 0.79	0.53 (0.003)	Excluded
4 days – 0 days	−1.46 ± 0.65	0.72 (0.000)	Excluded
7 day – 0 days	−1.38 ± 0.80	0.38 (0.049)	Excluded
14 days – 0 days	−2.24 ± 1.17	0.83 (0.000)	Excluded
2 days – 1 day	−0.55 ± 0.13	0.085 (0.67)	Excluded
4 days – 1 day	0.17 ± 0.25	0.22 (0.27)	Excluded
7 days – 1 day	0.44 ± 0.46	0.27 (0.19)	Excluded
14 days – 1 day	−0.42 ± 0.46	0.76 (<0.001)	Excluded
4 days – 2 days	0.61 ± 0.23	0.12 (0.54)	Excluded
7 days – 2 days	0.86 ± 0.50	0.12 (0.56)	Excluded
14 days – 2 days	0.11 ± 0.44	0.76 (<0.001)	Excluded
7 days – 4 days	−0.11 ± 0.17	0.047 (0.82)	Excluded
14 days – 4 days	−0.7 ± 0.37	0.78 (<0.001)	Excluded
14 days – 7 days	−0.86 ± 0.50	0.76 (<0.001)	0.667 (<0.0005)

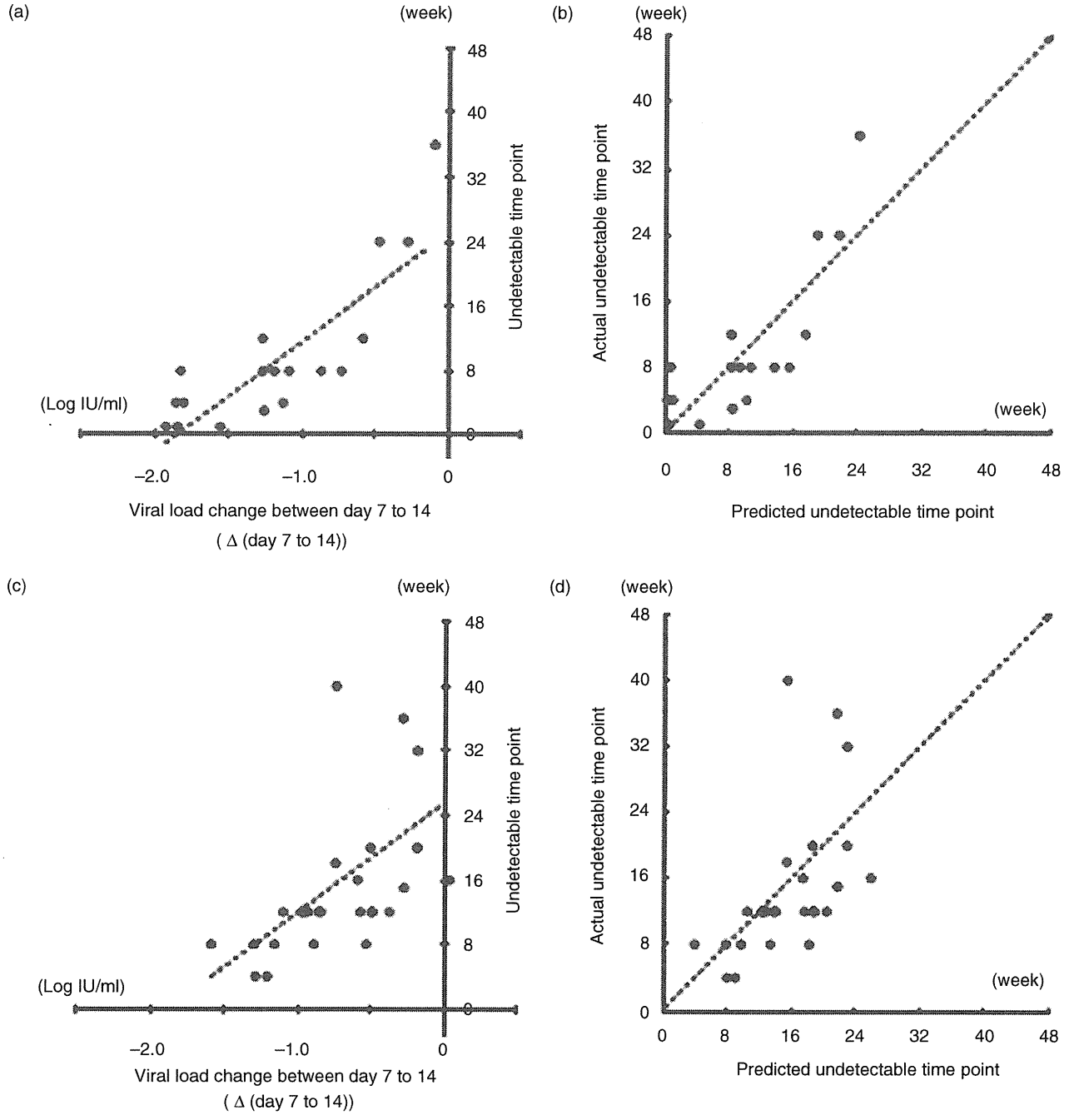

Figure 2 Correlation between the undetectable time point and the decrease in viral load from day 7 to 14 (a,b) and correlation between the actual and predicted undetectable time points (c,d). (a,c) Results of analyses for the model preparation group; and (b,d) analyses for the model validation group. Black circles, actual cases; dotted line, (a,c) estimate obtained from the prediction formula; (b,d) equal values of actual and predicted undetectable time points.

undetectable time point are plotted in Figure 2(a), which shows a very strong and a significant correlation ($r^2 = 0.67$, $P < 0.0005$).

The validity of the prediction formula was investigated in the validation group. Analysis was possible in 32 patients, as the other patients were excluded from the analysis due to the following reasons: therapy was discontinued before viral clearance in eight patients, PEG-IFN dosage was reduced before viral clearance in nine patients and viral clearance was achieved before day 14 in two patients. There were six cases of NVR, and incomplete blood collections from 13 patients on day 7 and/or 14. A strong and a significant correlation was demonstrated between the undetectable time points that were predicted using this formula and the actual undetectable time points (Fig. 2c, $r = 0.53$, $P = 0.005$).

Although only one case was predicted to achieve a rapid virological response (undetectable viral load at week 4)[13] in the model validation group, the actual undetectable time point of this patient was week 8 (Fig. 2d). In contrast, all nine cases who were predicted to achieve a complete early virological response (undetected viral load until week 12),[13] the actual undetectable time points of these patients were within week 12. Because the prediction formula was derived by the least squares method, half of the patients, who were predicted not to achieve the complete early virological response, actually achieved it.

DISCUSSION

NUMEROUS STUDIES HAVE documented that the undetectable time point is related to therapeutic responses, and its usefulness in predicting therapeutic efficacy is clear.[9–13] In the present study, we were able to derive a formula for predicting the undetectable time point for patients with HCV genotype 1b and high serum viral loads during PEG-IFN-α-2b/ribavirin combination therapy. Though the various parameters for the HCV dynamics were investigated, the change in viral load from day 7 to 14 was the only parameter that was useful for predicting the undetectable time point.

The standard length of PEG-IFN/ribavirin combination therapy is 48 weeks for patients with HCV genotype 1b and high serum viral loads; however, a 72-week administration is recommended to improve therapeutic response.[3,13,18] Therefore, when undetectable time points are predicted as from weeks 13–24 by our formula, the SVR rates could be improved by continuing the IFN therapy for longer periods. By prediction of the undetectable time point early during the treatment using our formula, the physician can make early modification and optimization of currently ongoing therapy.

Another important issue of PEG-IFN/ribavirin treatment is adherence to treatment. Because dose reductions may delay the time until serum viral clearance, patients in whom the dosage of IFN and ribavirin was reduced during therapy were excluded in the present study. However, there are many patients in whom the dosage of drugs has to be reduced during therapy for a wide variety of clinical reasons. If reducing dosage before the predicted undetectable time point, administration of IFN for longer periods should be considered.

In conclusion, we created a formula for predicting the undetectable time point in patients treated with PEG-IFN-α-2b/ribavirin combination therapy. Viral eradication is the ultimate objective of IFN-based therapy, but many patients failed to achieve viral eradication for some reason. Because our prediction formula for the undetectable time point was made with a small population, it is necessary to correct it by further analysis with a larger population. However, an early viral response reflects efficacy of the therapy, and the estimation of an undetectable time point by our formula would be useful for determining the optimal duration of treatment in the early period of the therapy for each chronic hepatitis C patient.

REFERENCES

1 Glue P, Rouzier-Panis R, Raffanel C *et al.*; The Hepatitis C Intervention Therapy Group. A dose-ranging study of pegylated interferon alfa-2b and ribavirin in chronic hepatitis C. *Hepatology* 2000; **32**: 647–53.
2 Reddy KR, Wright TL, Pockros PJ *et al.* Efficacy and safety of pegylated (40-kd) interferon alpha-2a compared with interferon alpha-2a in noncirrhotic patients with chronic hepatitis C. *Hepatology* 2001; **33**: 433–8.
3 Sánchez-Tapias JM, Diago M *et al.*; TeraViC-4 Study Group. Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; **131**: 451–60.
4 Tsubota A, Chayama K, Ikeda K *et al.* Factors predictive of response to interferon- therapy in hepatitis C virus infection. *Hepatology* 1994; **19**: 1088–94.
5 Chayama K, Tsubota A, Kobayashi M *et al.* Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. *Hepatology* 1997; **25**: 745–9.
6 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon

in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77–81.
7 Davis GL. Prediction of response to interferon treatment of chronic hepatitis C. *J Hepatol* 1994; **21**: 1–3.
8 Asahina Y, Izumi N, Hirayama I *et al.* Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response. *Gastroenterology* 2008; **134**: 1396–405.
9 Tong MJ, Blatt LM, McHutchison JG, Co RL, Conrad A. Prediction of response during interferon alfa 2b therapy in chronic hepatitis C patients using viral and biochemical characteristics: a comparison. *Hepatology* 1997; **26**: 1640–5.
10 Lee WM, Reddy KR, Tong MJ *et al.* Early hepatitis C virus-RNA responses predict interferon treatment outcomes in chronic hepatitis C. The Consensus Interferon Study Group. *Hepatology* 1998; **28**: 1411–5.
11 Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645–52.
12 Poordad F, Reddy KR, Martin P. Rapid virologic response: a new milestone in the management of chronic hepatitis C. *Clin Infect Dis* 2008; **46**: 78–84.
13 Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335–74.
14 Neumann AU, Lam NP, Dahari H *et al.* Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998; **282**: 103–7.
15 Powers KA, Dixit NM, Ribeiro RM, Golia P, Talal AH, Perelson AS. Modeling viral and drug kinetics: hepatitis C virus treatment with pegylated interferon alfa-2b. *Semin Liver Dis* 2003; **23** (Suppl 1): 13–8.
16 Halfon P, Bourlière M, Pénaranda G, Khiri H, Ouzan D. Real-time PCR assays for hepatitis C virus (HCV) RNA quantitation are adequate for clinical management of patients with chronic HCV infection. *J Clin Microbiol* 2006; **44**: 2507–11.
17 Gelderblom HC, Menting S, Beld MG. Clinical performance of the new rRoche COBAS TaqMan HCV Test and High Pure System for extraction, detection and quantitation of HCV RNA in plasma and serum. *Antivir Ther* 2006; **11**: 95–103.
18 Berg T, Wagner MV, Nasser S *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; **130**: 1086–97.

J Gastroenterol (2011) 46:1111–1119
DOI 10.1007/s00535-011-0412-z

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Pretreatment prediction of anemia progression by pegylated interferon alpha-2b plus ribavirin combination therapy in chronic hepatitis C infection: decision-tree analysis

Naoki Hiramatsu · Masayuki Kurosaki · Naoya Sakamoto · Manabu Iwasaki · Minoru Sakamoto · Yoshiyuki Suzuki · Fuminaka Sugauchi · Akihiro Tamori · Sei Kakinnuma · Kentaro Matsuura · Namiki Izumi

Received: 16 February 2011 / Accepted: 2 April 2011 / Published online: 17 June 2011

Abstract
Background This study aimed to develop a model to predict the development of severe anemia during pegylated interferon alpha-2b plus ribavirin combination therapy.
Methods Data were collected from 1081 genotype 1b chronic hepatitis C patients who were treated at 6 hospitals in Japan. These patients were randomly assigned to a model-building group ($n = 691$) or an internal validation group ($n = 390$). Factors predictive of severe anemia (hemoglobin, Hb < 8.5 g/dl) were explored using data-mining analysis.
Results Hb values at baseline, creatinine clearance (Ccr), and an Hb concentration decline by 2 g/dl at week 2 were used to build a decision-tree model, in which the patients were divided into 5 subgroups based on variable rates of severe anemia ranging from 0.4 to 11.8%. The reproducibility of the model was confirmed by the internal validation group ($r^2 = 0.96$). The probability of severe anemia was high in patients whose Hb value was <14 g/dl before treatment (6.5%), especially (a) in those whose Ccr was <80 ml/min (11.8%) and (b) those whose Ccr was ≥80 ml/min but whose Hb concentration decline at week 2 was ≥2 g/dl (11.5%). The probability of severe anemia was low in the other patients (0.4–2.5%).
Conclusions The decision-tree model that included Hb values at baseline, Ccr, and an Hb concentration decline by

N. Hiramatsu and M. Kurosaki contributed equally to this work.

N. Hiramatsu
Department of Gastroenterology and Hepatology,
Osaka University Graduate School of Medicine,
Osaka, Japan

M. Kurosaki · N. Izumi (✉)
Division of Gastroenterology and Hepatology,
Musashino Red Cross Hospital,
1-26-1 Kyonan-cho, Musashino,
Tokyo 180-8610, Japan
e-mail: nizumi@musashino.jrc.or.jp

N. Sakamoto · S. Kakinnuma
Department of Gastroenterology and Hepatology,
Tokyo Medical and Dental University, Tokyo, Japan

M. Iwasaki
Department of Computer and Information Science,
Seikei University, Tokyo, Japan

M. Sakamoto
First Department of Internal Medicine,
University of Yamanashi, Yamanashi, Japan

Y. Suzuki
Department of Hepatology, Toranomon Hospital,
Tokyo, Japan

F. Sugauchi
Department of Gastroenterology, Nagoya Koseiin
Medical Welfare Center, Nagoya, Japan

A. Tamori
Department of Hepatology,
Osaka City University Medical School, Osaka, Japan

K. Matsuura
Department of Gastroenterology and Metabolism,
Nagoya City University Graduate School of Medical Sciences,
Nagoya, Japan

2 g/dl at week 2 was useful for predicting the probability of severe anemia, and has the potential to support clinical decisions regarding early dose reduction of ribavirin.

Keywords Data mining · Decision tree · Severe anemia · Chronic hepatitis C · Pegylated interferon · Ribavirin

Abbreviations

Hb	Hemoglobin
PEG-IFN	Pegylated interferon
Ccr	Creatinine clearance
SVR	Sustained virological response
AFP	Alpha-fetoprotein

Introduction

The current standard therapy for chronic hepatitis C is 48 weeks of pegylated interferon (PEG-IFN) plus ribavirin [1]. Sustained virological response (SVR), defined as negative hepatitis C virus (HCV) RNA for 24 weeks after cessation of therapy, can be achieved by the current treatment regimen, but this outcome can be attained in only less than 50% of patients infected with genotype 1 HCV [2, 3]. Hemolytic anemia is a common side effect of ribavirin and is the major reason for dose reduction. Age, gender, baseline platelet level, baseline hemoglobin (Hb) level [4, 5], haptoglobin phenotype [6], drug dose [7], plasma concentration of ribavirin [8], apparent clearance of ribavirin (CL/F) [9], and an early decline in Hb concentration [10, 11] have been reported to contribute to ribavirin-induced anemia. Predicting the possibility of severe anemia before therapy or at the early phase of therapy can help modify ribavirin dosage, decrease the discontinuance rate for ribavirin, and raise the SVR rate.

Data mining is a method of predictive analysis that explores data 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 business fields. Recent publications concerning decision-tree analysis in the medical field 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 decision-tree analysis are presented in the form of a flow chart, which is easy to use in clinical practice [20]. This analysis was also used to predict early virological response (undetectable HCV RNA within 12 weeks of therapy) and SVR to PEG-IFN plus ribavirin combination therapy in chronic hepatitis C [21–24]. In the present study, we used decision-tree analysis to explore before- and during-treatment predictors of severe anemia during PEG-IFN alpha-2b/ribavirin combination therapy and used a prediction algorithm to try to identify chronic hepatitis C patients who are likely to develop severe anemia.

Materials and methods

Patients

This multicenter retrospective cohort study was supported by the Japanese Ministry of Health, Labour and Welfare. Data were collected from 1081 chronic hepatitis C patients who were treated with PEG-IFN alpha-2b plus ribavirin at Osaka University, Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Nagoya City University, Yamanashi University, and their related hospitals. The inclusion criteria applied in the present study were as follows: (1) infection by genotype 1b, (2) HCV RNA $\geq$ 100 KIU/ml by quantitative PCR (Cobas Amplicor HCV Monitor v 2.0, Roche Diagnostic Systems, CA, USA), (3) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (4) lack of other causes of liver diseases 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 g/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg). The dosage of ribavirin was reduced from 1000 to 600 mg, 800 to 600 mg, or 600 to 400 mg when the Hb concentration decreased to less than 10 g/dl, and was discontinued when the Hb concentration decreased to less than 8.5 g/dl, based on the recommendations in the package inserts. No patient received erythropoietin or blood transfusion for the treatment of anemia. Anemia with Hb < 8.5 g/dl was defined as severe anemia in this study.

For the analysis, patients were randomly assigned to either the model-building (n = 691) group or the internal validation (n = 390) 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. There were no significant differences between the clinical backgrounds of the two groups.

Laboratory tests

Blood samples were obtained before therapy and at least once every month during therapy, and were used for hematological tests, blood chemistry analyses, and determination of HCV RNA. Pretreatment levels of HCV RNA

Table 1 Comparison of clinical parameters of model-building and internal validation groups

	All patients ($N = 1081$)	Model building ($N = 691$)	Internal validation ($N = 390$)
Age (years)	55.6 ± 10.5	55.6 ± 10.8	55.6 ± 10.4
Gender (male/female)	612/469	393/298	219/171
Body mass index (kg/m^2)	23.2 ± 3.3	23.4 ± 3.8	23.1 ± 3.0
Creatinine (mg/dl)	0.73 ± 0.16	0.74 ± 0.17	0.73 ± 0.16
AST (IU/l)	62.0 ± 44.8	63.2 ± 48.6	61.4 ± 42.5
ALT(IU/l)	74.6 ± 56.1	75.4 ± 60.5	74.2 ± 53.5
GGT (IU/l)	58.6 ± 57.0	59.5 ± 58.5	58.0 ± 56.2
Albumin	4.0 ± 0.3	4.0 ± 0.3	4.0 ± 0.4
Total cholesterol	171.8 ± 31.7	171.5 ± 32.3	172.2 ± 30.8
HDL cholesterol	50.9 ± 14.5	51.1 ± 14.3	50.5 ± 15.0
LDL cholesterol	95.5 ± 27.7	96.1 ± 27.9	94.1 ± 27.2
Triglyceride	108.8 ± 55.4	107.8 ± 57.3	110.9 ± 51.7
Glucose	111.2 ± 39.0	111.7 ± 39.8	110.3 ± 37.6
Alpha-fetoprotein	14.5 ± 43.9	13.3 ± 37.7	16.8 ± 54.1
White blood cell count (/μl)	4946 ± 1427	4851 ± 1355	4999 ± 1464
Hemoglobin (g/dl)	14.2 ± 1.4	14.2 ± 1.4	14.2 ± 1.4
Platelets (10^9/mm^3)	166.1 ± 51.4	165.6 ± 51.7	166.4 ± 51.2
Ccr (ml/min)	95.1 ± 26.5	94.8 ± 25.9	95.4 ± 26.9
HCV RNA (KIU/ml)	1978 ± 1442	1937 ± 1382	2001 ± 1476
Fibrosis stage (F0–2/F3–4/ND)	695/148/238	454/85/152	241/63/86
Activity (A0–1/2–3/ND)	457/383/241	295/241/154	162/141/87
PEG-IFN alpha-2b dosage (μg/kg/body weight)	1.48 ± 0.13	1.49 ± 0.13	1.48 ± 0.13
Ribavirin dosage (600/800/1000 mg)	581/457/43	370/298/23	211/159/20
Decline of Hb at week 1	−0.2 ± 0.8	−0.2 ± 0.8	−0.2 ± 0.8
Decline of Hb at week 2	−1.2 ± 1.2	−1.2 ± 1.3	−1.2 ± 1.1
Decline of Hb at week 4	−2.3 ± 1.5	−2.2 ± 1.4	−2.4 ± 1.5
Decline of Hb at week 8	−2.8 ± 1.4	−2.8 ± 1.4	−2.7 ± 1.4

Data are expressed as median ± standard deviation unless otherwise indicated

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma-glutamyltransferase, *Ccr* creatinine clearance, *Hb* hemoglobin

were quantified by Cobas Amplicor (Roche Diagnostic Systems, CA, USA).

Database of variables and decision-tree analysis

A database of pretreatment variables was created containing 3 variables from hematological tests (Hb, white blood cells, and platelets), 11 variables from blood biochemical tests (creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, fasting blood glucose, and alpha-fetoprotein), creatinine clearance (Ccr), serum level of HCV RNA, liver histology (activity, fibrosis), 3 variables from patient characteristics (age, gender, and body mass index), 2 variables from therapeutic factors (PEG-IFN alpha-2b dosage, ribavirin dosage), and the level of decline of Hb concentration (at the end of 1, 2, 4, and 8 weeks from the start of treatment). Ccr levels were calculated using the Cockcroft–Gault formula [25]. Variables with data deficiency of greater than 15% were not included in the decision-tree analysis. Data deficiency was 21% in liver histology (activity, fibrosis) and 16% in the level of decline of Hb concentration at the end of 1 week. Accordingly, these variables were excluded from the database.

On the basis of this database, we implemented the recursive partitioning analysis algorithm referred to as the decision-tree analysis algorithm [26] to define subgroups of patients with respect to the possibility of severe anemia. The data-mining software used was IBM SPSS Modeler 13 (IBM SPSS Inc, Chicago, IL, USA), as reported previously [21–24]. In brief, the software searched the patient population for the most significant variables and cutoffs to be used for dividing the total population into 2 subgroups, having different probabilities of severe anemia. Thereafter,

the analysis was repeated on all subgroups in the same manner until either no additional significant variable was detected or the sample size was less than 20.

For other statistical analyses, including multivariable analysis, IBM SPSS Statistics software v.15.0 (IBM SPSS Inc, Chicago, IL, USA) was used. Differences in proportions were tested by the chi-squared test. Differences in continuous variables were compared by Student's t test. For univariate and multivariate analyses, logistic regression analysis was used to predict ribavirin-induced severe anemia. A value of $P < 0.05$ (two-tailed) was considered to indicate significance.

Results

Decision-tree analysis

Decision-tree analysis was carried out on the data of the model-building group using 27 variables, as described above. The analysis automatically selected 3 predictive variables to produce a total of 5 patient subgroups to build the decision tree (Fig. 1). Baseline Hb was selected as the first splitting variable, with an optimal cutoff of 14 g/dl. The possibility of severe anemia was 6.5% for patients with Hb levels <14 g/dl compared to 1.0% for patients with Hb levels ≥14 g/dl. Among patients with Hb ≥ 14 g/dl, the level of decline of Hb at the end of 2 weeks from the start of treatment, with an optimal cutoff of 2 g/dl, was selected as the second splitting variable. Patients with lower decline levels had a lower probability of developing severe anemia [<2 g/dl (group A) 0.4% vs. ≥2 g/dl (group B) 2.5%]. Among patients whose Hb was less than 14 g/dl, Ccr was selected as the second splitting variable, with an optimal cutoff of 80 ml/min. Patients with higher Ccr levels had a lower probability of developing severe anemia [≥80 ml/min, 2.4% vs. <80 ml/min (group C) 11.8%]. Among patients with a Ccr ≥ 80 ml/min, the level of decline of Hb at the end of 2 weeks from the start of the treatment was selected as the third splitting variable, with an optimal cutoff of 2 g/dl. Patients with lower decline levels had a lower probability of developing severe anemia [<2 g/dl (group D) 1.4% vs. ≥2 g/dl (group E) 11.5%].

The probabilities of severe anemia for the 5 subgroups derived by this process were highly variable. The subgroup of patients with higher Hb levels (≥14 g/dl) (groups A and B) had a low probability of developing severe anemia (0.4–2.5%). Also, the subgroup of patients with lower Hb (<14 g/dl) but with a higher Ccr (≥80 ml/min) and lower Hb decline levels at the end of 2 weeks from the start of the treatment (<2 g/dl) (group D) showed a low probability of developing severe anemia (1.4%). On the other hand, the

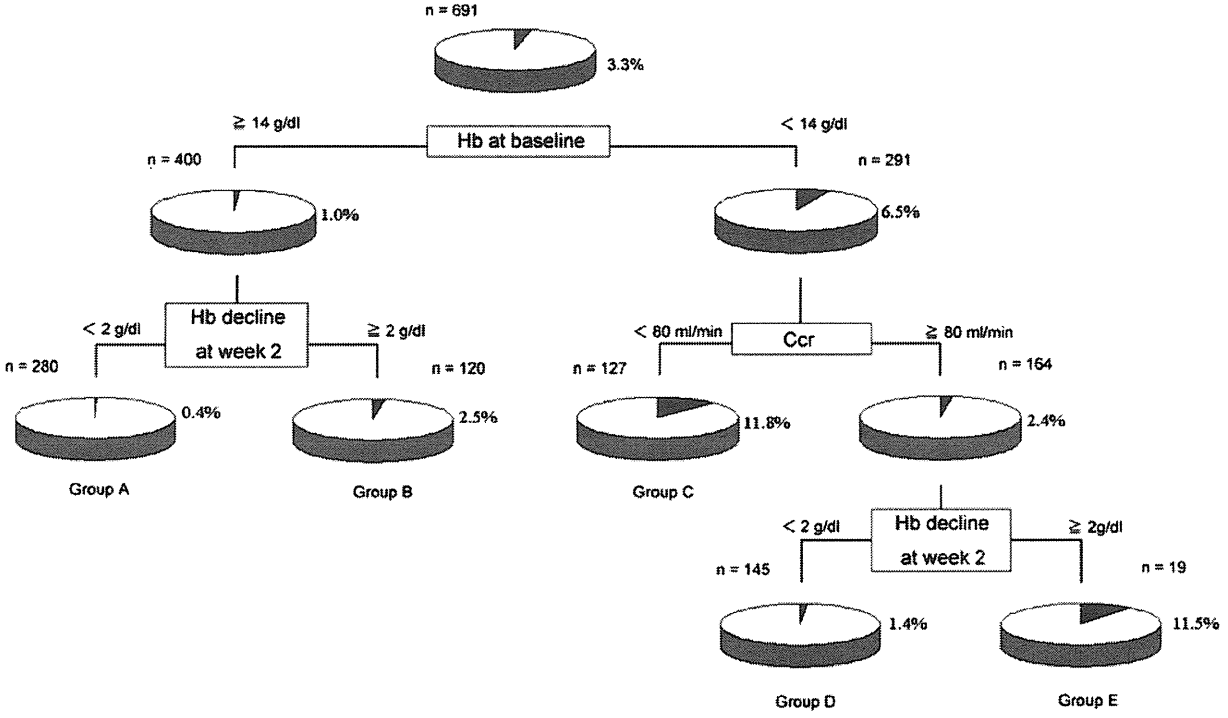

Fig. 1 Decision-tree analysis. *Boxes* indicate the splitting factors and the cutoff value for the split. *Pie charts* indicate the rate of severe anemia (Hb < 8.5 g/dl) for each group. Terminal groups classified by the analysis were labeled from A to E. *Hb* hemoglobin, *Ccr* creatine clearance

subgroup of patients with lower Hb (<14 g/dl) and lower Ccr (<80 ml/min) (group C) levels showed the highest probability of severe anemia (11.8%). Also, the subgroup of patients with lower Hb levels (<14 g/dl), higher Ccr (≥80 ml/min), and higher Hb decline levels at the end of 2 weeks from the start of treatment (≥2 g/dl) (group E) showed a high probability of developing severe anemia (11.5%).

Validation of the decision tree

The results of the decision tree were validated with the dataset of the internal validation group, which was independent of the model-building group dataset. Each patient in the validation group was allocated to groups A–E using the flow chart form of the decision tree. The rates of severe anemia (Hb < 8.5 g/dl) were 0.6% for group A, 3.0% for group B, 16.9% for group C, 2.3% for group D, and 11.0% for group E. The rates of severe anemia for each subgroup of patients were closely correlated between the model-building group and the internal validation group ($r^2 = 0.96$) (Fig. 2).

The efficiency and stability of the decision-tree model were validated using the discrimination efficiency curve (Fig. 3). The subgroups were sorted according to the order of incidence rate of severe anemia and validated using the correlation between the cumulative cases (%) and the cumulative incidence of severe anemia (%). The curve of the model-building group was located at the left upper part compared with the standard curve, indicating that the discrimination efficiency was high. Furthermore, the curve of the model-building group was extremely similar to the curve of the internal validation group, indicating that the stability was high.

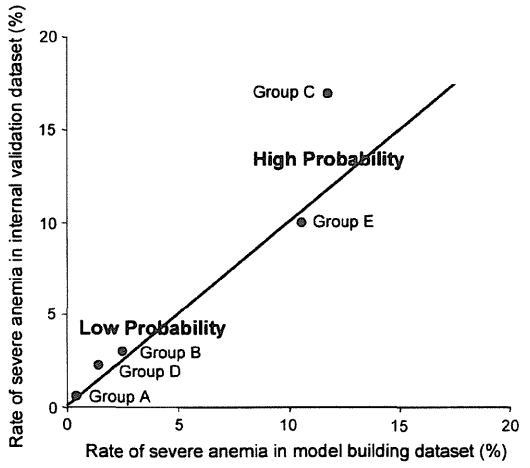

Fig. 2 Validation of decision-tree analysis with the internal validation dataset: subgroup-stratified comparison of the rate of severe anemia. The rate of severe anemia in each subgroup was plotted. The *X*-axis represents the model-building dataset and the *Y*-axis represents the internal validation dataset. There was a close correlation between the model-building and internal validation datasets ($r^2 = 0.96$)

Factors associated with severe anemia determined by multivariate logistic regression analysis

We also explored the factors associated with severe anemia using standard statistical analysis. By univariable analysis, age, creatinine, Hb, Ccr, fibrosis stage, and decline of Hb at 2, 4, and 8 weeks from the start of treatment were found to be associated with severe anemia (Table 2) and the odds ratio for these factors were 1.06, 9.61, 0.47, 0.95, 3.14, 0.76, 0.70, and 0.68, respectively, by univariable logistic regression analysis (Table 3). By multivariate analysis, Hb, Ccr, and decline of Hb at 2 weeks from the start of treatment were found to be independently associated with severe anemia (Table 3). Fibrosis was not included in the multivariable analysis because data were not available for 238 patients. Creatinine was not included in the multivariable analysis because creatinine and Ccr were confounding factors due to their close correlation. Decline of Hb at week 2, 4, and 8 were also closely correlated. We selected decline of Hb at week 2 in the multivariable analysis because we think that variables at earlier time points may be more useful in clinical use. As a result, decision-tree and multivariable logistic regression analyses identified the same factors for prediction of severe anemia.

Discussion

Hemolytic anemia, a major common side effect of ribavirin treatment, is one of the most important adverse effects of PEG-IFN and ribavirin combination treatment. Therefore, before- and during-treatment prediction of the likelihood of severe anemia can be very useful for physicians to support clinical decisions concerning the dose reduction of ribavirin. Reducing the dose of ribavirin has been shown to affect the HCV RNA negativity [27], and the discontinuation of ribavirin has been reported to lead to a marked decrease of SVR [9]. Therefore, averting ribavirin discontinuance, even if its dose must be reduced, can lead to an improvement in the SVR rate. It is important to identify patients prone to develop severe anemia leading to ribavirin dose reduction or discontinuance in the early phase of treatment.

Using decision-tree analysis, we constructed a simple model for predicting the incidence of severe anemia during therapy. The analysis highlighted 3 variables relevant to virological response: Hb, Ccr, and the decline of Hb concentration by 2 g/dl at the end of the 2 weeks from the start of treatment. Classification based on these variables identified subgroups of genotype 1b chronic hepatitis C patients with high probabilities of developing severe anemia. The