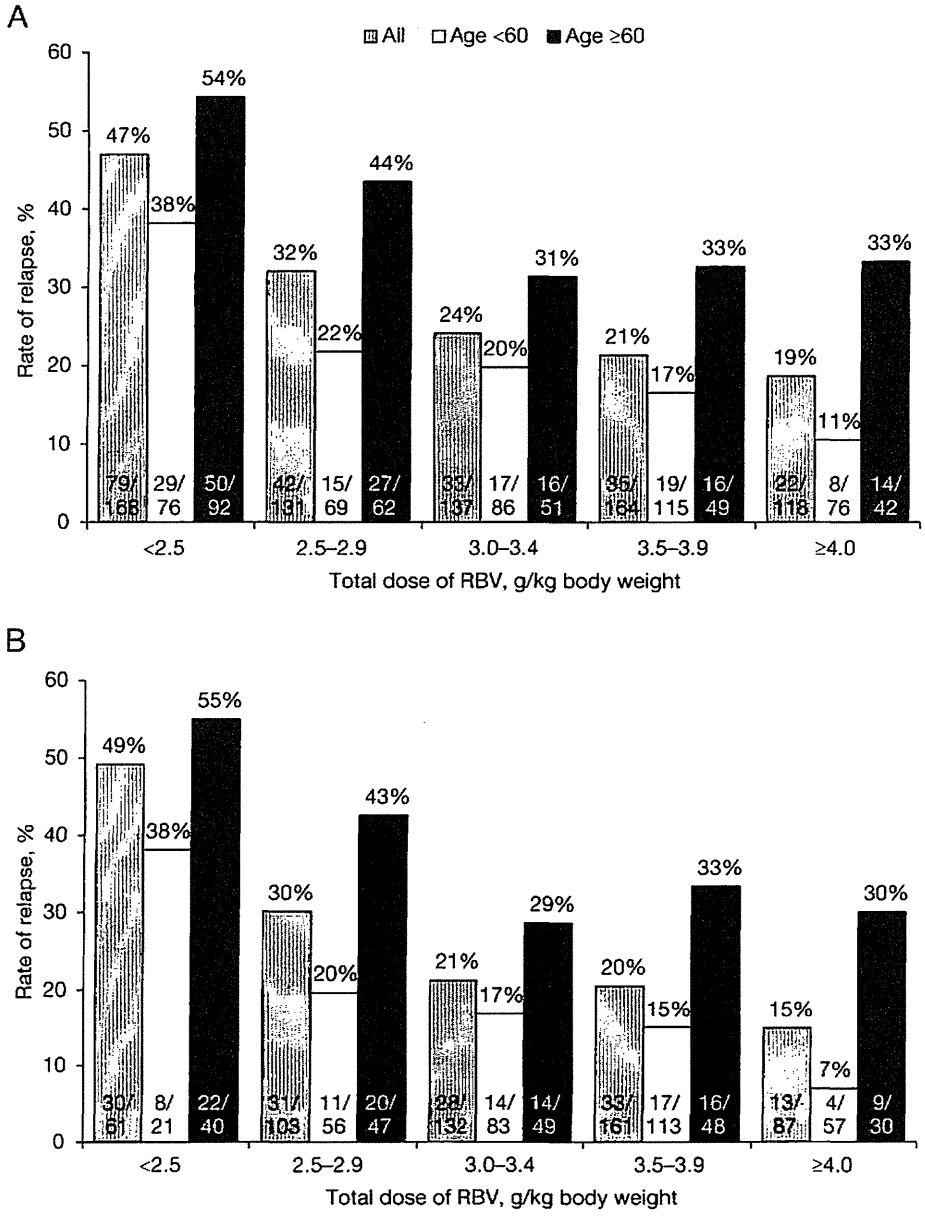
RBV dose (P=0.283 for RBV <2.5 g/kg, P=0.017 for RBV 2.5–2.9 g/kg, P=0.127 for RBV 3.0–3.4 g/kg, P=0.011 for RBV 3.5–3.9 g/kg and P=0.009 for RBV ≥4.0 g/kg).

Total dose of RBV was associated with relapse independently of PEG-IFN dose. The cutoff value of 58 zμg/kg of PEG-IFN was selected, which corresponds to the 80% of 1.5 μg/kg dose for 48 weeks. In patients who received <58 μg/kg of body weight of PEG-IFN, the rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 24% and 42%, respectively. In patients who received ≥58 μg/kg of body weight of PEG-IFN, the rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 21% and 38%, respectively.

The data mining analysis procedure did not select further split variables among RVR patients. However,

Figure 3. Correlation between the rate of relapse and total RBV dose among patients with cEVR after stratification by age



Association between the total ribavirin (RBV) dose and the rate of relapse among patients with complete early virological response (cEVR) is shown. **(A)** Higher dose of RBV was associated with reduced rate of relapse. **(B)** These associations were also confirmed in selected patients who received 42–54 weeks of therapy.

when analysed separately, the rate of relapse was also associated with age and total RBV dose among patients with RVR. The rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 5% and 14%, respectively. The rate of relapse for patients <60 and ≥60 years was 9% and 18%, respectively. Collectively, the rate of relapse for patients <60 years who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 2% and 11%, respectively, whereas the rate of relapse for patients ≥60 years who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 12% and 20%, respectively.

Discussion

The result of the present study shows that older age and insufficient dose of RBV are significant and independent risk factors for relapse among patients with cEVR to PEG-IFN plus RBV. Older patients (≥60 years) who received a total RBV dose <3.0 g/kg of body weight had the highest risk of relapse (52%), whereas younger patients who received a total RBV dose ≥3.0 g/kg of body weight had the lowest risk of relapse (16%). The rate of relapse decreased depending on the total RBV dose in younger patients, but remained stable in older patients despite a further increase in the RBV dose beyond 3.0 g/kg of body weight. These findings imply that the target dose of total RBV can be set at 3.0 g/kg of body weight in patients who achieved cEVR, and further increase in RBV dose up to 4.0 g/kg of body weight or greater may be recommended in patients <60 years.

The associations between the drug adherence and virological response had been reported with inconsistent results. In an earlier study, patients who received >80% of the planned dose of PEG-IFN plus RBV for >80% of the planned duration of therapy had a higher rate of SVR compared to those who received a lesser dose (51% versus 34%) [31]. Consistent results were obtained in a study reporting that patients who received >80% of the planned dose of PEG-IFN and RBV within the first 12 weeks of therapy had a higher rate of EVR compared with those who received a lesser dose of both drugs (80% versus 33%) [4]. By contrast, a large-scale multicentre study showed that reducing the PEG-IFN dose during the first 20 weeks reduced SVR; however, reducing RBV did not affect SVR as long as RBV was not prematurely discontinued [32]. The reason for these inconsistencies is unclear. One reason may be the differences in the backgrounds of patients enrolled in the study, and hence the last study was limited to patients with advanced fibrosis and prior non-responders to PEG-IFN therapy. Because the probability of SVR is affected by virological response and relapse after response, the effect of drug dosing should be analysed separately with respect to these two factors.

In the present study, we focused on factors predictive of relapse after early virological response. According to the decision tree model, relapse was less likely in patients with RVR compared with cEVR. Among patients with cEVR, older patients (≥60 years) had a higher risk of relapse compared to younger patients (41% versus 22%). In addition, our results emphasized the effect of RBV dose for the prevention of relapse. In our study, a total RBV dose of ≥3.0 g/kg of body weight was repeatedly associated with a suppressed rate of relapse in the model derivation and validation groups. The rate of relapse in patients <60 years who received an RBV dose of <3.0 versus ≥3.0 g/kg of body weight in the model derivation, internal validation and external validation groups were 32% versus 16%, 27% versus 16%, and 41% versus 16%, respectively. The rate of relapse in patients ≥60 years who received an RBV dose of <3.0 versus ≥3.0 g/kg of body weight in the model derivation, internal validation and external validation groups were 52% versus 26%, 45% versus 38%, and 44% versus 22%, respectively. It has been reported that the rate of relapse is suppressed in 48 weeks of IFN plus RBV combination therapy compared to IFN monotherapy, indicating that RBV contributes to the increase in SVR by reducing relapse [2,3]. Another study, focused on the associations between the drug dose reduction and relapse in patients with virological response, found that maintaining RBV dose ≥12 mg/kg/day during 48 weeks of treatment, which can be translated into a total dose of 4.0 g/kg of body weight, suppressed relapse [33]. Results of the present study are in accordance with this report.

The importance of drug dosing on reduction in relapse is also supported by the findings that extending therapy from 48 to 72 weeks in patients with delayed virological response improved SVR rates by reducing relapse [9–13]. Apart from these clinical studies, in the real world of clinical practice, duration of therapy is extended – even in patients with cEVR – at the physician's discretion. The relationship between duration of therapy or RBV dose, and relapse among patients with cEVR and treated with various lengths of therapy has not been examined. In the combined group of our study, extending the duration of therapy was not associated with a reduction in relapse rate. Rather, the rate of relapse decreased depending on the total RBV dose. These findings suggest that acquiring a sufficient total RBV dose, either within 48 weeks or by extending the duration of therapy, is essential to prevent relapse among patients with cEVR. The limitation of the present study was that the mean duration of therapy was only 56.3 weeks in patients whose duration of therapy was extended beyond 48 weeks. It is probable that extended duration of therapy was not long enough for the prevention of relapse. Further studies with

longer durations of therapy are necessary to confirm the effect of extended duration of therapy on reduction of relapse among patients with cEVR.

Previous reports did not consider the effects of age in setting the optimal dose of RBV. In the present study, the relapse rate decreased with an increase in RBV dose from <2.5 to 3.0–3.5 g/kg of body weight, but remained relatively stable despite a further increase in the RBV dose in older patients. Thus, a total RBV dose ≥3.0 g/kg of body weight should be the target dose for patients ≥60 years with cEVR. By contrast, ≥3.0 g/kg of body weight of RBV was associated with lower risk of relapse in patients <60 with cEVR (16% versus 32%), and a further increase in RBV dose led to a more profound reduction in relapse rates, as low as 11% in patients who received ≥4.0 g/kg of body weight. Thus, a total dose of ≥4.0 g/kg of body weight or even greater should be the target dose in patients <60 years.

In the near future, more potent therapies, such as direct antiviral agents [34,35], may become available. These drugs require RBV and PEG-IFN in combination. However, not all patients may be able to tolerate this triple combination therapy due to adverse drug reactions, such as severe anaemia or skin eruption. In particular, it may be difficult to administer a full dose of triple drugs to older patients. Thus, personalizing the PEG-IFN and RBV combination therapy based on this model may be beneficial to patients who were intolerant to triple combination therapy.

In the present study creatinine was an independent predictor of relapse by multivariable logistic regression analysis. However creatinine was not selected as a splitting variable in decision tree, which may be due to the unique property of data mining analysis. In data mining analysis, limitation is imposed to stop the analysis when the number of patients is <20. This limitation is used to avoid dividing patients into too small subgroups which lead to the generation of rules that only apply to the model derivation population and not reproduced when applied to other populations. This phenomenon is called the over-fitting of the model. Due to this limitation, the variables selected in the data mining analysis are not necessarily identical to the variables that are significant by ordinary multivariable analysis. In a separate analysis, lower level of creatinine was associated with higher rate of relapse in each subgroup of patients with cEVR. The reason for this association is not clear, but lower creatinine level may be related to more efficient clearance of RBV leading to lower serum level of RBV. Further research is needed to confirm this speculation.

A potential limitation of the present study is that data mining analysis has an intrinsic risk of showing relationships that fit to the original dataset, but are not reproducible in different groups. Although internal and external validations showed that our model had high reproducibility, we recognized that further validation on a larger external validation cohort, especially in groups other than Japanese, may be necessary to further verify the reliability of our model.

In conclusion, we built a decision tree model for the prediction of relapse among patients with EVR to PEG-IFN plus RBV. The result of the present study shows that older age and insufficient dose of RBV are significant and independent risk factors for relapse. The target dose of total RBV can be set at 3.0 g/kg of body weight in patients who achieved cEVR. A further increase in RBV dose up to 4.0 g/kg of body weight may be warranted in patients <60 years.

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Disclosure statement

The authors declare no competing interests.

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Research Article

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Data mining model using simple and readily available factors could identify patients at high risk for hepatocellular carcinoma in chronic hepatitis C

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Background & Aims: Assessment of the risk of hepatocellular carcinoma (HCC) development is essential for formulating personalized surveillance or antiviral treatment plan for chronic hepatitis C. We aimed to build a simple model for the identification of patients at high risk of developing HCC.

Methods: Chronic hepatitis C patients followed for at least 5 years (n = 1003) were analyzed by data mining to build a predictive model for HCC development. The model was externally validated using a cohort of 1072 patients (472 with sustained virological response (SVR) and 600 with nonSVR to PEG-interferon plus ribavirin therapy).

Results: On the basis of factors such as age, platelet, albumin, and aspartate aminotransferase, the HCC risk prediction model identified subgroups with high-, intermediate-, and low-risk of HCC with a 5-year HCC development rate of 20.9%, 6.3–7.3%, and 0–1.5%, respectively. The reproducibility of the model was confirmed through external validation ($r^2 = 0.981$). The 10-year HCC development rate was also significantly higher in the high- and intermediate-risk group than in the low-risk group (24.5% *vs.* 4.8%; $p < 0.0001$). In the high-and intermediate-risk group, the incidence of HCC development was significantly reduced in patients with SVR compared to those with nonSVR (5-year rate, 9.5% *vs.* 4.5%; $p = 0.040$).

Conclusions: The HCC risk prediction model uses simple and readily available factors and identifies patients at a high risk of HCC development. The model allows physicians to identify patients requiring HCC surveillance and those who benefit from IFN therapy to prevent HCC.

Keywords: Decision tree; Prediction; Pegylated interferon; Ribavirin; Risk.
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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide [1] and its incidence is increasing in many countries [2]. Chronic viral hepatitis is responsible for 80% of all HCC cases [2]. The need to conduct HCC surveillance should be determined according to the risk of HCC development because this surveillance is cost-effective only in populations with an annualized cancer development rate of ⩾1.5% [3]. The annualized rate of developing HCC from type C liver cirrhosis is 2–8% [4–6], indicating that this population with type C liver cirrhosis needs surveillance. However, the annualized rate of HCC development is <1.5% in patients with chronic hepatitis C but without cirrhosis and the benefit of surveillance for all patients with chronic hepatitis has not yet been established [3]. HCC surveillance may be needed for patients with advanced fibrosis because the risk of HCC development increases in parallel with the progression of liver fibrosis [7,8]. Liver biopsy is the most accurate means of diagnosing fibrosis, but a single liver biopsy cannot indicate long-term prognosis because liver fibrosis progresses over time. Serial liver biopsies are not feasible because of the procedure's invasiveness. Moreover, factors other than fibrosis, such as advanced age, obesity, sex, lower albumin, and low platelet counts, also contribute to the development of HCC from chronic hepatitis C [8–11]. Therefore, these factors must be considered while assessing the risk of HCC development.

A meta-analysis of controlled trials [12] has shown that interferon (IFN) therapy reduced the rate of HCC development in patients with type C liver cirrhosis. However, there was a marked heterogeneity in the magnitude of the prevention effect

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of IFN on HCC development among the studies, probably due to the large differences in the baseline rate of HCC development among the different trials [12]. Whether the incidence of HCC development could be reduced in all patients with chronic hepatitis C, especially in those without liver cirrhosis, remains to be elucidated.

Data mining analysis, unlike conventional statistical analysis, is performed in an exploratory manner without considering a predefined hypothesis. Decision tree analysis, the major component of data mining analysis, is used to extract relevant factors from among various factors. These relevant factors are then combined in an orderly sequence to identify rules for predicting the incidence of the target outcome [13]. Data mining analysis has been used to define prognostic factors in various diseases [14–20]. In the field of hepatic diseases, data mining analysis has proven to be a useful tool for predicting early response [21], sustained virological response (SVR) [22–25], relapse [26], and adverse events [27] in patients with chronic hepatitis C treated with pegylated interferon (PEG-IFN) plus ribavirin (RBV). The findings of data mining analysis are expressed as flowcharts and are therefore easily understood [28] and readily available for clinical use, even by physicians without a detailed understanding of statistics.

In the present study, data mining analysis was used to identify risk factors for HCC development in a cohort of patients with chronic hepatitis C who had been followed for at least 5 years. An HCC risk prediction model was constructed on the basis of simple and generally available tests because the goal was to make the model easy to use in the clinic. The suitability, reproducibility, and generalizability of the results were validated using the data of an external cohort that was independent of the model derivation cohort.

Materials and methods

Patients

The model derivation cohort consisted of 1003 chronic hepatitis C patients without cirrhosis who had a non-sustained virological response (nonSVR) to previous IFN administered at the Musashino Red Cross Hospital and were followed for at least 5 years. Patients who had SVR or those who were followed for less than 5 years were not included. An analytical database on age, body mass index, albumin, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, γ-glutamyltransferase (GGT) levels, total bilirubin levels, total cholesterol levels, hemoglobin levels, and platelet count at the start of the observation was created. Histological data such as fibrosis stage, activity grade, or degree of steatosis was not included in the database because the goal of the present study was to make the model on the basis of simple and generally available tests. The patients who developed HCC more than 5 years after the start of the observation were considered not to have developed HCC by the 5-year point because the model was intended to predict HCC development within 5 years. The 1072 chronic hepatitis C patients included in the external validation cohort were treated with PEG-IFN and RBV at the University of Yamanashi, Tokyo Medical and Dental University, Osaka University, Osaka City University, Nagoya City University, or Toranomon Hospital and followed for at least 5 years. Among them, 600 had nonSVR and 472 had SVR. Data from nonSVR patients in this external cohort were used for external validation of the HCC prediction model. To assess the preventive effect of PEG-IFN plus RBV therapy on HCC development, the cumulative HCC development rate was compared between SVR and nonSVR patients in the external validation cohort after stratification by the risk of HCC development as determined by data mining analysis. Informed 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 committees of all concerned hospitals.

HCC surveillance and diagnosis

HCC surveillance was conducted by performing abdominal ultrasonography every 4–6 months. Contrast-enhanced computer tomography, magnetic resonance imaging, or angiography were performed when abdominal ultrasonography suggested a new lesion suspicious for HCC. Classical HCC was diagnosed for tumors showing vascular enhancement with washout on at least two types of diagnostic imaging. Tumor biopsy was used to diagnose tumors with non-classical imaging findings.

Statistical analysis

The IBM-SPSS Modeler 13 (IBM SPSS Inc., Chicago, IL, USA) was used for decision tree analysis. The statistical methods used have been described previously [21,22,24–27]. In brief, the software searched the analytical database for the factor that most effectively predicted HCC development and for its cutoff value. The patients were divided into two groups according to that predictor. Each divided group was repeatedly assessed and divided according to this 2-choice branching method. Branching was stopped when the number of patients decreased to $\leqslant 20$ to avoid over fitting. Finally, an HCC risk prediction model was created through this analysis. The model classified patients into subgroups with different HCC development rates in a flowchart form. For model validation, nonSVR patients from an external cohort were individually fitted into the model and classified into the subgroups and the HCC development rates of those subgroups were then calculated. The suitability and reproducibility of the model were validated by comparing the subgroup HCC development rates of the model derivation group to those of the validation group.

On univariate analysis, Student's *t*-test was used for continuous variables and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. A log-rank test for Kaplan–Meier analysis was used to statistically test HCC development rates over time. *p*-Values of <0.05 were considered significant. SPSS Statistics 18 (IBM SPSS Inc.) was used for these analyses.

Results

Univariate and multivariate analysis of factors associated with HCC development

The baseline characteristics of patients are shown in Table 1. The 5-year HCC development rate in the model derivation group was 6.2%, which did not differ significantly from the rate of 6.0% in the nonSVR group of the external cohort, but the rate of 2.0% in the SVR group of the external cohort was significantly lower than that in the model derivation group ($p = 0.0003$) and the nonSVR group of the external cohort ($p = 0.0012$). On univariate analysis, the factors found to be associated with HCC development in the model derivation cohort were age, AST levels, albumin levels, total cholesterol levels, and platelet count. On multivariate analysis, age (odds ratio 1.086), albumin levels (odds ratio 0.248), and platelet count (odds ratio 0.842) were significant predictors of HCC development (Table 2).

HCC risk prediction model by data mining analysis

The results of decision tree analysis are presented in Fig. 1. Age was selected as the first predictor. The 5-year HCC development rate was 3.4% in younger patients (<60 years) and 8.6% in older patients ($\geqslant$60 years). The second predictor for younger patients (<60 years) was platelet count. The HCC development rate was 6.9% in patients with a lower platelet count ($<150 \times 10^9$/L) and 0.8% in patients with a higher count ($\geqslant 150 \times 10^9$/L). The second predictor for older patients ($\geqslant$60 years) was also platelet count. The HCC development rate was 13.1% in patients with a lower platelet count ($<150 \times 10^9$/L) and 1.8% in patients with a higher count ($\geqslant 150 \times 10^9$/L). The third predictor was albumin levels,

Table 1. Baseline characteristics of patients for model deviation and external validation.

	Model derivation (n = 1003)	External cohort, non-SVR (n = 600)	External cohort, SVR (n = 472)
Sex: Male/Female*	463 (46%)/540 (54%)	306 (51%)/294 (49%)	299 (63%)/173 (37%)
Age (yr)	57.3 (11.1)	55.9 (9.6)	51.4 (10.6)
Body mass index (kg/m^2)	23.5 (3.2)	23.4 (3.3)	23.3 (3.1)
Albumin (g/dl)	4.1 (0.3)	4.0 (0.4)	4.0 (0.3)
AST (IU/L)	64.2 (36.5)	67.3 (43.8)	62.5 (48.3)
ALT (IU/L)	80.6 (55.1)	81.2 (62.3)	88.6 (82.1)
GGT (IU/L)	59.3 (50.5)	67.6 (65.1)	55.7 (71.2)
Total cholesterol (mg/dl)	172.1 (31.5)	168.2 (31.0)	174.3 (33.7)
Platelet (10^9/L)	154.0 (53.0)	153.7 (53.2)	176.6 (49.7)
Hemoglobin (g/dl)	13.3 (1.5)	14.2 (1.5)	14.4 (1.4)
HCC development within 5 years: n (%)*	62 (6.2%)	36 (6.0%)	10 (2.0%)

Data expressed as mean (standard deviation) unless otherwise indicated.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.
*Data expressed as number of patients (percentage).

whose cutoff value was 3.75 g/dl in patients with a higher platelet count ($\geq 150 \times 10^9$/L). The HCC development rate was 6.3% when albumin levels were lower (<3.75 g/dl) and 1.5% when levels were higher ($\geq$3.75 g/dl). The cutoff value for albumin levels was 4.0 g/dl in patients with a lower platelet count ($<150 \times 10^9$/L). The HCC development rate was 20.9% when albumin levels were lower (<4.0 g/dl) and 6.4% when levels were higher ($\geq$4.0 g/dl). The fourth and final predictor was AST levels. The HCC development rate was 7.3% when AST levels were at least 40 IU/L and 0% when the levels were <40 IU/L. On the basis of this analysis, seven subgroups with a 5-year HCC development rate of 0–20.9% were identified. The area under the receiver operating characteristic curve according to the HCC risk prediction model was 0.817.

External validation of the HCC risk prediction model with an independent external cohort

Six hundred nonSVR patients from an external cohort were fitted into the HCC risk prediction model and classified into the seven subgroups. The 5-year HCC development rate of these subgroups was 0–17.9%. The HCC development rate in the individual subgroups of the model derivation group was closely correlated to that in the corresponding subgroups of the external validation group (Fig. 2; correlation coefficient $r^2 = 0.981$). The HCC development rate in the subgroup of patients with the highest risk of HCC development (high-risk group) according to the model older age ($\geq$60 years) with a lower platelet count ($<150 \times 10^9$/L) and lower albumin levels (<4.0 g/dl) was 20.9% in the model derivation group and 17.9% in the external validation group. The intermediate-risk group or the patients with an HCC development rate of at least 5% consisted of the following three subgroups: (1) older age ($\geq$60 years), lower platelet count ($<150 \times 10^9$/L), higher albumin levels ($\geq$4.0 g/dl), and higher AST levels ($\geq$40 IU/L); (2) older age ($\geq$60 years), higher platelet count ($\geq 150 \times 10^9$/L), and lower albumin levels (<3.75 g/dl); and (3) younger age (<60 years) and lower platelet count ($<150 \times 10^9$/L). In these intermediate-risk groups, the 5-year HCC development rate was 6.3–7.3% in the model derivation group and 5.3–7.9% in the external validation group. The low-risk group consisted of the following three subgroups: (1) younger age (<60 years) and higher platelet count ($\geq 150 \times 10^9$/L); (2) older age ($\geq$60 years), lower platelet count ($<150 \times 10^9$/L), higher albumin levels ($\geq$4.0 g/dl), and lower AST levels (<40 IU/L); and (3) older age ($\geq$60 years), higher platelet count ($\geq 150 \times 10^9$/L), and higher albumin levels ($\geq$3.75 g/dl). In these low-risk groups, the 5-year HCC development rate was 0–1.5% in the model derivation group and 0–2.9% in the external validation group.

Table 2. Multivariable analysis of factors associated with subsequent development of HCC within 5 years.

	Odds ratio	95% CI	*p* value
Age	1.086	1.029-1.146	0.003
Albumin	0.248	0.100-0.613	0.003
Platelet	0.842	0.769-0.921	<0.0001

CI, confidence interval.

Predictability of the HCC risk prediction model on HCC development rate beyond 5 years

Cumulative HCC development rates in the high-, intermediate-, and low-risk groups were compared over time using the Kaplan–Meier method. The 10-year rates were 28.9% in the high-risk group, 22.9% in the intermediate-risk group, and 4.8% in the low-risk group (Fig. 3A). The high and intermediate-risk group created by pooling data from the high- and intermediate-risk groups had a significantly higher cumulative HCC development rate than the low-risk group beyond 5 years (Fig. 3B; 5-year rate, 11.6% *vs.* 1.0%; 10-year rate, 24.5% *vs.* 4.8%; p <0.0001).

Effect of response to PEG-IFN plus RBV therapy in the reduction of HCC development: analysis stratified by the HCC risk prediction model

The 600 nonSVR patients and 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and

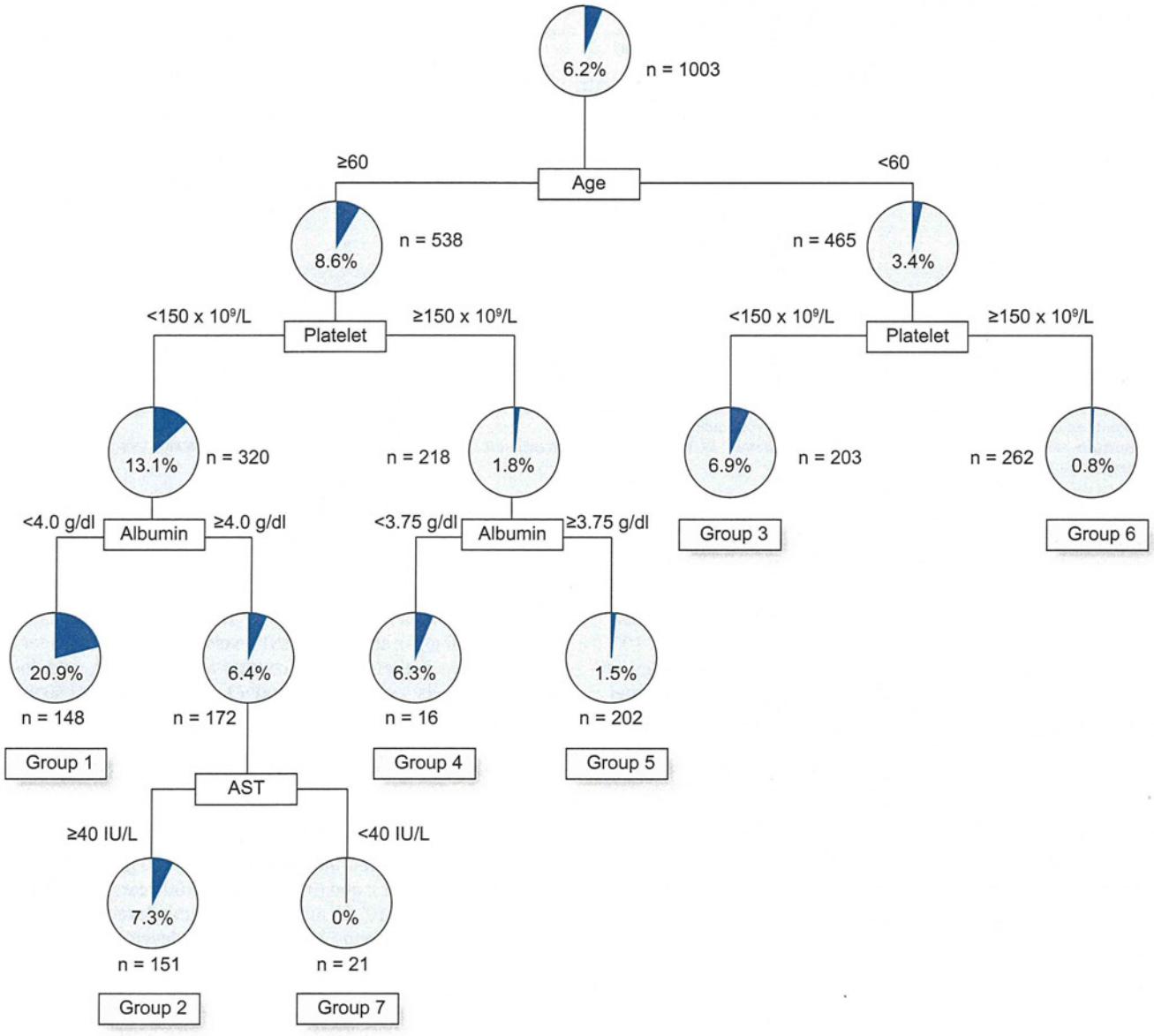

Fig. 1. The decision tree model of HCC development within 5 years. Boxes indicate the factors used to differentiate patients and the cutoff values for those different groups. Pie charts indicate the HCC development rate within 5 years for each group of patients after differentiation. Terminal groups of patients differentiated by analysis are numbered from 1 to 7.

classified into the high-and intermediate-risk group or the low-risk group, as defined above. The HCC development rate was significantly lower in SVR patients than in nonSVR patients in the high-and intermediate-risk group (5-year HCC rate, 9.5% *vs.* 4.5%; $p = 0.040$, log-rank test). In the low-risk group, the 5-year rate was 1.8% in nonSVR patients and 0.9% in SVR patients. Both rates were low and not significantly different ($p = 0.331$, log-rank test) (Fig. 4).

Discussion

An awareness of the risk of HCC development in the context of routine care for chronic hepatitis C is essential for formulating an HCC surveillance plan personalized for individual patients. The risk of developing HCC from chronic hepatitis is lower than that from cirrhosis [7]; therefore, across-the-board surveillance for chronic hepatitis C is not recommended [3]. A method to easily determine this risk, without performing serial liver biopsies, would be extremely significant clinically. In the present study, an HCC risk prediction model that included the factors such as age, platelet count, albumin levels, and AST levels was constructed. The model was found to have excellent reproducibility when validated with an external cohort. This model could identify subgroups of chronic hepatitis C patients at high risk of HCC development; the 5-year HCC development rate for the high- and intermediate-risk groups was 11.6%, yielding an annual incidence of 2.3%. This HCC risk prediction model requires only

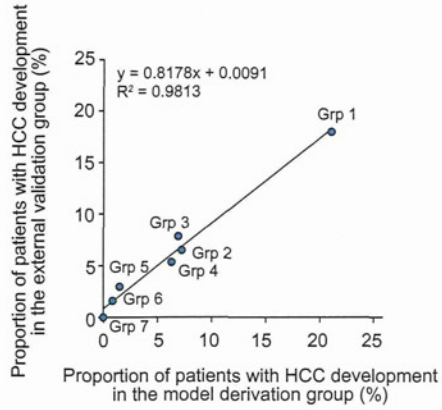

Fig. 2. External validation of the decision tree model with an independent cohort. Each patient in the external validation group was allocated to groups 1–7 following the flowchart of the decision tree. The HCC development rates were then calculated for each group and the graph plotted. The *x*-axis represents the HCC development rate in the model derivation group, and the *y*-axis represents the HCC development rate in the external validation group. The HCC development rates in each subgroup of patients are closely correlated between the model derivation group and the external validation group (correlation coefficient: $R^2 = 0.981$).

simple test values that are readily obtained in routine care and can therefore be easily used at the patient bedside. The model can be used to identify patients with a high risk of HCC development and therefore requiring surveillance, thereby allowing the formulation of surveillance plans personalized for individual patients.

Advanced fibrosis has been reported as independent risk factors for HCC development [7,8]. Platelet counts and albumin levels, which were factors selected for discrimination of the risk of HCC development, are closely related to the stage of fibrosis. Their correlation with the HCC risk has been repeatedly demonstrated [9–11,29–31]. The present study confirmed the impact of old age and advanced fibrosis, as reflected by low platelet counts and albumin levels. These results are consistent with our previous report [32]. What is unique to the present study was the study design to build a simple and reliable model for

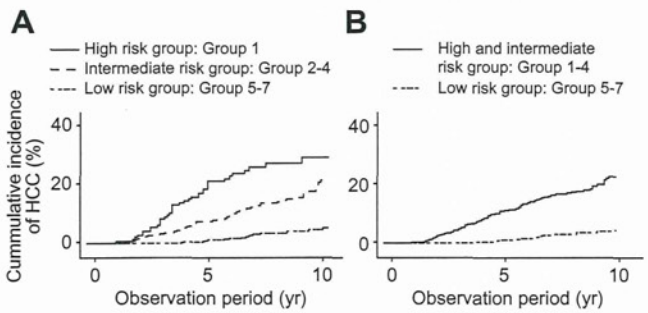

Fig. 3. Cumulative incidence of HCC development beyond 5 years in subgroups of patients defined by the decision tree model. Cumulative incidences of HCC in the groups classified by the decision tree model are compared. (A) The cumulative HCC development rate beyond 5 years is higher in the high- (group 1) and intermediate-risk (groups 2–4) groups compared to the low-risk group (groups 5–7). (B) The high and intermediate-risk group created by pooling data from the high- and intermediate-risk groups has a significantly higher cumulative HCC development rate than the low-risk group (5-year rate, 11.6% *vs.* 1.0%; 10-year rate, 24.5% *vs.* 4.8%; $p < 0.0001$).

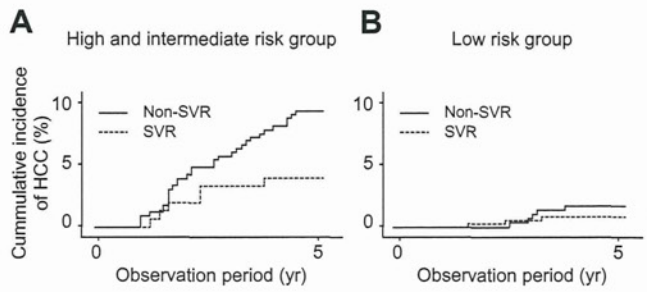

Fig. 4. Sustained virological response to PEG-IFN plus RBV therapy reduces the incidence of HCC development after stratification by the HCC risk. The 600 nonSVR patients and the 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and classified into the high and intermediate-risk group or the low-risk group. The HCC development rate is significantly lower in SVR patients than in nonSVR patients in the high and intermediate-risk group (groups 1–4) (5-year HCC rate, 9.5% *vs.* 4.5%; $p = 0.040$). In the low-risk group (groups 5–7), the 5-year rate is 1.8% in nonSVR patients and 0.9% in SVR patients. Both rates are low and not significantly different ($p = 0.331$).

the prediction of HCC development that could be easily used in the clinic. For this purpose, a novel statistical method was used, histological factors were excluded in the analysis, the model derivation cohort was restricted to those who had nonSVR and had a long follow-up period duration (5 years), and the reproducibility of the model was independently validated by an external cohort. These are the major differences of the present study compared to our previous report. Many researchers have put a lot of efforts to formulate regression models for HCC prediction [9,10,33]. These prediction models are useful for identifying high-risk patients but are somewhat complicated to use at the bedside because they require calculations to be performed. Our prediction model is used simply by incorporating patients' data obtained through simple tests into the decision tree and following the flowchart. These prediction models based on factors easily accessible in routine clinical settings help physicians identify high-risk patients out of chronic hepatitis.

Viral eradication is the short-term goal of IFN therapy, but the ultimate goal is the prevention of HCC occurrence. Previous reports have shown that SVR to IFN therapy suppresses HCC occurrence in patients with type C liver cirrhosis and chronic hepatitis [7,12,30,34,35]. However, there is a marked heterogeneity in the magnitude of the treatment effect on the risk of HCC among studies, probably due to differences in the baseline risk of HCC among different trials [12]. Thus, the question remains whether the preventive effect of IFN therapy on HCC development could apply to all patients with chronic hepatitis C, especially those without liver cirrhosis. The result of the present study indicated that among high- and intermediate-risk patients, as assessed with our HCC risk prediction model, the cumulative HCC development rate was significantly reduced in SVR patients compared with nonSVR patients. This finding suggests that patients with chronic hepatitis, in whom disease has not yet progressed to hepatic cirrhosis but who are at a high risk of HCC development, benefit from antiviral treatment. The preventive effect of IFN on HCC development was not evident in low-risk patients within 5 years of observation. A longer observation term may be required to analyze the possible effect of antiviral therapy in these patients. Application of the present model on treatment decision may have limitations in that effect to prevent HCC development may differ in newer therapeutic agents such as protease

inhibitors [36,37], and that low-risk patients may also benefit from therapy after a longer term observation period such as 15–20 years.

Patients with chronic hepatitis often have no subjective symptoms accompanying their disease and therefore have a low consciousness of the disease. The broad array of adverse reactions and the high cost of IFN therapy are frequent hurdles in motivating patients to undergo therapy. However, patients may be convinced to undergo therapy or remain motivated for continued therapy if they are made aware of their risk of HCC development and the preventive effect of IFN on HCC development.

In conclusion, a reproducible HCC risk prediction model, which includes the factors such as age, platelet count, albumin levels, and AST levels, was constructed to predict the 5-year HCC development rate in patients with chronic hepatitis C. The model requires only a combination of readily available test values and can therefore be easily used at the bedside. The information provided by the model allows the physician to identify patients requiring IFN therapy for the prevention of HCC and formulate plans for imaging HCC surveillance.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Anemia and thrombocytosis induced by ribavirin monotherapy in patients with chronic hepatitis C

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Abstract
Background An inosine triphosphatase (*ITPA*) single-nucleotide polymorphism (SNP) is associated with anemia induced by pegylated interferon and ribavirin (RBV) combination therapy in patients with chronic hepatitis C (CHC). However, there are very few reports on the hematological effects of RBV monotherapy. Here, hematological changes were monitored in patients with CHC who received RBV monotherapy, and the mechanism of these changes was investigated.
Methods Patients with CHC ($n = 30$) received RBV monotherapy for 4 weeks. The RBV dose was determined on the basis of body weight. Complete blood count, and serum erythropoietin (EPO) and thrombopoietin (TPO) levels were assessed. The associations between these parameters and the *ITPA* SNP (*rs1127354*) were analyzed.
Results Over the 4 weeks, the median hemoglobin level of all patients decreased significantly, from 13.6 (10.5–16.6) to 11.7 (9.4–14.9) g/dl ($P < 0.001$), and the platelet counts increased, from 14.0×10^4 ($8.9–37.4 \times 10^4$) to 15.8×10^4 ($10.2–40.6 \times 10^4$) /mm^3 ($P = 0.003$). At week 4, hemoglobin levels differed between patients with the *ITPA* CC genotype and those with the AA or AC genotypes [11.1 (9.4–13.5) vs. 12.9 (12.5–14.9) g/dl, $P = 0.001$]. The platelet change ratio (i.e., platelet count at week 4/platelet count at baseline) in the patients with developing anemia was correlated with the increase in the serum EPO level over 4 weeks ($r = 0.88$, $P = 0.002$), but not with the increase in the serum TPO level over 4 weeks.
Conclusions RBV monotherapy induced anemia and affected thrombocytosis in patients with CHC. Elevated endogenous EPO may stimulate platelet production.

Keywords Ribavirin · Anemia · Erythropoietin · Thrombocytosis · *ITPA* SNP

Abbreviations

ITPA	Inosine triphosphatase
SNP	Single-nucleotide polymorphism
PEG-IFN	Pegylated interferon
RBV	Ribavirin
CHC	Chronic hepatitis C
EPO	Erythropoietin
TPO	Thrombopoietin
HCV	Hepatitis C virus
GWASs	Genome-wide association studies
IL28B	Interleukin 28B
DDRGK1	DDRGK domain-containing protein 1

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Introduction

Hepatitis C virus (HCV) infection currently affects an estimated 160 million individuals, or 2.35 % of the world population [1]. Of the patients with a primary HCV infection, 70–80 % develop chronic infection and are consequently at significant risk for progressive liver fibrosis, which can lead to liver cirrhosis (LC) and/or hepatocellular carcinoma (HCC) [2, 3].

Current antiviral treatment for chronic hepatitis C (CHC) patients is pegylated interferon alfa (PEG-IFN) and ribavirin (RBV) combination therapy. However, despite advances in the treatment of CHC, the sustained viral response (SVR) rate of patients infected with HCV genotype 1 and with a high viral load is <50 %; these patients have the most difficulty achieving SVR [4, 5].

In the 1970s, RBV, a guanosine analog, was demonstrated to have antiviral activity against a broad spectrum of DNA and RNA viruses in tissue culture cells [6]. RBV monotherapy has transient antiviral effects in patients with HCV, but the treatment response improves markedly when RBV is combined with IFN [4].

Drug tolerance is an important factor associated with the treatment response. Side effects induced by PEG-IFN/RBV combination therapy lead to dose reduction and sometimes to discontinuation of the combination therapy. Treatment-induced anemia is a common cause of RBV dose reduction. Reportedly, patients receiving less than 60 % of the planned RBV dose have a lower response rate and a higher relapse rate than patients receiving a higher dose [7, 8].

In recent years, genome-wide association studies (GWASs) have demonstrated a marked association between particular single-nucleotide polymorphisms (SNPs) near the interleukin 28B (*IL28B*) gene and treatment outcome with PEG-IFN/RBV combination therapy in patients with CHC [9].

In addition, some studies indicate that inosine triphosphatase (*ITPA*) SNPs are associated with anemia induced by PEG-IFN/RBV combination therapy [10, 11].

Tanaka et al. [12] reported that the *ITPA rs1127354* genotype was associated with the outcome of PEG-IFN/RBV combination therapy in a Japanese population, and Ochi et al. [11] reported a marginally significant association between the *ITPA* SNP and treatment outcomes of combination therapy, based on univariate analysis. Taken together, these findings indicate that there is a correlation between the *ITPA* SNP and the outcome of combination therapy in a Japanese population. Furthermore, it was surmised that the *ITPA* SNP may be associated with some treatment outcomes because this SNP affected RBV dose reduction and may have contributed to treatment failures.

Tanaka et al. [12] have demonstrated that *DDRGK1* (DDRGK domain-containing protein 1) SNPs are also associated with treatment-induced anemia and treatment-induced thrombocytopenia associated with PEG-IFN/RBV combination therapy.

IFN/RBV combination therapy leads to thrombocytopenia primarily because of the administration of IFN. However, in most studies of hematological changes associated with CHC treatments, patients received IFN/RBV or PEG-IFN/RBV combination therapy. Therefore, these studies did not address the hematological effects of RBV monotherapy.

Here, we assessed hematological changes in patients with CHC who received RBV monotherapy, and we studied factors associated with these changes, including *ITPA* SNPs and hematopoietic hormones.

Patients and methods

Patients and treatment protocol

Patients ($n = 30$; 14 males and 16 females; median age 56 years; age range 31–71) with chronic HCV infection who received RBV monotherapy at our hospital between April 2002 and March 2004 were enrolled in this study; the RBV monotherapy was administered for 4 weeks. All patients received IFN alfa-2b/RBV combination therapy after the RBV monotherapy.

The characteristics of the patients are shown in Table 1. The initial diagnosis was made using a second-generation enzyme-linked immunosorbent assay (ELISA) for antibodies against HCV and confirmed by quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) amplification of HCV from serum samples.

Patients who were positive for hepatitis B surface antigen or HIV antibodies were excluded from the study. The dose of RBV (Rebetol™; MSD, Tokyo, Japan) was determined based on body weight: the daily dose was 600 mg for patients <60 kg, 800 mg for those between 60 and 80 kg, and 1000 mg for those ≥80 kg. Complete blood counts were assessed at weeks 0, 1, 2, 3, and 4. The daily RBV dose was reduced by 200 mg if hemoglobin was <10 g/dl or if there was a 2 g/dl decline from the week-0 baseline; additionally, RBV treatment was withheld if the hemoglobin level was <8.5 g/dl. Serum samples were collected at weeks 0, 1, 2, 3, and 4 of RBV monotherapy and stored at −30 °C.

This protocol was approved by the Ethics Committee of Hokkaido University Hospital (Sapporo, Japan) and written informed consent was obtained from all patients before starting the trial.

Of the 30 patients who were enrolled in the study, 26 received all the planned dose of RBV. Owing to anemia, three patients received 70 % of the planned RBV dose, and

Table 1 Characteristics of the patients enrolled in this study

Characteristic	No. of patients or median	Range
Gender (male/female)	14/16	
Age (years)	56	31–71
BMI (kg/m^2)	24.5	19.4–32.0
rs8099917 (TT/TG or GG)	25/5	
rs1127354 (AA/AC or CC)	7/23	
rs11697186 (TT/TA or AA)	7/23	
WBC (/mm^3)	4500	3100–7700
Hemoglobin (g/dl)	13.6	10.5–16.6
Hematocrit (%)	40.8	32.0–48.6
Platelets ($\times 10^4$/mm^3)	14.0	8.9–37.4
AST (IU/l)	55	17–228
ALT (IU/l)	81	14–397
γ-GT (IU/l)	43	11–219
LDH (IU/l)	339	135–594
Albumin (g/dl)	4.1	2.6–5.0
T-bilirubin (mg/dl)	0.8	0.5–1.4
Creatinine (mg/dl)	0.7	0.4–1.1
HCV-RNA ($\log_{10}$ IU/ml)	6.0	3.7–6.6
Fibrosis (0/1/2/3/4)	3/6/11/9/1	
Activity (0/1/2/3)	1/9/20/0	

The data shown are medians and ranges unless otherwise specified

BMI body mass index, *WBC* white blood cell, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GT* gamma-glutamyl transpeptidase, *HCV* hepatitis C virus, *LDH* lactate dehydrogenase

one patient received just 53 %. No patients required a blood transfusion or administration of recombinant human erythropoietin (rhEPO).

SNP genotyping

To determine the *IL28B*, *ITPA*, and *DDRGK1* genotypes at select SNPs, genomic DNA was extracted from 200 µl of whole blood, using the QIAamp DNA Blood Mini Kit (QIAGEN Sciences, Germantown, MD, USA). SNP genotypes were determined using the real-time PCR method (TaqMan™ SNP Genotyping Assay; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Genotypes at three SNPs—*rs8099917*, *IL28B* (Assay ID: C_11710096_10); *rs1127354*, *ITPA* (Assay ID: C_27465000_10); and *rs11697186*, *DDRGK1* (Assay ID: C_11815649_20)—were determined. The genotype of *DDRGK1* could be determined by this method in all patients, but the genotypes of *ITPA* and *IL28B* could not be determined by this method in some patients. Therefore, when the genotype of a patient could not be determined by this method, the genotype was determined using standard PCR (ExTaq Hot Start version; Takara Bio, Otsu, Japan) and direct sequencing (BigDye Terminator; Applied Biosystems). A 2-µl sample of the genomic DNA extracted from a whole blood sample was amplified over 40 cycles of PCR. The PCR thermal profile comprised an initial denaturation at 95 °C for 10 min and 40 cycles of amplification (denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s). The forward primer for *rs8099917* was TTTGTCACTGTTCCTCCTTTTG and the reverse primer was TGCTGGGCCCTAACTGATAC. The forward primer for *rs1127354* was ATGAGAAAGG CGGATGACAG and the reverse primer was CGGCACT TATCAGGGAAACA.

Measurement of serum EPO and thrombopoietin (TPO) levels

Serum levels of EPO were measured using an ELISA (EPO ELISA; Roche, Mannheim, Germany) in stored blood samples taken from patients at weeks 0, 1, 2, 3, and 4. Serum TPO levels were measured using an ELISA (Quantikine™ Human TPO; R&D Systems, Minneapolis, MN, USA) in patient blood samples taken at 0, 2, and 4 weeks. Both assays were performed according to the manufacturers' instructions.

Pathological findings

Baseline liver biopsies were performed on all patients prior to the treatment, to determine METAVIR activity and fibrosis score. The METAVIR scoring system grades fibrosis on a 5-point scale (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous bridging septa without cirrhosis; F4, cirrhosis) and grades activity on a 4-point scale (A0, no activity; A1, mild activity; A2, moderate activity; A3, severe activity).

Measurement of serum ribavirin concentration

Serum concentrations of RBV after 4 weeks of monotherapy were measured using high-performance liquid chromatography (HPLC) as described previously [13].

Statistical analyses

All results are presented as medians and ranges. Statistical tests were performed based on Friedman's test to assess the change in a parameter over time, the Mann–Whitney test and Chi-square test to assess differences between groups, and the Spearman test to assess the correlation between hematological changes and hematopoietic hormones. The degree of platelet increase was measured using the platelet change ratio, specifically the platelet count at week 4/platelet count at week 0.

P values of <0.05 were considered significant. All statistical analyses were performed using PASW statistics 18 software (IBM, Armonk, NY, USA).

Results

Changes in hemoglobin, platelet count, serum alanine aminotransferase (ALT), and HCV RNA level during RBV monotherapy

Changes in values during RBV monotherapy are shown in Table 2. During 4 weeks of RBV monotherapy, the median hemoglobin level of the patients decreased significantly, from 13.6 (10.5–16.6) to 11.7 (9.4–14.9) g/dl ($P < 0.001$). The median platelet count increased significantly, from 14.0×10^4 ($8.9–37.4 \times 10^4$) to 15.8×10^4 ($10.2–40.6 \times 10^4$) /mm^3 ($P = 0.003$). The median mean corpuscular volume (MCV) increased from 98.3 (88.3–104.1) to 99.6 (89.9–105.3) fl ($P = 0.009$), and the median reticulocyte count increased from 9.2 (6.1–40.2) to 29.5 (9.0–80.2) ‰ ($P = 0.002$). There were no significant differences between baseline and week 4 in WBC, neutrophil counts, or lymphocyte counts. The median ALT level decreased significantly, from 81 (14–397) IU/l at baseline to 50 (12–312) IU/l at week 4 ($P = 0.007$), and the level of HCV RNA decreased significantly, from 6.0 (3.7–6.6) at baseline to 5.6 (3.3–6.5) $\log_{10}$ IU/ml at week 4 ($P = 0.045$). Serum EPO increased significantly during 4 weeks of RBV monotherapy, whereas serum TPO did not change significantly.

Association between *ITPA* SNP and hematological changes and hematopoietic hormones during RBV monotherapy

The 30 enrolled patients were divided into two groups based on *ITPA* genotype. Based on this grouping, baseline TPO level was significantly associated with the *ITPA* genotype, but other parameters, including gender, age, and renal function, were not (Table 3). Although the difference was not statistically significant, during the first 2 weeks of RBV monotherapy, hemoglobin levels in patients with the *ITPA* CC genotype tended to be lower than levels in those with the *ITPA* AA or AC genotypes [12.2 (9.8–15.9) vs. 13.2 (12.4–15.1) g/dl, $P = 0.07$]. After 4 weeks of RBV monotherapy, there was a significant difference in hemoglobin levels between the patients with the *ITPA* CC genotype and those with the AA or AC genotypes [11.1 (9.4–13.5) vs. 12.9 (12.5–14.9) g/dl, respectively, $P = 0.001$] (Fig. 1). Reticulocyte counts in patients with the *ITPA* CC genotype increased from 9.7 (6.1–40.4) to 31.0 (15.8–70.0) ‰ ($P = 0.001$) over the 4 weeks, while reticulocyte counts did not change significantly in the group of patients with the *ITPA* AA or AC genotypes [baseline, 8.8 (8.0–16.9) ‰; 4 weeks, 11.3 (9.0–20.5) ‰, not significant (NS)]. Serum concentrations of RBV were not different between the patients with the *ITPA* CC genotype and those with the AA or AC genotypes. The *DDRGK1* SNP was also analyzed. Because the *DDRGK1* TT or TA genotypes showed linkage with the *ITPA* AA or AC genotypes in all patients enrolled in the present

Table 2 Hematological changes and changes of ALT and HCV-RNA levels over a 4-week course of RBV monotherapy

	Week 0	Week 2	Week 4	*P* value
WBC (/mm^3)	4500 (3100–7700)	4800 (3800–8700)	4400 (2900–7500)	NS
Neutrophils (/mm^3)	2162 (1473–4068)	2355 (1867–4219)	2501 (1334–4219)	NS
Lymphocytes (/mm^3)	1659 (707–3796)	1678 (1092–2642)	1548 (616–2688)	NS
Hemoglobin (g/dl)	13.6 (10.5–16.6)	12.3 (9.8–15.9)	11.7 (9.4–14.9)	<0.001
MCV (fl)	98.3 (88.3–104.1)	97.2 (90.2–106.1)	99.6 (89.9–105.3)	0.009
Reticulocytes (‰)	9.2 (6.1–40.4)	23.3 (7.0–54.1)	29.5 (9.0–80.2)	0.002
Platelets ($\times 10^4$/mm^3)	14.0 (8.9–37.4)	15.3 (9.2–32.8)	15.8 (10.2–40.6)	0.003
ALT (IU/l)	81 (14–397)	58 (17–254)	50 (12–312)	0.007
HCV-RNA ($\log_{10}$ IU/ml)	6.0 (3.7–6.6)	5.9 (4.0–6.7)	5.6 (3.3–6.5)	0.045
EPO (pg/ml)	2.9 (0–35.8)	11.9 (0–114.8)	16.8 (0–184.2)	<0.001
TPO (fmol/ml)	1.84 (0.94–2.50)	1.95 (0.66–2.57)	1.93 (0.82–2.51)	NS
Serum RBV concentration (ng/ml)	–	1868 (1087–4656)	2266 (1157–4366)	0.004

The significance of the changes in each parameter was analyzed using Friedman's test

WBC white blood cell, *MCV* mean corpuscular volume, *ALT* alanine aminotransferase, *EPO* erythropoietin, *TPO* thrombopoietin, *NS* not significant, *RBV* ribavirin

Table 3 Characteristics of the patients grouped according to inosine triphosphatase (*ITPA*) SNP genotype

	ITPA (*rs1127354*)		
	CC allele ($n = 23$)	AA or AC allele ($n = 7$)	*P* value
Age (years)	56 (32–67)	60 (31–71)	NS
Gender (M/F)	10/13	4/3	NS
BMI (kg/m^2)	25.2 (19.4–32.0)	23.5 (20.5–27.6)	NS
rs8099917 (TT/non-TT)	18/5	7/0	NS
WBC (/mm^3)	4550 (3400–7500)	4500 (3100–7000)	NS
Hemoglobin (g/dl)	13.5 (10.5–16.6)	13.7 (11.8–15.6)	NS
Platelets ($\times 10^4$/mm^3)	13.2 (8.9–26.9)	15.5 (12.3–37.4)	NS
T-bilirubin (mg/dl)	0.8 (0.5–1.4)	0.8 (0.5–1.1)	NS
Albumin (g/dl)	4.0 (2.6–5.0)	4.0 (3.9–4.6)	NS
ALT (IU/l)	80 (14–176)	88 (18–397)	NS
γ-GT (IU/l)	46 (11–156)	36 (23–219)	NS
Creatinine (mg/dl)	0.7 (0.4–1.1)	0.6 (0.5–1.0)	NS
HCV-RNA ($\log_{10}$ IU/ml)	6.0 (4.5–6.6)	6.3 (3.7–6.6)	NS
EPO (pg/ml)	3.4 (0–35.8)	2.4 (0–12.2)	NS
TPO (fmol/ml)	1.75 (0.94–2.50)	2.03 (1.94–2.33)	0.038
Fibrosis (0–1/2–4)	6/17	7/0	NS
RBV concentration at 2 weeks (ng/ml)	1960 (1246–4656)	1395 (1087–2286)	NS
RBV concentration at 4 weeks (ng/ml)	2256 (1157–4366)	2551 (1349–3304)	NS

P values were calculated using the Mann–Whitney test

BMI body mass index, *WBC* white blood cell, *ALT* alanine aminotransferase, *γ-GT* gamma-glutamyl transpeptidase, *EPO* erythropoietin, *TPO* thrombopoietin, *NS* not significant, *RBV* ribavirin, *SNP* single-nucleotide polymorphism

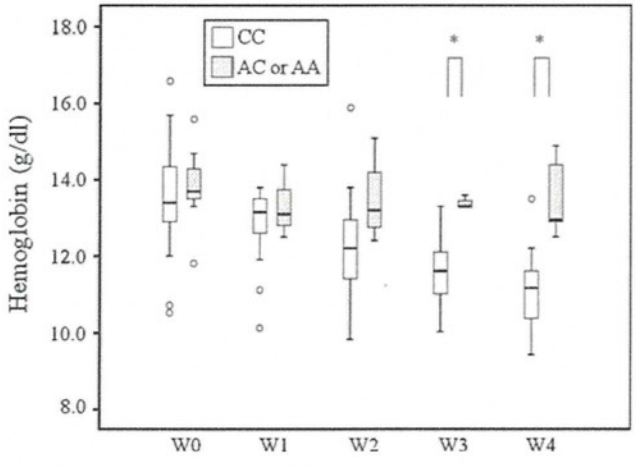

Fig. 1 Changes in hemoglobin according to inosine triphosphatase (*ITPA*) single-nucleotide polymorphism (SNP) genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum values for hemoglobin in patients divided into two groups based on *ITPA* SNP genotype. *P* values were calculated using the Mann–Whitney test. The *white boxes* represent the patients with the CC genotype, and the *gray boxes* represent patients with the AC or AA genotype. $*P < 0.05$

study, the association between *DDRGK1* SNP and changes in platelet counts were not further examined.

The median serum EPO level in patients with the *ITPA* CC genotype increased significantly, from 3.4 (0.0–35.8) to 26.1 (3.1–154.2) pg/ml ($P = 0.005$), over the 4 weeks. In contrast, serum EPO levels in patients with the *ITPA* AA or AC genotypes did not change significantly [2.4 (0.0–12.2) pg/ml at baseline and 4.7 (0.0–17.3) pg/ml at week 4, NS] (Fig. 2). There were no significant differences in WBC, neutrophil, lymphocyte, or platelet counts (Fig. 3) or TPO levels between the patients with the *ITPA* CC genotype and those with the AA or AC genotypes.

Correlation between hemoglobin levels, platelet counts, and EPO levels

There was a significant negative correlation between hemoglobin levels at week 2 and the increase in serum EPO over those 2 weeks ($r = -0.758$, $P = 0.003$) and between hemoglobin levels at week 4 and the increase in serum EPO over those 4 weeks ($r = -0.622$, $P = 0.004$) (Fig. 4).

Next, the association between EPO and the degree of platelet increase as measured by the platelet change ratio (i.e., platelet count at week 4/platelet count at baseline) was analyzed. Although not statistically significant, the platelet change ratio for 4 weeks tended to be correlated with the increase of EPO for 4 weeks ($r = 0.426$, $P = 0.056$). There was no significant correlation between the platelet change ratio and serum TPO over the 4 weeks. Similarly,

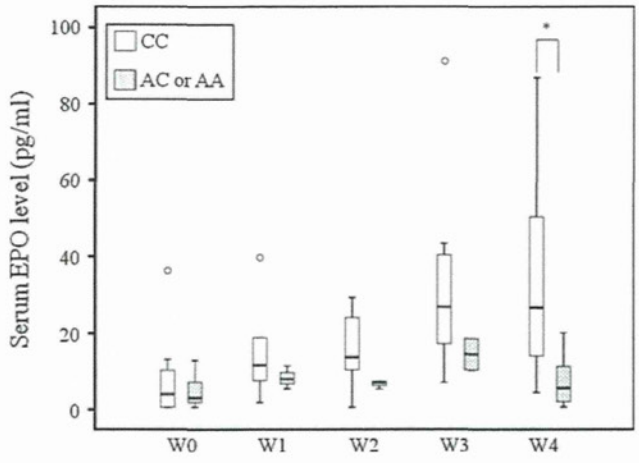

Fig. 2 Changes in serum erythropoietin (*EPO*) according to *ITPA* SNP genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum values for serum EPO in patients divided into two groups based on the *ITPA* SNP genotype. *P* values were calculated using the Mann–Whitney test. The *white boxes* represent patients with the CC genotype, and the *gray boxes* represent patients with the AC or AA genotype. $*P < 0.05$

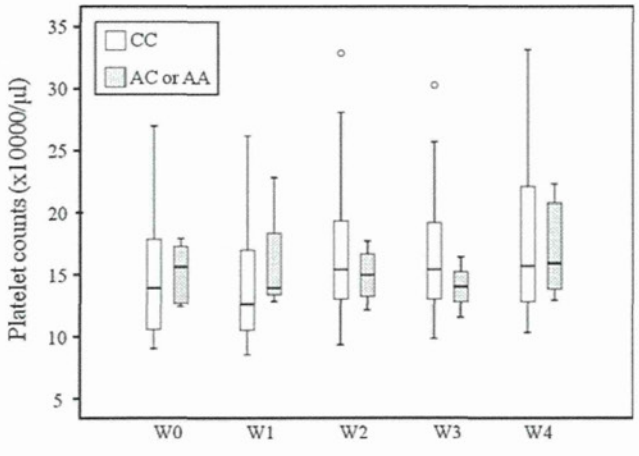

Fig. 3 Changes in platelet counts according to *ITPA* SNP genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum value for platelet counts in patients divided into two groups based on *ITPA* SNP genotype. The *white boxes* represent patients with the CC genotype, and the *gray boxes* represent patients with the AC or AA genotype

there was no significant correlation between hemoglobin levels and the platelet change ratio, or between the increase in serum EPO and the increase in serum TPO (Fig. 5).

Association between serum EPO and platelet counts according to anemia

Because there was a correlation between serum EPO and the platelet count, it was expected that platelet counts would not increase in patients who had not developed anemia.

Therefore, the correlation between serum EPO and platelet count was determined in patients with and without anemia. Here, anemia was defined as a decrease in hemoglobin of >2 g/dl or a hemoglobin level of <10 g/dl. All patients with anemia ($n = 15$) had the *ITPA* CC genotype, while the group of patients who did not develop anemia ($n = 15$) included 8 patients with the CC allele and 7 patients with the AA or AC genotype. Among the group of patients with anemia, platelet counts increased significantly from baseline over the 4 weeks ($P = 0.001$). However, there was no significant increase in platelet counts among the patients who did not develop anemia. There was a significant correlation between serum EPO and the platelet change ratio from baseline to week 4 in the anemia group ($r = 0.88$, $P = 0.002$), but there was no such correlation in the non-anemia group ($r = 0.39$, $P = 0.27$) (Fig. 6).

Factors associated with increase in platelet count

The patients were divided into two groups based on the degree of platelet increase as measured by the platelet change ratio (i.e., platelet count at week 4/platelet count at baseline); specifically, patients with a platelet change ratio greater than or equal to the median of 1.05 were placed in one group, and those with a ratio below the median were placed in the other group (Table 4). The factors that contributed to a platelet increase were examined. The group with a ratio of ≥1.05 tended to be younger than the other group ($P = 0.062$) in univariate analyses. A multivariate analysis could not be performed because of the small number of patients enrolled in this study.

Furthermore, factors that contributed to the platelet increase were examined in the patients without anemia (Table 5). The patients who did not have anemia and had a ratio of platelet increase of ≥1.05 were significantly younger [age 48 years (range 31–56) vs. 61 years (range 54–71), $P < 0.001$] and tended to have higher platelet counts at baseline [17.1×10^4 ($9.1–37.1 \times 10^4$) vs. 12.4×10^4 ($8.9–15.5 \times 10^4$) /mm^3] than those who had a platelet ratio of <1.05.

Discussion

Although RBV has antiviral activity against a broad spectrum of DNA and RNA viruses, RBV itself has only transient effects on HCV. In spite of the minimal antiviral effect of RBV on HCV, some studies show that IFN alpha and RBV combination therapy has significantly better treatment outcomes than IFN monotherapy [6, 14]. Furthermore, in recent years, direct-acting antiviral agents (DAAs), such as telaprevir, were shown to have a strong antiviral effect on HCV. However, in clinical trials of IFN

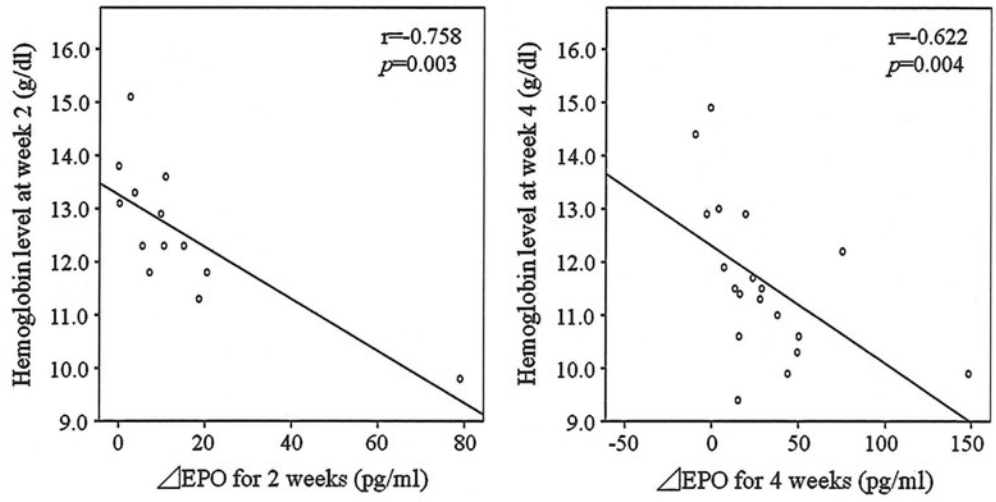

Fig. 4 Correlation between hemoglobin levels and increases in serum EPO. Correlation coefficients and *P* values were calculated using Spearman's rank correlation coefficient test

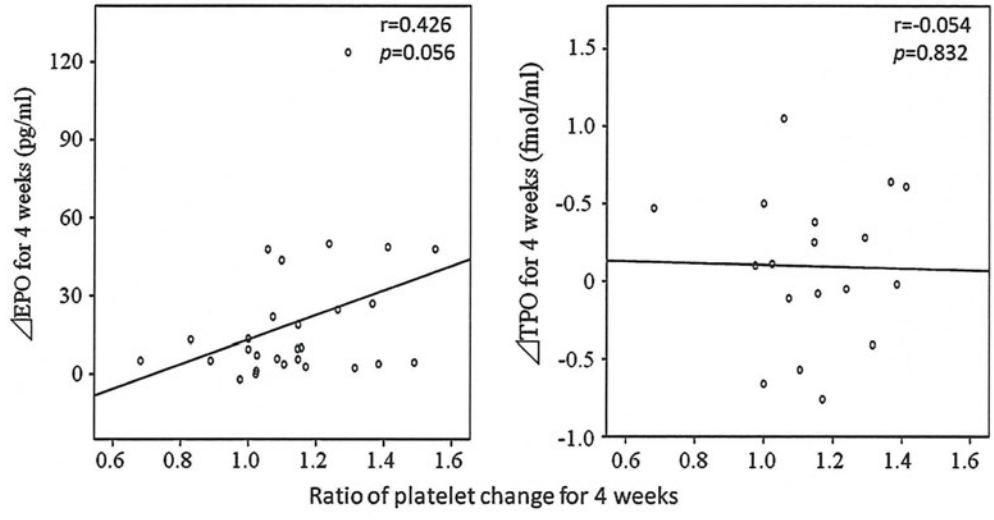

Fig. 5 Correlations between platelet counts and hematopoietic hormones. Correlation coefficients and *P* values were calculated using Spearman's rank correlation coefficient test. *TPO* thrombopoietin

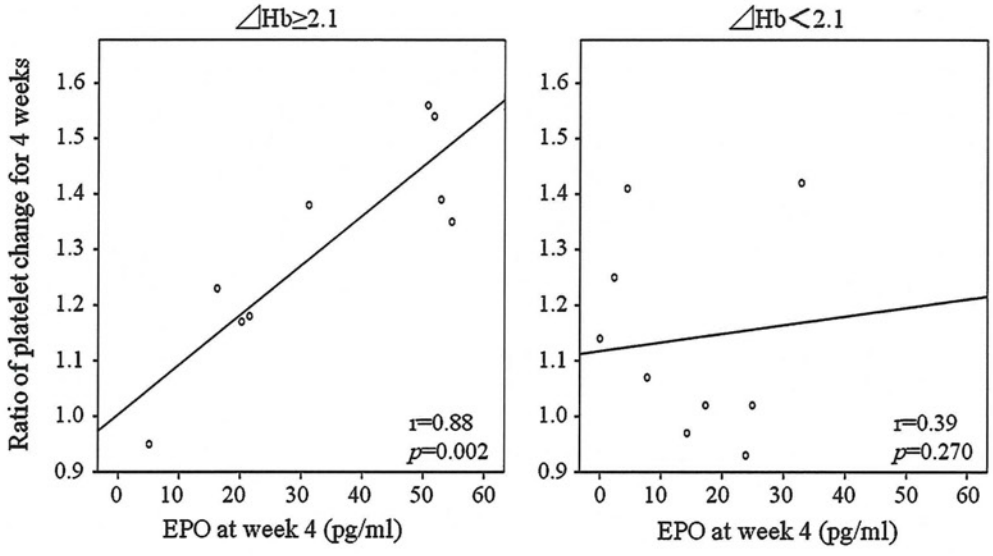

Fig. 6 Correlation between the platelet change ratio and EPO based on the presence/absence of treatment-induced anemia. The platelet change ratio was defined as the platelet count at week 4/platelet count at baseline. Correlation coefficients and *P* values were calculated using Spearman's rank correlation coefficient test. *Hb* hemoglobin

Table 4 Associations between hematological parameters and platelet counts

	Ratio of platelet increase for 4 weeks		
	<1.05 (n = 11)	≥1.05 (n = 19)	P value
Age (years)	61 (41–71)	55 (31–67)	0.062
Gender (M/F)	6/5	8/11	NS
BMI (kg/m^2)	25.2 (19.4–28.1)	24.0 (19.8–32.0)	NS
rs8099917 (TT/non-TT)	10/1	13/4	NS
rs1127354 (CC/non-CC)	7/4	16/3	NS
WBC (/mm^3)	4750 (3800–7400)	4500 (3100–7700)	NS
Hemoglobin (g/dl)	14.1 (10.5–16.6)	13.5 (11.8–15.7)	NS
Platelets ($\times 10^4$/mm^3)	13.5 (10.0–26.9)	13.8 (8.9–37.4)	NS
T-bilirubin (mg/dl)	0.7 (0.5–1.4)	0.8 (0.5–1.2)	NS
Albumin (g/dl)	4.0 (2.6–4.6)	4.1 (3.4–5.0)	NS
ALT (IU/l)	49 (18–397)	93 (14–176)	NS
γ-GT (IU/l)	43 (15–219)	48 (11–156)	NS
Creatinine (mg/dl)	0.7 (0.5–1.1)	0.7 (0.4–0.9)	NS
HCV-RNA ($\log_{10}$ IU/ml)	6.2 (3.7–6.6)	6.0 (4.5–6.6)	NS
EPO (pg/ml)	2.0 (0.0–12.2)	2.9 (0.0–35.8)	NS
TPO (fmol/ml)	1.96 (1.41–2.33)	1.75 (0.94–2.5)	NS
Fibrosis (0–1/2–4)	4/7	5/14	NS

P values were calculated using the Mann–Whitney test

BMI body mass index, *WBC* white blood cell, *ALT* alanine aminotransferase, *γ-GT* gamma-glutamyl transpeptidase, *EPO* erythropoietin, *TPO* thrombopoietin, *NS* not significant

Table 5 Associations between increases in hematological parameters and platelet counts in patients without RBV-induced anemia

	Ratio of platelet increase for 4 weeks		
	<1.05 (n = 8)	≥1.05 (n = 6)	P value
Age (years)	61 (54–71)	48 (31–56)	<0.01
Gender (male/female)	3/5	4/2	NS
BMI (kg/m^2)	23.5 (19.4–27.6)	23.0 (19.8–25.8)	NS
rs8099917 (TT/non-TT)	8/0	5/1	NS
rs1127354 (CC/non-CC)	4/4	3/3	NS
WBC (/mm^3)	4400 (3500–7400)	5000 (3100–7700)	NS
Hemoglobin (g/dl)	13.2 (10.5–15.6)	13.6 (11.8–14.1)	NS
Platelets ($\times$ 10^4/mm^3)	12.4 (8.9–15.5)	17.1 (9.1–37.4)	0.052
T-bilirubin (mg/dl)	0.7 (0.5–1.1)	0.8 (0.5–1.1)	NS
Albumin (g/dl)	4.0 (2.6–4.6)	4.0 (3.9–4.6)	NS
ALT (IU/l)	52.5 (18–219)	107 (30–119)	NS
γ-GT (IU/l)	47.5 (21–219)	43 (19–96)	NS
Creatinine (mg/dl)	0.65 (0.40–1.00)	0.70 (0.50–1.90)	NS
HCV-RNA ($\log_{10}$ IU/ml)	6.0 (3.7–6.6)	5.9 (5.4–6.6)	NS
EPO (pg/ml)	6.6 (0.0–35.8)	1.94 (0.0–8.3)	NS
TPO (fmol/ml)	2.1 (1.15–2.33)	1.85 (0.94–2.09)	NS
Fibrosis (0–1/2–4)	2/6	1/5	NS

P values were calculated by Mann–Whitney test

BMI body mass index, *WBC* white blood cell, *ALT* alanine aminotransferase, *γ-GT* gamma-glutamyl transpeptidase, *EPO* erythropoietin, *TPO* thrombopoietin, *NS* not significant

and telaprevir with or without RBV, response rates were lower when the treatment regimen did not include RBV. This finding indicates that RBV is a key drug in treatments that achieve SVR for patients with CHC [15].

It is well known that RBV induces anemia, but few reports have shown that RBV monotherapy induced anemia. In 1984, Canonico et al. [16] reported that RBV administration to rhesus monkeys led to anemia, increased platelet counts, and increased megakaryocytes in the bone marrow, indicating that RBV influences bone marrow function. Bone marrow aspiration was not performed in the present study, but our findings confirmed that RBV monotherapy can lead to anemia and increases in platelet counts. Decreases in hemoglobin and increases in serum EPO were evident just 1 week after the start of RBV monotherapy. Increases in platelet counts were evident 2 weeks after the start of RBV monotherapy. However, RBV did not affect serum TPO levels. The patients who did

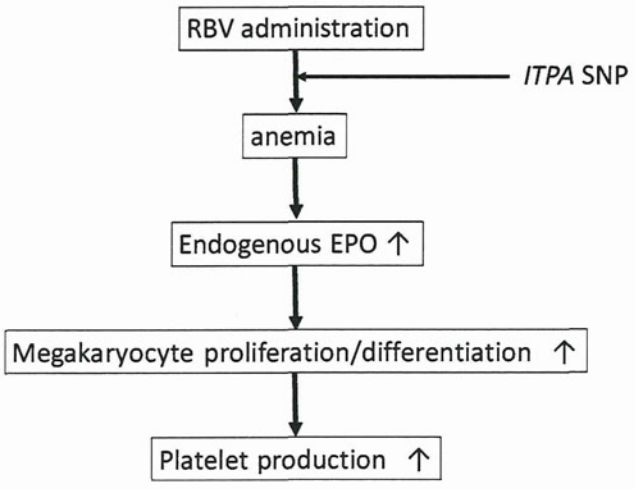

Fig. 7 Model of the mechanism leading to increases in platelet numbers during ribavirin (*RBV*) monotherapy

not develop anemia did not show an increase in serum EPO; this finding indicated that RBV-induced anemia led to an increase in endogenous EPO secretion, which subsequently resulted in increases in platelet counts. While there was no apparent association between the TPO level and the platelet count, there was a significant positive correlation between serum EPO levels and increased platelet counts. Thus, the present study revealed that the thrombocytosis effects of RBV were caused by an RBV-induced increase in EPO level (Fig. 7).

Although EPO is the hematopoietic growth hormone that regulates red blood cell, not platelet, production, some studies indicate that EPO can affect platelet production. Streja et al. [17] reported that the administration of recombinant human (rh) EPO led to relative thrombocytosis. Homoncik et al. [18] reported that rhEPO increased platelet activity and platelet counts in patients with alcoholic liver cirrhosis (LC). Dessypris et al. [19] showed the ability of EPO to stimulate the growth and differentiation of megakaryocytes in vitro. Regarding the mechanisms of the increase in platelet counts induced by EPO, some investigators have suggested that EPO acts similarly to TPO because of the sequence homology between TPO and EPO [20, 21]. Other studies have indicated that rhEPO administration leads to iron deficiency, which is associated with antioxidant defense and increased oxidative stress, and that iron deficiency subsequently results in a tendency toward platelet aggregation [22, 23]. Though some studies support these hypotheses, the effects of EPO on platelets remain controversial.

Many studies have addressed the hematological changes that occur during IFN monotherapy or PEG-IFN/RBV combination therapy. Schmid et al. [24] demonstrated that anemia, increases in serum EPO levels, and decreases in platelet counts were milder in patients receiving PEG-IFN/RBV combination therapy than in those receiving IFN monotherapy. Their data indicate that endogenous EPO contributes to the increases in platelet counts, but that it cannot completely compensate for IFN-induced thrombocytopenia. However, the patients enrolled in their study received PEG-IFN/RBV combination therapy. PEG-IFN may have different effects from RBV on leukocytes, erythrocytes, and thrombocytes. In particular, RBV often leads to anemia. The patients enrolled in the Schmid et al. [24] study experienced increases in serum TPO and serum EPO levels. TPO might affect or mediate changes in platelet numbers. Studies involving PEG-IFN/RBV combination therapies have some limitations for examining the separate and distinct effects of RBV and IFN on hematological parameters. In contrast, the present study of RBV monotherapy has overcome this limitation.

In recent years, GWASs have revealed an association between *ITPA* SNPs and anemia among patients receiving PEG-IFN/RBV combination therapy [10–12]. Fellay et al. [10] showed that two SNPs, *rs1127354* and *rs7270101*, located in the *ITPA* gene on chromosome 20, were strongly associated with treatment-induced anemia in the population enrolled in the IDEAL study, which included European, African, and Hispanic populations. Ochi et al. [11] reported that an SNP in the *ITPA* region, *rs1127354*, was associated with treatment-induced anemia, and that there were no variants at *rs7270101* in the Japanese population. Therefore, we analyzed only the *rs1127354* SNP in the present study.

De Franceschi et al. [25] have suggested that RBV-induced anemia is caused by the accumulation of RBV-triphosphate (TP) in erythrocytes and that this build-up results in oxidative damage to erythrocyte membranes and extravascular erythrophagocytic destruction. Vanderheiden [26] reported that an *ITPA* deficiency caused a strong accumulation of inosine triphosphate (ITP) in erythrocytes. In patients with an *ITPA* genotype that protects against treatment-induced anemia, ITP may compete with RBV-TP in erythrocytes and thereby protect cells from the hemolytic effects of RBV-TP. Therefore, *ITPA* SNPs are definitively associated with RBV-induced anemia. However, until the present study, no report has revealed an association between *ITPA* SNPs and RBV-induced anemia in patients who have received RBV monotherapy. The present study, however, showed a strong association between an *ITPA* SNP and the anemia induced by RBV monotherapy.

In our study, we assessed associations between the *ITPA* *rs1127354* SNP and increases in platelet counts because there were strong associations between anemia and *ITPA* SNPs and between the serum levels of EPO and changes in platelet counts. However, no significant association was found between the *ITPA* genotype and increases in platelet