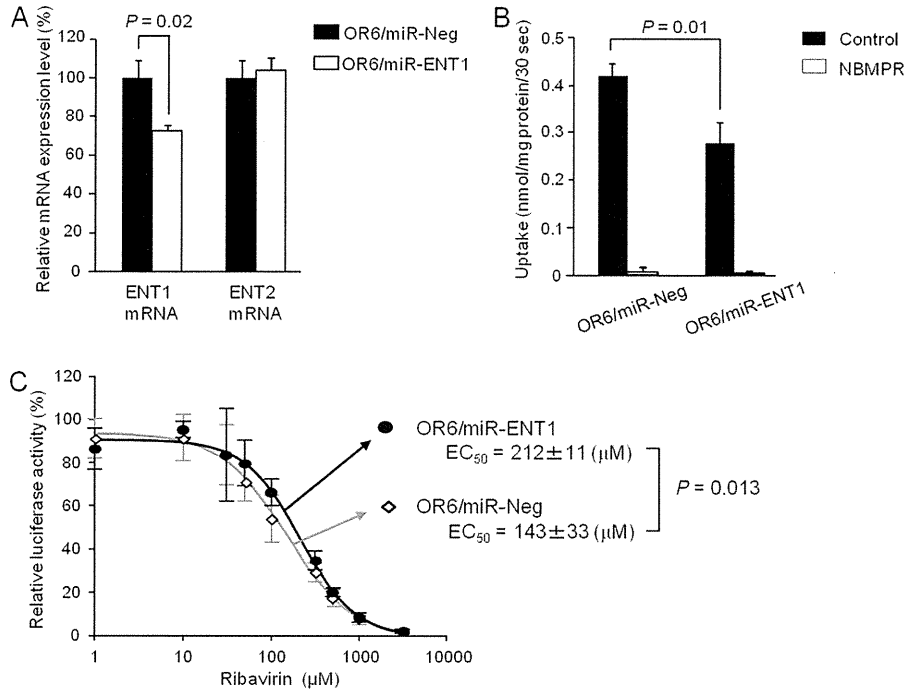

FIG 4 Effect of ENT1 mRNA knockdown on anti-HCV activity of ribavirin. (A) The expression levels of ENT1 and ENT2 mRNA were determined by real-time PCR. Abundance is shown relative to the level of ENT1 or ENT2 mRNA in OR6/miR-Ng cells. Each value is the mean plus SD from three independent experiments, each performed in duplicate. (B) Ribavirin (100 μM) uptake by OR6/miR-ENT1 and OR6/miR-Ng cells was analyzed in Na^+-free KHB in the absence (control) or presence of 100 μM NBMPR. Each value is the mean plus SD of transport activity from three independent experiments, each performed in duplicate. (C) The concentration dependency of ribavirin in OR6/miR-Ng and OR6/miR-ENT1 cells was then examined. The ribavirin concentrations used are shown in the legend to Fig. 1A. The relative luciferase activity value in the absence of ribavirin in each cell line was set to 100%. Each value is the mean ± SD of relative luciferase activity from four independent experiments, each performed in triplicate.

the other findings, indicate that ENT1 plays an indispensable role in ribavirin antiviral activity.

The importance of ENT1 in ribavirin antiviral activity was further underscored by the results of both the ENT1 knockdown and uptake inhibition experiments using NBMPR. It is noteworthy that even a small reduction of ENT1 activity significantly weakened ribavirin's antiviral potency. These results indicate that increasing or decreasing ENT1 activity level in the cells results in stronger or weaker ribavirin efficacy by increasing or reducing the uptake of the drug, even if extracellular ribavirin concentrations and exposure durations are constant. Therefore, it can be concluded that the ENT1-mediated ribavirin uptake level determines the level of ribavirin antiviral activity in OR6 cells and, presumably, in human hepatocytes.

The above-mentioned findings and suppositions prompt us to propose the following two possibilities (see Fig. S7 in the supplemental material). One is that patients with higher ENT1 activity levels in hepatocytes could more likely attain RVR (defined as a faster and stronger ribavirin antiviral effect in the early stage of the treatment) than those with lower ENT1 activity levels, when other factors affecting the treatment outcome are similar. The mechanisms underlying the interindividual difference in the hepatic ENT1 activity level remain unclear, but SNPs are promising candidates for the causal factors that result in the difference. Since two intronic SNPs have been revealed to be associated with RVR (and SVR) (12, 18), investigations should be conducted to determine whether these SNPs have a positive effect on the hepatic ENT1 expression level.

The other possibility is that the hepatic uptake of ribavirin by ENT1 could be hindered by coadministered chemicals, thus resulting in attenuation of the treatment response in some patients, as shown in Fig. 3. Although there have been no clinical reports supporting this possibility, preceding studies have been performed to determine whether hepatic uptake inhibition of pravastatin and metformin, which are hepatocyte-targeting drugs, reduces their effectiveness (1). These drugs are known substrates for hepatic organic ion transporters, and it has been shown that aberrations in these transporters significantly impair their *in vivo* functions (2, 15). Since, due to attendant complications or other chronic diseases, several drugs are often coprescribed along with ribavirin during treatment regimens, it may be worth considering whether interactions between ribavirin and other drugs at the point of ENT1-mediated uptake can affect the treatment response.

Exploration of these possibilities must await further studies aimed at clarification of the factors affecting the hepatic ENT1 activity level, including the above-described SNP studies and ribavirin-drug interaction studies. The results obtained from such studies could contribute not only to a better understanding of the mode of action of ENT1 on ribavirin antiviral activity but also to identification of the associated markers for RVR or null responses in clinical settings.

It should be noted that, unexpectedly, ENT1 activity was found to be insensitive to inhibition by NBMPR in the nanomolar range in OR6 cells. This was not due to nucleotide alterations in ENT1 cDNA of OR6 cells (Iikura, unpublished). Since OR6 cells were

derived from Huh-7 cells, we examined the sensitivity of ENT1 to inhibition of NBMPR using Huh-7 cells and obtained results similar to those obtained with OR6 cells (Iikura, unpublished). Therefore, the lower sensitivity of ENT1 to NBMPR in OR6 cells was thought to have originated from the Huh-7 cells. Although the reason for the altered sensitivity of ENT1 to NBMPR remains unknown at this time, it is believed that the cell-specific posttranslational modification might be involved. It has been reported that defective glycosylation of ENT1 leads to decreased affinity for NBMPR (19). Therefore, it can be speculated that the type or structure of glycochain and/or other modifications could be responsible for decreased affinity of ENT1 of OR6/Huh-7 cells for NBMPR. Further studies aimed at ascertaining the reason might provide novel insights into the biology of ENT1.

Finally, we briefly discuss the static cytotoxic effects of ribavirin and NBMPR on OR6 cells. According to the results of toxicological analyses, these reagents (at most concentrations tested) did not cause severe toxicity in OR6 cells (less than 10%), and only marginal toxicity was found in treatment of the reagents at the highest concentrations tested in an MTS assay. In contrast, blasticidin S treatment (20 ng/ml) significantly damaged the cells (>50% in the MTS assay [Iikura, unpublished]). Therefore, it is assumed that OR6 cells possess inherent resistance to ribavirin and NBMPR, and this factor might be related to the relatively high EC_{50} of ribavirin. Although we do not know the reason for the behavior of the cells, it is unlikely that the limited toxicity would give rise to a question regarding the present results. In actuality, 100 μM NBMPR treatment, which caused marginal toxicity, did not affect HCV replication activity (see Fig. S4 in the supplemental material).

In conclusion, we have clearly demonstrated that ENT1 plays an indispensable role in ribavirin antiviral activity by facilitating the uptake and accumulation of the drug in OR6 cells, thereby indicating that ENT1 provides a gate that is essential to the success of ribavirin's mission. Our study limitations include an *in vitro* HCV model system using hepatoma cells and no *in vivo* evidence of association between hepatic ENT1 activity and ribavirin efficacy. Nevertheless, our results, together with the literature, strongly suggest that ENT1 also plays the determinant role in the antiviral efficacy of ribavirin in the human liver during the course of anti-HCV therapy. Accordingly, it is believed that our results, as well as the ideas described in this paper, will encourage further studies aimed at the clarification of the clinical importance of ENT1 in anti-HCV therapy.

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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 $\times$ 10^4 (8.9–37.4 $\times$ 10^4) to 15.8 $\times$ 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

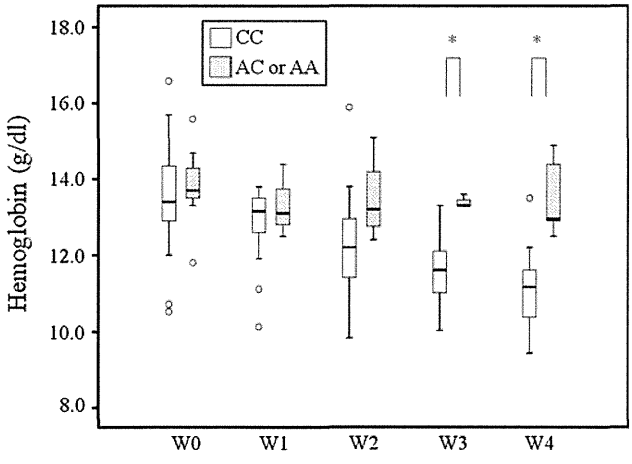

Fig. 1 Changes in hemoglobin according to inosine triphosphatase (*ITPA*) single-nucleotide polymorphism (SNP) genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum values for hemoglobin in patients divided into two groups based on *ITPA* SNP genotype. *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,

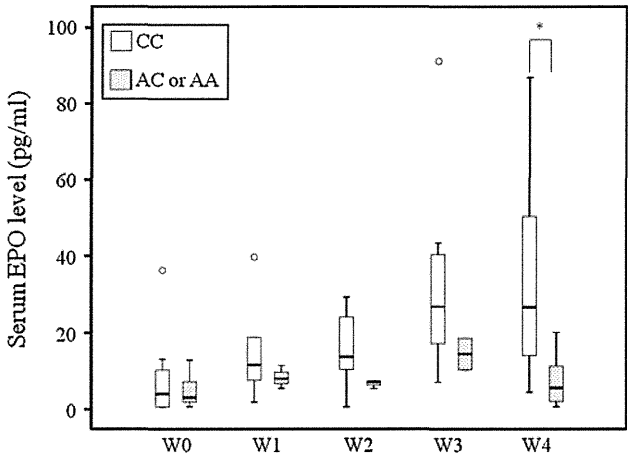

Fig. 2 Changes in serum erythropoietin (*EPO*) according to *ITPA* SNP genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum values for serum EPO in patients divided into two groups based on the *ITPA* SNP genotype. *P* values were calculated using 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. $*P < 0.05$

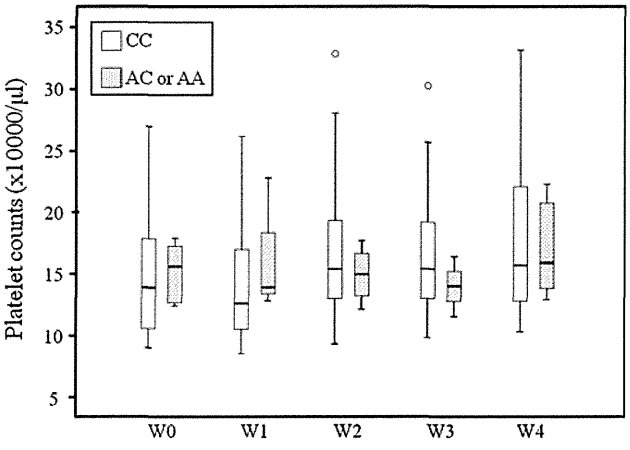

Fig. 3 Changes in platelet counts according to *ITPA* SNP genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum value for platelet counts in patients divided into two groups based on *ITPA* SNP genotype. The *white boxes* represent patients with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

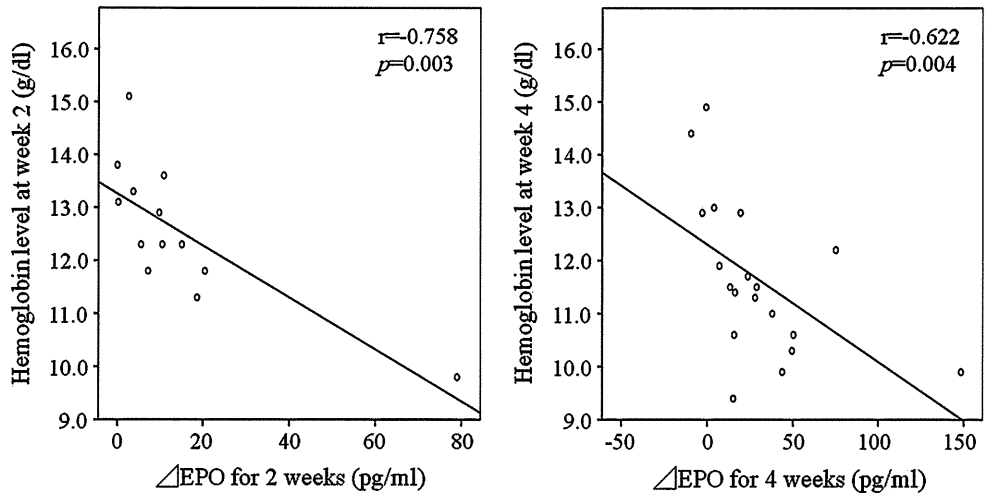

Fig. 4 Correlation between hemoglobin levels and increases in serum EPO. Correlation coefficients and *P* values were calculated using Spearman's rank correlation coefficient test

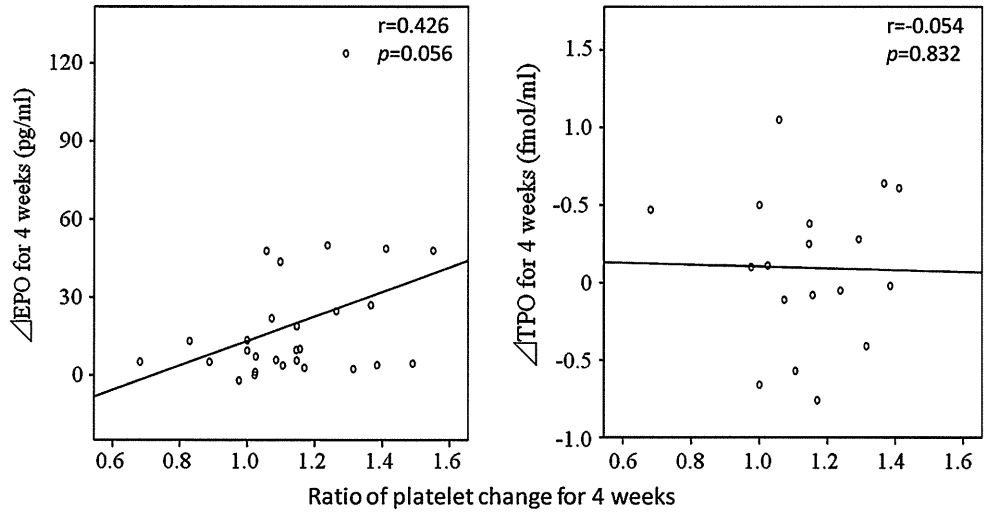

Fig. 5 Correlations between platelet counts and hematopoietic hormones. Correlation coefficients and *P* values were calculated using Spearman's rank correlation coefficient test. *TPO* thrombopoietin

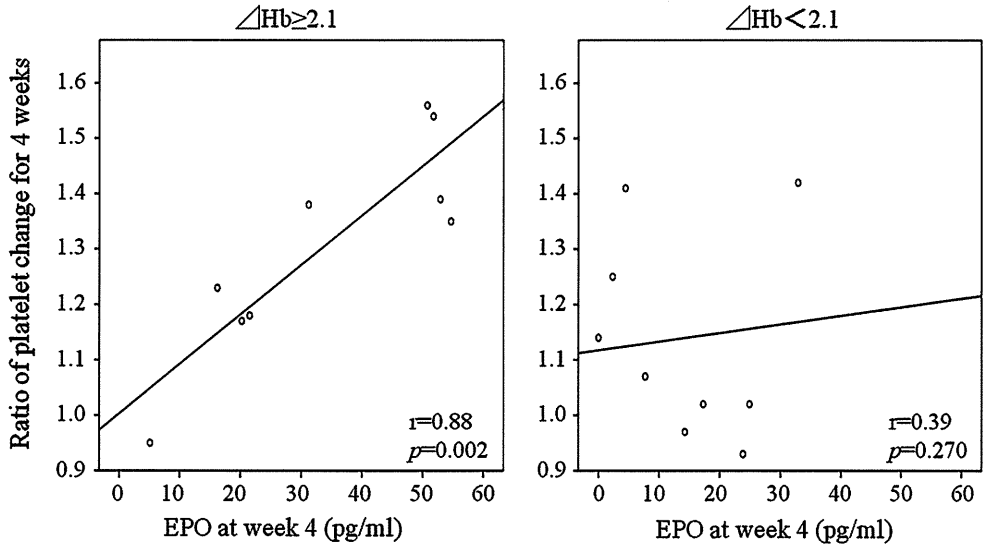

Fig. 6 Correlation between the platelet change ratio and EPO based on the presence/absence of treatment-induced anemia. The platelet change ratio was defined as the platelet count at week 4/platelet count at baseline. Correlation coefficients and *P* values were calculated using Spearman's rank correlation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

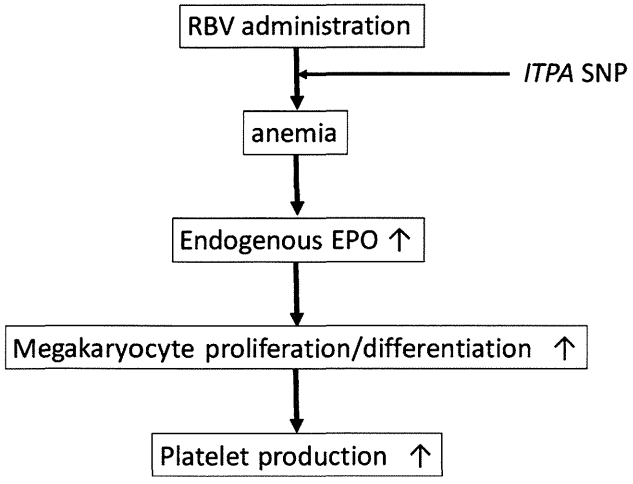

Fig. 7 Model of the mechanism leading to increases in platelet numbers during ribavirin (*RBV*) monotherapy

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

counts. The lack of a significant association may reflect an indirect, rather than a direct, relationship between *ITPA* genotype and platelet physiology.

Tanaka et al. [12] showed that a *DDRGK1* SNP near the *ITPA* gene, like the *ITPA* SNP, was associated with treatment-induced anemia; moreover, the *DDRGK1* SNP was associated with treatment-induced thrombocytopenia during PEG-IFN/RBV combination therapy. Because a protective *DDRGK1* allele showed linkage with a protective *ITPA* allele in all the patients enrolled in the present study, the association between *DDRGK1* SNP and changes in platelet counts could not be further examined.

In conclusion, an association was found between *ITPA* SNP genotype and treatment-induced anemia during a 4-week course of RBV monotherapy. This RBV-induced anemia may have led to increases in endogenous serum EPO that, in turn, resulted in the stimulation of platelet production. However, the sample size in this study was small; therefore, further investigations are needed to elucidate the effects of RBV on hematopoietic parameters.

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Conflict of interest Shuhei Hige has received a research grant from MSD. The other authors have declared that no conflict of interest exists.

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Upregulation of nuclear PA28γ expression in cirrhosis and hepatocellular carcinoma

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Abstract. We previously reported that proteasome activator 28γ (PA28γ) is an oncogenic protein in hepatitis C virus (HCV) core protein transgenic mice. The aim of this study was to determine the role of PA28γ expression at the protein level in the development and progression of human hepatocarcinogenesis and hepatocellular carcinoma (HCC). Samples from tissues representing a wide spectrum of liver disease were analyzed, including histologically normal livers (n=5), HCV-related chronic hepatitis (CH) (n=15) and cirrhosis (n=31). The level of nuclear PA28γ increased with the progression of liver disease from CH to cirrhosis. The majority of cirrhotic livers (68%; 21/31) displayed high nuclear PA28γ expression. However, in half of the HCCs (50%; 18/36), little or no nuclear PA28γ expression was observed, while the remaining 50% (18/36) of the cases displayed high levels of nuclear PA28γ expression. A clinicopathological survey demonstrated a significant correlation between nuclear PA28γ expression and capsular invasion in HCC (P=0.026); a striking difference was found between nuclear PA28γ expression in non-tumor tissues and shorter disease-free survival (P<0.01). Moreover, nuclear PA28γ expression in non-tumor tissues correlated with the expression of molecules related to the genesis of hepatic steatosis and HCC, such as sterol regulatory element binding protein-1c mRNA. The findings suggest the involvement of nuclear PA28γ expression in the progression and relapse of HCC, and suggest that nuclear PA28γ is a potentially suitable target for the prevention and/or treatment of HCC.

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Abbreviations: CH, chronic hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PA, proteasome activator; PBGD, porphobilinogen deaminase; RT-PCR, reverse transcription-polymerase chain reaction

Key words: proteasome activator 28γ, hepatocellular carcinoma, cirrhosis, western blotting, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for approximately 6% of all human carcinomas and 1 million deaths annually, with an estimated number of new cases of over 500,000/year (1). Clinical and experimental evidence suggests a link between infection with hepatitis C virus (HCV) and/or hepatitis B virus (HBV), chronic hepatitis (CH) and cirrhosis, as well as the progression of HCC. Liver cirrhosis is observed in up to 90% of patients with HCC, and HCV is the causative factor in 80% and HBV in 10% of cases in Japan (2-5). In the United States, almost 4 million individuals are infected with HCV each year which progresses to chronic hepatitis C, which could potentially progress to liver cirrhosis. The results are often liver failure or HCC. Chronic hepatitis C is the nation's leading cause of HCC, and according to the American Liver Foundation, is also the leading reason for liver transplantation. In Japan, HCV and/or HBV-based hepatitis and cirrhosis are also serious problems since they progress to HCC at a ratio of 5 to 7% per year (4,5). These findings strongly suggest the existence of a link between hepatocarcinogenesis and HCV/HBV infection and chronic liver inflammation.

Various therapies are currently in use for HCC. These include surgical resection, percutaneous ethanol injection (PEI), systemic or arterial chemotherapy using either single or combination drugs, transcatheter arterial chemoembolization (TACE), hormonal therapy and selective radiotherapy. However, the prognosis of patients with HCC remains poor, as they often develop intrahepatic and/or multicentric tumor recurrence, at a rate of 20-40% within 1 year, and ~80% within 5 years of therapy even when curative treatment is applied (6-9). Liver transplantation offers the best prognosis for patients with small HCC, although its use is limited due to the scarcity of donor organs. Therefore, an effective therapeutic strategy against HCC is required.

In a previous study, we reported that proteasome activator 28γ (PA28γ) directly enhances the degradation of the HCV core protein and plays a key role in the genesis of hepatic steatosis and HCC in HCV core protein transgenic mice (10). Furthermore, the above events were not observed in PA28γ-knockout mice. The present study is an extension of our previous study and was designed to assess the utility of PA28γ expression as a biological marker for HCV-related human liver disease and HCC. The findings showed the presence of high levels of nuclear PA28γ in multistep hepatocarcinogenesis and HCC invasion, suggesting that selective inhibitors of nuclear PA28γ may be useful in the prevention and/or treatment of this disease.

Materials and methods

Tissue samples. The study protocol was approved by the Human Ethics Review Committee of Osaka University, and a signed consent form was obtained from each subject for the use of tissue samples for medical research. Tissue samples were obtained from 51 patients with liver tumors, who underwent hepatectomy at the Department of Gastroenterological Surgery, Osaka University Hospital. All patients had HCV infection (28 patients) and some had HCV plus HBV infection (18 patients), but none had only HBV infection. The mean post-treatment follow-up period was 6.2±2.5 years ± standard deviation (SD). The excised hepatic tissue samples were examined immunohistochemically for PA28γ expression, including 46 paired HCCs. Non-tumor tissues were also examined, which comprised 15 CH-based livers (5 chronic active hepatitis and 10 chronic inactive hepatitis) and 31 cirrhotic livers. Prior to hepatectomy for HCC, 10 patients were treated with transarterial embolization (TAE). In these cases, histopathological examination showed complete hepatic necrosis. Histologically normal livers were also obtained from patients negative for hepatic viral infections who had liver metastasis secondary to colorectal cancer.

For immunohistochemistry, the tissue samples were fixed in 10% neutral buffered formalin, processed through graded ethanol and embedded in paraffin. The samples were frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis by reverse transcription-polymerase chain reaction (RT-PCR).

Histopathological examination. Tissue sections (4 μm thick) were deparaffinized in xylene, rehydrated and stained with hematoxylin and eosin solution. Separation of the tissues into non-tumor and tumor tissues was determined by a pathologist (K.W.) who was blinded to the clinical background. For non-tumor tissues, the presence of inflammation or cirrhotic nodules was examined. Tumor tissues were examined for the following characteristics: cell differentiation (well, moderate, poorly differentiated), number of tumors, capsular formation, septal formation, capsular invasion, portal vein tumor thrombus formation and hepatic vein invasion.

Preparation of anti-human PA28γ antibody. Chicken anti-human PA28γ antibody was prepared by immunization using the synthetic peptides of residues from 75 to 88, SHDGLDGPTYKKRR, of human PA28γ. The antibody was purified by affinity chromatography using beads conjugated with the antigen peptide.

Immunohistochemistry and evaluation of PA28γ immunostaining. Formalin-fixed tissues were embedded in paraffin according to the standard procedures. For immunohistochemistry, formalin-fixed tissue sections were boiled in Target Retrieval Solution (Dako, Glostrup, Denmark) and then treated with 3% H_2O_2. The activated sections were washed twice with phosphate-buffered saline (PBS), blocked with PBS containing 5% bovine serum albumin, and incubated overnight with the purified chicken antibody to PA28γ, followed by incubation with horseradish peroxidase-conjugated anti-chicken IgG antibody (ICN, Biomedicals, Inc., Aurora, OH, USA) as a secondary antibody. Immunoreactive antigen was visualized with 3,3'-diaminobenzidine substrate. The resulting sections were counterstained with hematoxylin. Staining of endogenous PA28γ with the antibody was identified in normal mouse liver sections but not in the liver sections from PA28γ-deficient mice. Pre-immune purified antibody did not react with any other antigen in these sections under the experimental conditions.

For evaluation of PA28γ immunostaining, each section was scored for nuclear and cytoplasmic staining using a scale from 0 to 2 where 0 represented negative or faint staining, 1 represented moderate staining, and 2 represented strong staining. In general, the nuclei of the bile ducts faintly expressed PA28γ (Fig. 1a). Thus, the staining level was used as a nuclear inner control within the sample, which was designated arbitrarily as intensity level 0. Also, slightly higher expression was designated arbitrarily as intensity level 1 and clearly higher expression was designated arbitrarily as intensity level 2. PA28γ expression was very faint or undetectable in the vascular epithelia and nuclei (Fig. 1a), whereas the cytoplasm of bile duct epithelial cells and nuclei devoid of significant inflammation generally expressed faint levels of PA28γ (Fig. 1a). For semi-quantitative analysis, the latter level of staining was used as a cytoplasmic inner control within the sample, and designated arbitrarily as intensity level 0. Furthermore, a slightly higher expression was designated arbitrarily as intensity level 1 whereas clearly higher expression was designated arbitrarily as intensity level 2. PA28γ expression was generally heterogeneous in each sample. For assessment of nuclear and cytoplasmic PA28γ, 4 high-power fields in each specimen were selected at random, and staining was examined under high power magnification. More than 1,000 cells were counted to determine the labeling index, which represented the percentage of immunostained cells relative to the total number of cells. The tissue samples were also categorized as positive (levels 1 and 2) and negative (level 0) for evaluation of the relationship between immunostaining and various clinicopathological factors.

Semi-quantitative RT-PCR. RNA extraction was carried out with TRIzol reagent using the single-step method, and the cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described previously (11). Sterol regulatory element binding protein-1c (SREBP-1c) mRNA expression was analyzed semi-quantitatively using the multiplex RT-PCR method. In this assay, the

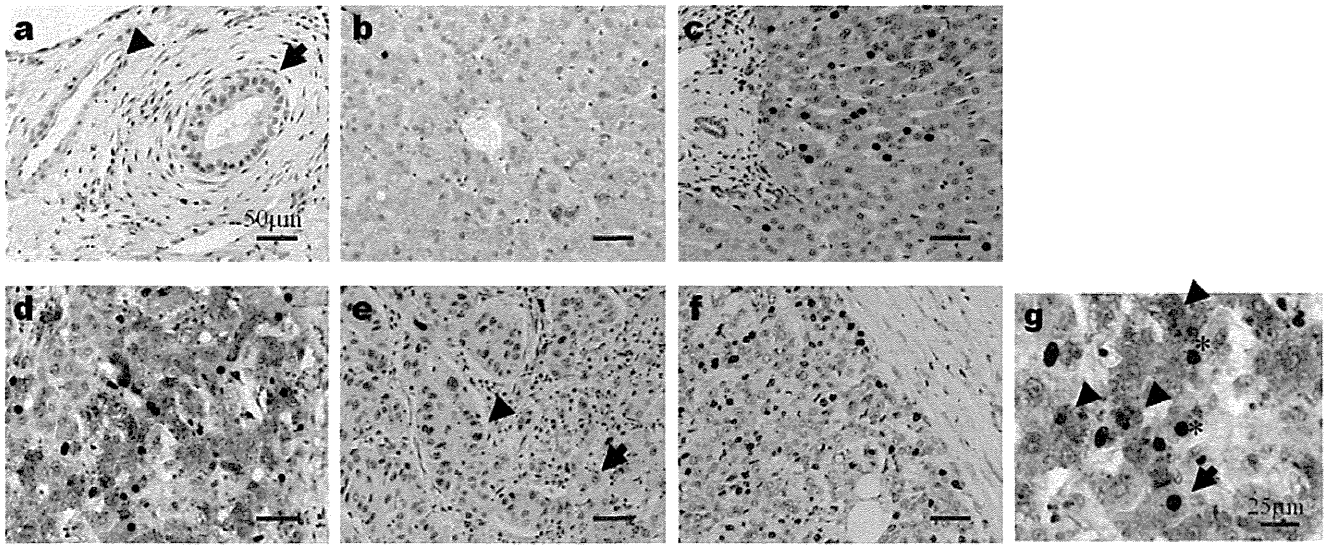

Figure 1. Immunohistochemical staining for PA28γ. (a-f) Representative samples for bile duct (inner control), vascular epithelium and various liver pathologies; (a) bile duct (arrow), vascular epithelium (arrowhead); (b) normal liver; (c) chronic hepatitis; (d) cirrhotic liver; (e) HCC with high nuclear PA28γ expression (arrowhead; left side) and non-tumor liver tissue with low nuclear PA28γ expression (arrow; right side); (f) HCC with low expression of nuclear PA28γ. Magnification, x200. (g) High-power view of liver section shown in (d). Note the faint staining of hepatocytes with high expression of nuclear PA28γ (arrow; hepatocytes, level 0 and nucleus, level 2), moderate staining of hepatocytes with high expression of nuclear PA28γ (asterisk; hepatocyte, level 1 and nucleus, level 2) and strong staining of hepatocytes with low expression of nuclear PA28γ (arrowhead; hepatocyte, level 2 and nucleus, level 0). Magnification, x400. No staining was observed when the primary antibody was substituted by non-immunized rabbit IgG or TBS (data not shown). PA28γ, proteasome activator 28γ; HCC, hepatocellular carcinoma; IgG, immunoglobulin G; TBS, Tris-buffered saline.

housekeeping gene, porphobilinogen deaminase (PBGD), was used as the internal control. This gene is favored over β-actin or glyceraldehyde-3-phosphate dehydrogenase as a reference gene for competitive PCR amplification as the presence of pseudogenes for the latter housekeeping genes may produce false-positive signals from genomic DNA contamination (12,13). In addition, in order to minimize possible inter-PCR differences, PCR was performed with SREBP-1c and PBGD primers in an identical tube, under unsaturated conditions. PCR was performed in a 25-μl reaction mixture containing 1 μl of the cDNA template, 1X Perkin-Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.8 μM of each primer for SREBP-1c and 80 nM PBGD, and 1 unit of TaqDNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc.). The PCR primers used for the detection of SREBP-1c and PBGD cDNAs were synthesized as described previously (14,15). The conditions for multiplex PCR were one cycle of denaturation at 95˚C for 12 min, followed by 40 cycles at 95˚C for 1 min, 62˚C for 1 min and 72˚C for 1 min, and a final extension at 72˚C for 10 min. The electrophoresed PCR products were scanned by densitometry, and the relative value of the SREBP-1c band relative to that of PBGD was calculated for each sample.

Statistical analysis. Data were expressed as the means ± SD. The Chi-square test and Fisher's exact probability test, or the log-rank test, were used to examine the association between PA28γ expression and the clinicopathological parameters or prognosis. A P-value of <0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using the StatView-J-5.0 program (SAS Institute, Cary, NC, USA).

Results

Immunohistochemical analysis of PA28γ. Immunohistochemical assays were performed on a series of 46 paired HCCs and their matched non-tumor tissues, and 5 normal livers. The labeling index of nuclear PA28γ showed a wide spectrum and increased from low in the normal livers to strong in the cirrhotic livers (Fig. 1b-d). Specifically, the nuclear PA28γ labeling index was generally low in the normal liver tissues, but was moderate-strong in HCV-related liver tissues. The nuclear labeling index was markedly higher in the majority of cirrhotic liver tissues. Fig. 2 summarizes the above results and the analysis of cytoplasmic expression of PA28γ. The difference in the PA28γ-nuclear labeling index between normal and cirrhotic livers was significant (P<0.0001) as was that between CH and cirrhosis (P<0.0001) (Fig. 2A). Also, the difference in the proportion of the PA28γ-cytoplasmic expression labeling index between normal and cirrhotic livers was significant (P<0.05) (Fig. 2B). The mean labeling indexes of nuclear PA28γ expression was 42% in both HCC and HCV-related livers.

To evaluate the relationship between immunohistochemical staining and various clinicopathological factors, we divided the samples into nuclear PA28γ high index (≥42%) and low index (<42%) groups. The labeling index was low in half of the examined HCC cases (50%; 18/36) and markedly high in the other half (50%; 18/36) (Table I). The labeling index was low in 30% (14/46) of HCV-related cases and markedly higher in the remaining 70% (32/46) (Table II). The samples were also divided into 2 groups according to the labeling index of cytoplasmic staining. The mean PA28γ-labeling index of the HCC and HCV-related cases was 58 and 80%, respectively. The labeling index was low in 47% (17/36) and high in 53% (19/36)

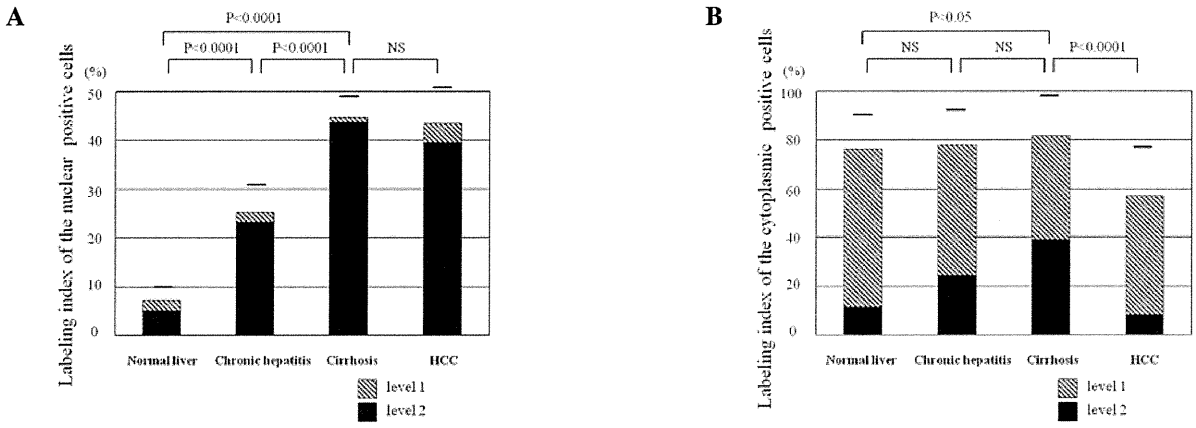

Figure 2. (A) Nuclear PA28γ expression in multistep hepatocarcinogenesis. The labeling index increased in a stepwise manner with the severity of liver damage and carcinogenesis. Quantitative analysis showed that 25, 10 and 1% of cells of the normal liver, CH and cirrhosis, respectively, were moderately positive (level 1). In HCCs, 10% of cells were evaluated as moderately positive (level 1). (B) Cytoplasmic PA28γ expression in multistep hepatocarcinogenesis. The expression increased slightly in a stepwise manner. Quantitative analysis showed that 80, 68 and 50% of cells of the normal liver, CH and cirrhosis, respectively, were moderately positive (level 1). In HCCs, 82% of cells were evaluated as moderately positive (level 1). PA28γ, proteasome activator 28γ; CH, chronic hepatitis; HCC, hepatocellular carcinoma. NS, not significant.

Table I. Correlation between nuclear PA28γ expression and various clinicopathological parameters in patients with HCC.

		PA28γ		
	n	Low (<42%)	High (≥42%)	P-value
Age (years)				
≥60	15	7	8	
<60	21	11	10	NS
Gender				
Male	21	10	11	
Female	15	8	7	NS
Tumor size				
≤2 cm	8	4	4	
>2 cm	28	14	14	NS
Histological type				
Well/moderately differentiated	5	2	3	
Poorly differentiated	31	16	15	NS
Hepatic vein invasion				
Yes	6	2	4	
No	30	16	14	NS
Portal vein tumor thrombus				
Yes	5	2	3	
No	31	16	15	NS
Number of tumors				
Multiple[a]	3	1	2	
Solitary	33	17	16	NS
Septum formation				
Yes	15	8	7	
No	21	10	11	NS
Capsular formation				
Yes	14	6	8	
No	22	12	10	NS
Capsular invasion				
Yes	8	1	7	
No	6	5	1	0.026

[a]This category includes intrahepatic metastasis and multicentric carcinogenesis. PA28γ, proteasome activator 28γ; HCC, hepatocellular carcinoma; NS, not significant.

Table II. Correlation between nuclear PA28γ expression and various clinicopathological parameters in non-tumor liver tissues.

		PA28γ		
	n	Low (<42%)	High (≥42%)	P-value
Age (years)				
≥60	22	5	17	
<60	24	9	15	NS
Gender				
Male	27	6	21	
Female	19	8	11	NS
HCV	28	9	19	
HBV	0			
HCV plus HBV	18	5	13	NS
Inflammatory status (HAI score)				
Absent-mild (0-3)	22	12	10	
Moderate-severe (>4)	24	2	22	0.0007
Degree of fibrosis (HAI score)				
Absent-moderate (0-2)	12	11	1	
Severe-cirrhosis (>3)	34	3	31	<0.0001

NS, not significant; PA28γ, proteasome activator 28γ; HCV, hepatitis C virus; HBV, hepatitis B virus; HAI, histological activity index.

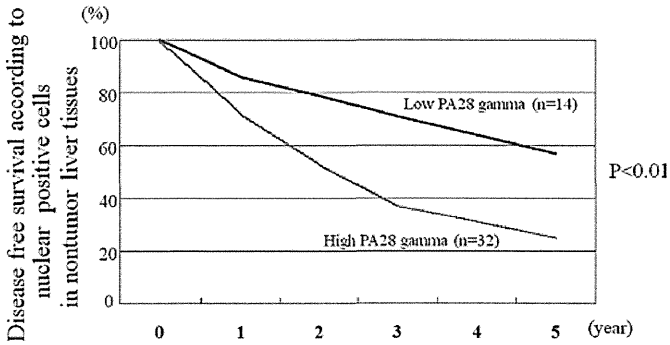

Figure 3. Disease-free survival based on nuclear PA28γ expression in non-tumor tissues. The disease-free survival was significantly different between patients with high nuclear PA28γ expression (levels 1 and 2) and those with low nuclear PA28γ expression (level 0) (P<0.01). PA28γ, proteasome activator 28γ.

of the HCC cases. The respective values for HCV-related cases were 28% (13/46) and 72% (33/46). All cut-off values used were according to the mean labeling index.

Correlation between nuclear PA28γ expression and clinicopathological parameters. We examined the correlation between PA28γ nuclear expression analyzed in 36 HCCs (10 samples with complete necrosis by TAE were excluded from this analysis) and various clinicopathological features (Table I). The cases were divided into two groups based on the labeling index of nuclear expression of PA28γ, using a cut-off mean value of 42%. There was a significant difference in PA28γ expression based on capsular invasion (Table I). We also analyzed the relationship between nuclear PA28γ expression in non-tumor tissues (15 CH and 31 cirrhosis) and disease-free survival, as the pathologic status of non-tumor tissues has been shown to correlate with the relapse of HCC (16-18). The disease-free survival, but not overall survival (P=0.052), was significantly different between high and low nuclear PA28γ expressors (P<0.01) (Fig. 3). In addition, PA28γ expression in non-tumor tissues correlated closely with active inflammation and fibrosis (Table II).

In univariate analysis, PA28γ expression in non-tumor liver tissues, portal vein tumor thrombus, inflammatory status and degree of fibrosis in the non-cancerous liver tissue were significant factors for disease-free survival. These variables were subsequently entered into multivariate analysis. The results identified nuclear PA28γ expression level [95% confidence interval (CI), 1.82-3.22; P<0.01], portal vein tumor thrombus (95% CI, 1.33-6.38; P=0.023), inflammatory status (95% CI, 2.11-3.58; P=0.012) and degree of fibrosis (95% CI, 1.99-7.21; P<0.01) as independent factors for disease-free survival (Table III).

SREBP-1c expression. Five CH and five cirrhotic liver tissues were selected to analyze the correlation between nuclear PA28γ expression and SREBP-1c gene expression in non-tumor liver tissues. Fig. 4 shows a clear correlation between nuclear PA28γ expression and SREBP-1c gene expression.

Discussion

The present study shows that non-tumor liver tissues commonly express high levels of nuclear PA28γ protein relative to those of carcinoma tissues. These results are contradictory to those from other studies on other types of cancer, such as thyroid carcinoma; the nuclear PA28γ level was higher in these tumors compared to non-tumor tissues (19). While the exact reason for

Table III. Multivariate analysis of clinicopathological factors for disease-free survival in patients with HCC.

	n	Relative risk	95% confidence interval	P-value
PA28γ				
High	32	2.67	1.82-3.22	<0.01
Low	14			
Portal vein tumor thrombus				
Yes	5	2.21	1.33-6.38	0.023
No	31			
Inflammatory status (HAI score)				
Absent-mild (0-3)	22	2.59	2.11-3.58	0.012
Moderate-severe (>4)	24			
Degree of fibrosis (HAI score)				
Absent-moderate (0-2)	12	2.68	1.99-7.21	<0.01
Severe-cirrhosis (>3)	34			

HCC, hepatocellular carcinoma; PA28γ, proteasome activator 28γ; HAI, histological activity index.

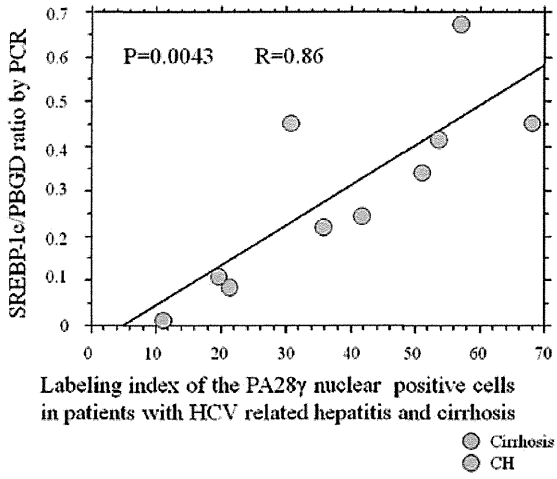

Figure 4. Linear correlation analysis of nuclear PA28γ expression and SREBP-1c gene expression in patients with cirrhosis and chronic hepatitis (CH) (P=0.0043). PA28γ, proteasome activator 28γ; HCV, hepatitis C virus. SREBP-1c, sterol regulatory element binding protein-1c.

the different results is not known at present, it is likely to be related to the type of control tissue used in the present study; the non-tumor tissues were mostly not normal, consisting of HCV-infected CH or cirrhotic tissues. In support of this conclusion, normal liver tissues from patients with metastatic liver tumors from patients with colorectal carcinoma who were negative for HCV/HBV showed low expression of nuclear PA28γ.

In non-neoplastic liver tissues, we found a wide spectrum of nuclear PA28γ expression from normal liver to cirrhosis. Our results also show that active inflammation with hepatitis virus induces nuclear PA28γ in CH and cirrhotic livers (Table II). This is reasonable considering the fundamental action of nuclear PA28γ as a mediator of inflammation. Another mechanism for the high induction of nuclear PA28γ in cirrhosis might be related to the degradation of the HCV core protein by PA28γ and its translocation from the cytoplasm to the nucleus, based on the results of our previous study (10). In fact, nuclear PA28γ-expressing cells had no or faint-to-moderate cytoplasmic PA28γ expression (Fig. 1c and g). Furthermore, the nuclear overexpression could be due to the relatively hypoxic microenvironment in the cirrhotic liver. In this regard, we hypothesized that hypoxia might directly induce PA28γ, which in turn enhances angiogenesis via the enhanced release of a battery of angiogenic growth factors, such as vascular endothelial growth factor (VEGF). Since the VEGF level is increased in cirrhosis (20), it is possible that nuclear PA28γ may improve the ischemic/hypoxic microenvironment in the cirrhotic liver through upregulation of angiogenesis. Although cirrhotic nodules occasionally show p53 mutation and increased telomerase activity (21,22), cirrhosis is not considered a premalignant lesion. However, it is apparent from a number of etiological studies that cirrhosis is a strong risk factor for HCC. In this context, nuclear PA28γ expression in cirrhosis might be a prerequisite for the genesis of premalignant dysplastic nodules or early cancer.

From a clinical point of view, it is interesting to note the correlation between high nuclear PA28γ expression in non-tumor tissues and the relapse of HCC. The prognosis of HCC is generally unfavorable. Although primary tumors are curatively resected, 50-60% of patients develop relapse within 5 years. This is due to either a newly established tumor from the remnant liver, a process termed multicentric carcinogenesis, or recurrence of the original tumor. One possible mechanism for a link between nuclear PA28γ and disease relapse is that high expression of PA28γ in the remnant liver may contribute to carcinogenesis. Nuclear PA28γ expression highly correlated with the presence of active inflammation (P<0.0001). Furthermore, active inflammation in non-tumor tissues has been reported to be associated with relapse of HCC (17,23,24).

In the present study, a clinicopathological survey demonstrated a significant correlation between nuclear PA28γ protein expression and capsular invasion of the cancer tissue. This

finding is in agreement with a recent study that showed increased expression of PA28γ protein during cancer progression and its correlation with PCNA labeling index (19). Thus, the results suggest the possible involvement of PA28γ in HCC progression. Further studies of larger population samples are required to confirm the clinical significance of nuclear PA28γ in HCC. This is particularly important, as the overall survival of patients with high nuclear PA28γ expression was worse than that of those with low expression level (P=0.052) (data not shown).

Also in our series, the labeling index of cytoplasmic expression of PA28γ significantly increased from normal liver to cirrhotic liver (Fig. 2b). Further extended studies are required to determine the importance of cytoplasmic expression of PA28γ in HCC and HCV-related liver.

In conclusion, the present study demonstrates a close correlation between nuclear PA28γ expression in liver tissue and the development and progression of HCC, as well as its possible involvement in HCC relapse. Further studies are required to examine the therapeutic benefits of the suppression of nuclear PA28γ expression in HCV-related CH, cirrhosis or HCC.

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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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