Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV ($\leq$40%, 41–60%, 61–80%, and >80%), which showed that patients with a high risk of anemia were predominantly in subgroups with a lower adherence to RBV ($\leq$40%, 41–60%, and 61–80%), whereas patients with a low risk of anemia were predominantly in subgroups with a higher adherence to RBV (>80%) (Fig. 4, upper panel). The percentage of patients who received >80% of the planned dose of RBV was significantly higher in the low-risk group for anemia than in the high-risk group (74% vs. 55%, $P < 0.0001$).

Within the groups with high and low risks of anemia, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV (Fig. 4, lower panel). The rate of sustained virological response was higher in patients who received >80% of the planned dose of RBV than those who received less, for both high-risk patients (71% vs. 47%, $P = 0.016$) and low-risk patients (81% vs. 60%, $P = 0.072$). Within the same subgroup of RBV adherence, however, the rate of sustained virological response did not differ between patients with a high risk and a low risk of anemia. Taken together, these results suggest that patients with a high risk of anemia have a disadvantage because they are likely

to be intolerant to RBV, leading to reduced adherence to RBV throughout the 48 weeks of therapy and a reduced rate of sustained virological response. However, if >80% adherence to RBV could be obtained, the rate of sustained virological response would increase by 24%.

DISCUSSION

This study confirmed previous reports that the *IL28B* genotype is the most significant predictor of a sustained virological response to PEG-IFN plus RBV therapy in chronic hepatitis C patients at baseline [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010; Kurosaki et al., 2011c] and at week 4 [Thompson et al., 2010b], but it had no impact on the rate of sustained virological response among those patients who achieved a complete early virological response [Thompson et al., 2010b; Kurosaki et al., 2011c]. In contrast, the risk of anemia, assessed by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was found to be associated with a sustained virological response in patients who achieved a complete early virological response. Generally, a complete early virological response is the hallmark of a high probability of a sustained virological response, but the rate of sustained virological responses in patients who achieved a complete early virological response and had a high risk of anemia was as low as 59%. This reduced rate of sustained virological response in these patients was attributable to poor adherence to RBV throughout the 48 weeks of therapy. Because administration of >80% of the planned RBV dose increased the rate of sustained virological response by 24%, it may be postulated that personalizing the treatment schedule to achieve a sufficient dose of RBV, such as extension of treatment duration, may improve sustained virological response rates in these patients. Clearly, this postulate needs to be confirmed in future study. Thus, the findings presented here may have the potential to support selection of the optimum, personalized treatment strategy for an individual patient, based on the risk of anemia.

The degree of hemolytic anemia caused by RBV varies among individuals. A reduction of the Hb concentration early during therapy predicts the likely development of severe anemia [Hiramatsu et al., 2008, 2011] but there are no reliable predictors at baseline. A breakthrough came from the results of a genome-wide association study that revealed that variants of the *ITPA* gene are protective against hemolytic anemia [Fellay et al., 2010]. The *ITPA* genotype has been shown repeatedly to be associated with the degree of hemolytic anemia and dose reduction of RBV [Fellay et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a; Seto et al., 2011; Tanaka et al., 2011; Kurosaki et al., 2011d]. However, factors other than the *ITPA* gene, such as baseline Hb concentrations [Ochi et al., 2010; Kurosaki et al., 2011d], platelet counts [Ochi
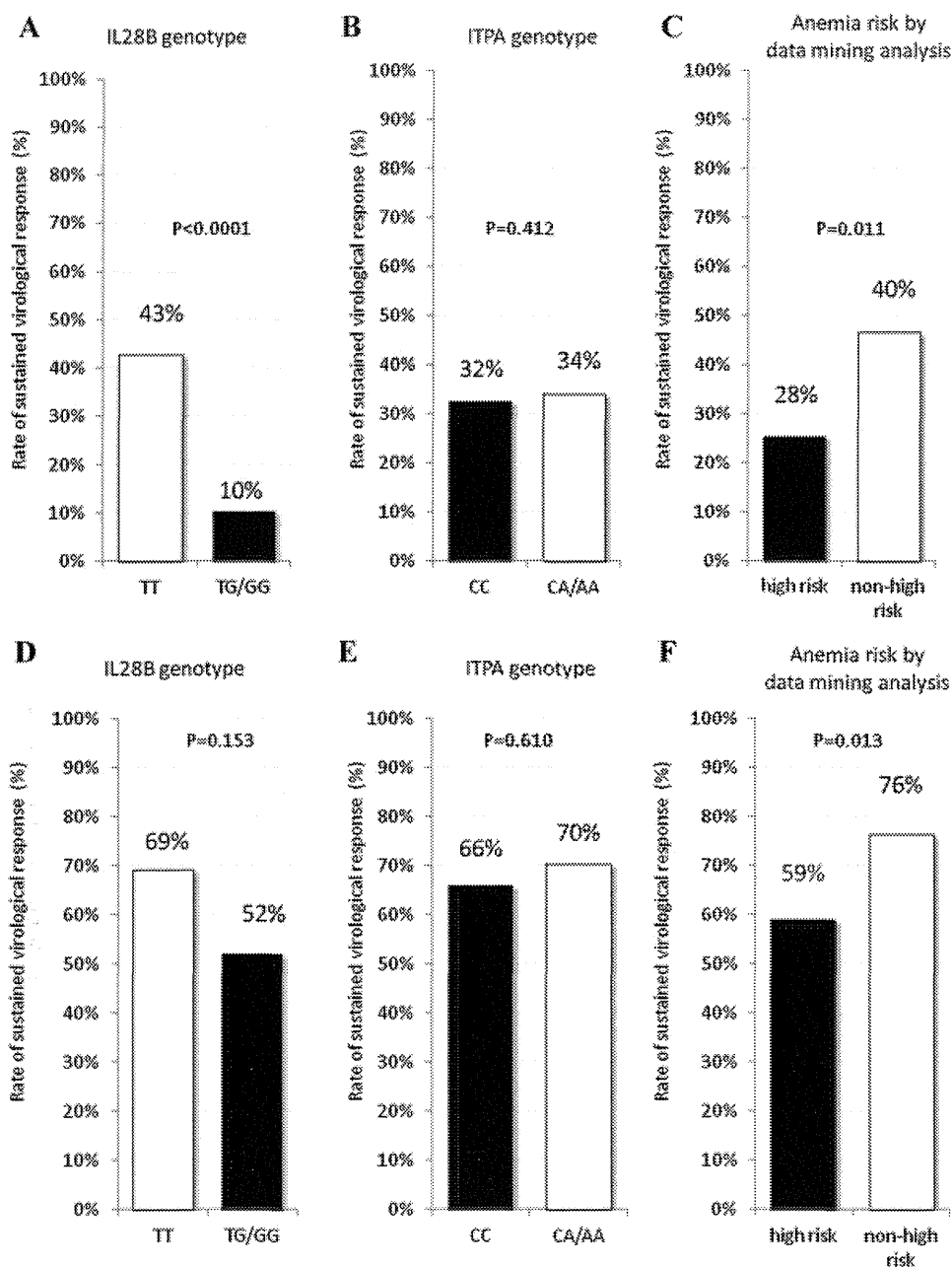
Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (**A–C**) and among those with complete early virological responses (defined as undetectable HCV RNA at week 12) (**D–F**). At baseline, those with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele and the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group. Among patients with complete early virological responses, the *IL28B* genotype was not associated with a sustained virological response, while the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group.

et al., 2010], and CLcr [Kurosaki et al., 2011d], also contribute to the risk of severe anemia or RBV dose reduction. In the present study, the predictive model of anemia based on the data mining analysis selected the *ITPA* genotype, baseline Hb concentration, and baseline CLcr as predictive factors and identified six subgroups of patients with a variable rate of severe anemia, ranging from 17% to 76%. The specificity of the prediction of severe anemia was improved by 25.7% in the predictive model, compared to *ITPA*

TABLE II. Logistic Regression Analysis for Factors Associated With Sustained Virological Response at Baseline, Week 4 and Week 12

	Multi-variable		
	Odds	95% CI	P-value
Pre-treatment			
Sex: female	0.42	0.26–0.68	<0.0001
Platelet (10^9/L)	1.09	1.04–1.15	<0.0001
Fibrosis: F3-4	0.49	0.27–0.91	0.024
HCV RNA: <600,000 IU/L	4.14	2.27–7.55	<0.0001
IL28B rs8099917: TT	9.88	5.01–19.48	<0.0001
At week 4			
Non-RVR patients			
Sex: female	0.45	0.28–0.72	0.001
Platelet (10^9/L)	1.10	1.05–1.16	0.000
IL28B rs8099917: TT	7.16	3.60–14.25	<0.0001
At week 12			
cEVR patients			
Platelet (10^9/L)	1.09	1.02–1.17	0.015
HCV RNA: <600,000 IU/L	3.21	1.39–7.55	0.007
High-risk of anemia[a]	0.47	0.24–0.91	0.026
At week 12			
Non-cEVR patients			
Platelet (10^9/L)	1.11	1.02–1.21	0.017
IL28B rs8099917: TT	9.13	2.02–41.3	0.004

RVR: rapid virological response, defined as undetectable HCV RNA at week 4.
cEVR: complete early virological response, defined as undetectable HCV RNA at week 12.
[a]High-risk of anemia defined by decision tree analysis includes the following groups: (1) baseline hemoglobin <14.0 g/dl and creatinine clearance <90 ml/min, (2) baseline hemoglobin <14.0 g/dl, creatinine clearance ≥90 ml/min and ITPA rs1127354 genotype CC, and (3) baseline hemoglobin ≥14.0 g/dl, ITPA rs1127354 genotype CC, and creatinine clearance <85 ml/min.

genotyping alone. Because hemolytic anemia induced by RBV is one of the major adverse events leading to premature termination of therapy [Fried et al., 2002], a method to predict the risk of severe anemia before treatment is important clinically. A predictive model of anemia may have the potential to support individualized treatment strategies; patients at high risk of anemia may be tested intensively for anemia or may be candidates for erythropoietin therapy, whereas those with a low risk of anemia may be treated with a higher dose of RBV. Prediction of anemia will remain important in the era of direct antiviral agents for chronic hepatitis C, because these newer therapies still require RBV and PEG-IFN in combination, and the degree of anemia complicating these therapies may be even greater than with the current combination therapy [McHutchison et al., 2009; Kwo et al., 2010].

Studies of the impact of the ITPA genotype on treatment outcome have produced conflicting results. Previous studies of American [Thompson et al., 2010a] and Italian [Thompson et al., 2011] cohorts did not find any association between the ITPA genotype and treatment outcome, whereas a marginal difference was observed in a report from Japan [Ochi et al., 2010]. Moreover, with a subgroup analysis of Japanese patients, the variant of the ITPA gene was

associated with a sustained virological response in patients with the IL28B major genotype [Kurosaki et al., 2011d], in patients infected with HCV other than genotype 1[Sakamoto et al., 2010], and in patients with pre-treatment Hb concentrations between 13.5 and 15 g/dl [Azakami et al., 2011]. These inconsistent results may be because the impact of anemia may be greater on a cohort of aged patients, such as in Japan. Another reason may be that the ITPA genotype is not the sole determinant of anemia; the ITPA genotype alone was not associated with treatment outcome in the present study but a high-risk of anemia, defined by the combination of the ITPA genotype, baseline Hb concentration, and baseline CLcr, was associated with sustained virological responses by patients with complete early virological responses, even after adjustment for the IL28B genotype and other relevant factors. This is in contrast to the finding that the IL28B genotype is an independent and significant predictor at baseline of a sustained virological response by patients without a rapid virological response and those without a complete early virological response, but not those with a complete early virological response. These results indicate that the IL28B genotype could be used to predict a sustained virological response at baseline or during therapy in patients in whom HCV RNA has not yet become undetectable, but it has no predictive value in patients in whom HCV RNA has become undetectable. The risk of anemia may be used to predict sustained virological responses in a selected subgroup of patients who achieve a complete early virological response.

Patients who received more than 80% of the planned dose of PEG-IFN or RBV had a higher rate of sustained virological responses than those who received a lower cumulative dose [McHutchison et al., 2002; Davis et al., 2003]. Patients who achieve a complete early virological response usually have a good chance of a sustained virological response and the treatment duration is not extended beyond 48 weeks. However, reduced adherence to drugs in these patients was related to relapse after the completion of 48 weeks of therapy [Hiramatsu et al., 2009; Kurosaki et al., 2012]. In the present study, the rate of sustained virological response was 59% in patients who achieved a complete early virological response but had a high risk of anemia, 17% lower than in patients with a low risk of anemia. However, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV, and the rate of sustained virological response was higher in high-risk patients who received >80% of the planned dose of RBV (71% vs. 47%). This 24% increase in sustained virological response was observed among the patients in the present study who received 48 weeks of treatment. These findings suggest that receiving a sufficient RBV dose is essential for patients with a complete early virological response to attain a sustained virological response and that the treatment strategy should be personalized for patients with a
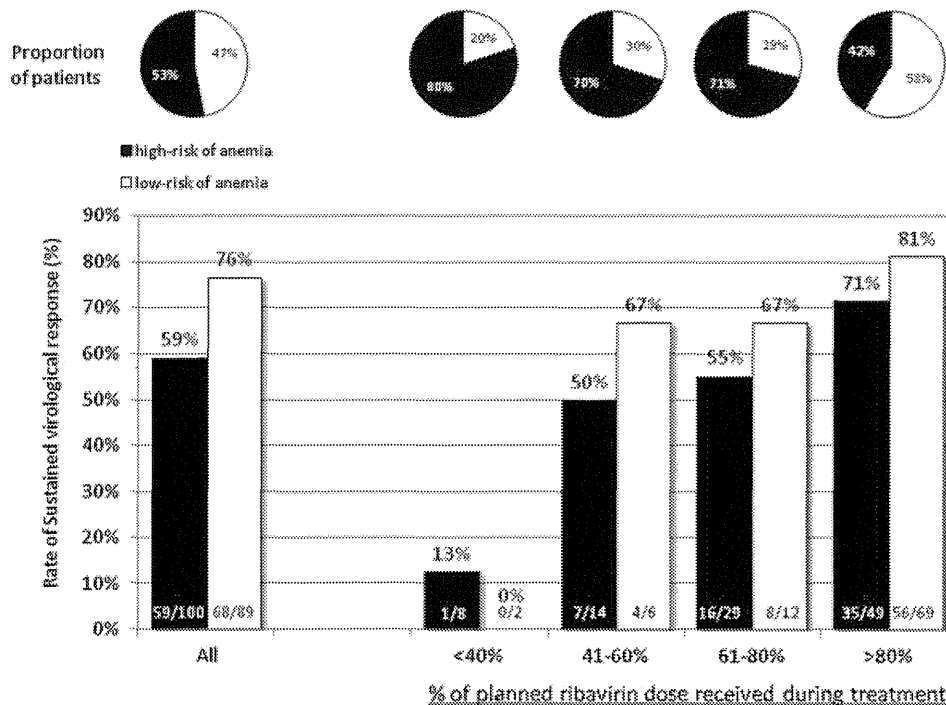
Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of patients with a high risk and a low risk of anemia is shown in the upper panel by pie charts, and the rates of sustained virological responses, stratified by high risk and low risk of anemia, are shown in the lower panel by bar graphs. The black and white bars or charts represent patients with high and low risks of anemia, respectively.

high risk of anemia to extend the duration of treatment, even those patients with a complete early virological response, to obtain >80% adherence to RBV.

In conclusion, the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr could be used as a pre-treatment predictor of anemia. The risk of anemia thus identified is associated with adherence to RBV and impacts on the treatment outcome of patients who achieve a complete early virological response. This is in contrast to the major role of the *IL28B* genotype in the prediction of sustained virological responses at baseline and among non-responders at weeks 4 and 12. Patients who achieve a complete early virological response generally have a high probability of a sustained virological response but those who have a high risk of anemia have a high rate of relapse because of reduced adherence to RBV. To improve the rate of sustained virological responses in these patients, it may be postulated that the treatment schedule may be personalized to obtain >80% adherence to RBV. Clearly, this postulate needs to be confirmed in a future study.

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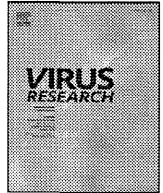
Short communication

Suppression of hepatitis C virus replicon by adenovirus vector-mediated expression of tough decoy RNA against miR-122a

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ABSTRACT

Recent studies have demonstrated that the liver-specific microRNA (miRNA) miR-122a plays an important role in the replication of hepatitis C virus (HCV). Antisense nucleotides against miR-122a, including locked nucleic acid (LNA), have shown promising results for suppression of HCV replication; however, a liver-specific delivery system of antisense nucleotides has not been fully developed. In this study, an adenovirus (Ad) vector that expresses tough decoy (TuD)-RNA against miR-122a (TuD-122a) was developed to suppress the HCV replication in the liver hepatocytes. Ad vectors have been well established to exhibit a marked hepatotropism following systemic administration. An in vitro reporter gene expression assay demonstrated that Ad vector-mediated expression of TuD-122a efficiently blocked the miR-122a in Huh-7 cells. Furthermore, transduction with the Ad vector expressing TuD-122a in HCV replicon-expressing cells resulted in significant reduction in the HCV replicon levels. These results indicate that Ad vector-mediated expression of TuD-122a would be a promising tool for treatment of HCV infection.

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Hepatitis C virus (HCV) is a hepatotropic human virus belonging to a member of the family *Flaviviridae* and possessing a 9.6-kb positive-sense RNA genome. HCV infection causes chronic hepatic inflammation and fibrosis, leading to hepatocellular carcinoma (Hoofnagle, 2002). Currently, 170 million people worldwide are infected with HCV, and suffering from or at risk for the diseases described above. In order to suppress the replication of HCV, PEGylated interferon alpha and ribavirin, which is a nucleotide analogue, have been used as standard-of-care therapy; however, the therapeutic efficiency has been limited, in spite of relatively severe side effects, including fever and malaise (Chisari, 2005; Feld and Hoofnagle, 2005). Another therapeutic strategy should be developed to efficiently suppress the HCV infection and HCV-caused diseases.

Among several host factors involved in HCV infection, the abundant liver-specific microRNA (miRNA), miR-122a has been demonstrated to be crucial for efficient replication and/or

translation of the HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007). The HCV genome has two closely spaced miR-122a-binding sites in the 5′-untranslated region (UTR), which contains overlapping *cis*-acting signals involved in translation and RNA synthesis (Jopling et al., 2005). Although the mechanism of the miR-122a-mediated enhancement of HCV replication is controversial (Henke et al., 2008; Jopling et al., 2005; Machlin et al., 2011; Roberts et al., 2011; Wilson et al., 2011), antisense oligonucleotides complementary to miR-122a, including locked nucleic acid (LNA) oligonucleotides, have been shown to significantly inhibit miR-122a and reduce the HCV genome, and thereby to exhibit superior therapeutic effects (Henke et al., 2008; Jopling et al., 2005; Krutzfeldt et al., 2005; Lanford et al., 2010). Intravenous administration of LNA oligonucleotides against miR-122a into HCV-infected chimpanzees resulted in the long-lasting suppression of HCV viremia without viral resistance or severe side effects (Lanford et al., 2010). In addition, the 5′-UTR of the HCV genome is composed of highly conserved structural domains, suggesting that a mutant lacking the miR-122a-binding sites in the genome is unlikely to appear. These results indicate that miR-122a is a promising target for the treatment of HCV-related diseases; however, LNA oligonucleotides accumulate in the kidney immediately after intravenous administration and are excreted into the urine (Fluiter et al., 2003).

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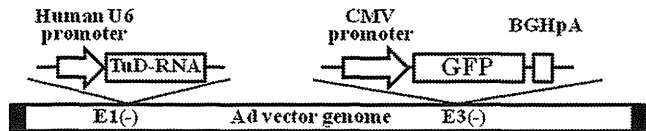E-mail address: mizuguch@phs.osaka-u.ac.jp (H. Mizuguchi).

Fig. 1. Structure of Ad vectors used in this study. The human U6 promoter-driven TuD-RNA expression cassette was inserted into the E1-deleted region of the Ad vector genome. The CMV promoter-driven GFP expression cassette was inserted into the E3-deleted region of the Ad vector genome.

Systems which efficiently deliver or express anti-miR-122a drugs in the liver are necessary to efficiently treat HCV-related diseases.

Recently, tough decoy (TuD)-RNAs against miRNAs, which efficiently and specifically inhibit miRNAs, were developed by Haraguchi et al. (2009). TuD-RNAs are composed of two miRNA-binding sequence (MBS) regions and two stem structures with 3-nucleotide linkers. The MBS in the TuD-RNA is considered to tightly bind to miRNAs, leading to the inhibition of miRNAs. The inhibition activity of the TuD-RNA against miRNAs is higher than that of LNA oligonucleotides and miRNA sponges (Haraguchi et al., 2009). Another advantage of the TuD-RNA is that it can be expressed by viral and non-viral vectors. miRNAs can be persistently suppressed by lentivirus vector- and retrovirus vector-mediated expression of the TuD-RNA. Furthermore, liver-specific expression of the TuD-RNA is thought to be achievable by an adenovirus (Ad) vector and adeno-associated virus vector, because these vectors can express transgenes in a liver-specific manner after systemic administration. These properties of the TuD-RNA are highly promising for inhibition of miR-122a in the liver and suppression of HCV replication.

In the present study, we developed an Ad vector expressing the TuD-RNA against miR-122a (TuD-122a) to efficiently inhibit miR-122a and to suppress the HCV replication. Transduction with an Ad vector expressing TuD-122a efficiently inhibited miR-122a in vitro. In HCV replicon-expressing cells, HCV replicon levels were significantly reduced by Ad vector-mediated TuD-122a expression.

First, in order to examine the transduction efficiencies of the Ad vectors constructed in this study in the HCV replicon-expressing cells, Huh-7.5.1 1bFeo cells, which is a genotype 1b HCV replicon cell line (Yokota et al., 2003), were transduced with an Ad vector expressing TuD-122a (Ad-TuD-122a) or the control TuD-RNA (Ad-TuD-NC). Ad-TuD-122a and Ad-TuD-NC were prepared as described in Supplemental materials and methods. Structure of Ad vectors used in this study is shown in Fig. 1. The ratio of particles-to-biological titer was between 6 and 9 for each Ad vector used in this study. Both Ad-TuD-122a and Ad-TuD-NC carry the TuD-RNA expression cassette and the green fluorescence protein (GFP) expression cassette in the E1-deleted and E3-deleted region, respectively (Fig. 1). Both Ad-TuD-NC and Ad-TuD-122a efficiently transduced Huh-7.5.1 1bFeo cells (Fig. 2). More than 80% of the cells were found to be GFP-positive following transduction with Ad-TuD-122a and Ad-TuD-NC, respectively, at a multiplicity of infection (MOI) of 100. The averages of GFP-positive cells following transduction with Ad-TuD-NC were slightly higher than those with Ad-TuD-122a; however, statistically significant differences were not found for either group. Apparent cellular toxicity was not found following transduction with Ad-TuD-122a or Ad-TuD-NC (data not shown). These results indicate that Ad-TuD-122a and Ad-TuD-NC efficiently transduce Huh-7.5.1 1bFeo cells.

Next, in order to examine the inhibitory effects of TuD-122a expressed by the Ad vector on miR-122a, a reporter gene assay using the miR-122a complementary sequence-encoded plasmid, psiCheck-122aT, was performed in Huh-7 cells. Huh-7 cells endogenously express a high level of miR-122a (Suzuki et al., 2008). Huh-7 cells were transduced with the Ad vectors at MOIs of 25 and 100 for 1.5 h. After a 24-h incubation, the cells were transfected with
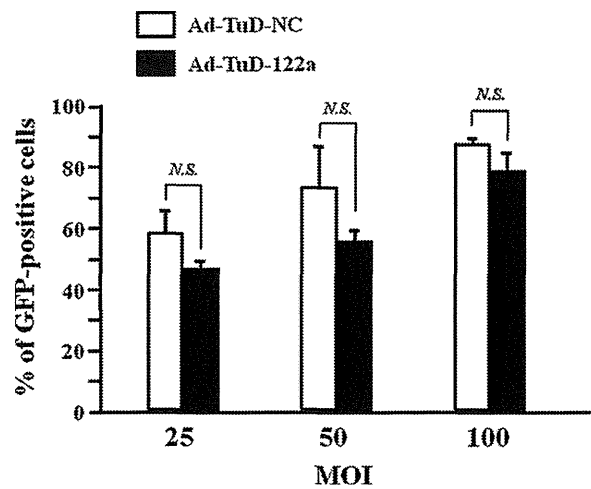

Fig. 2. Transduction efficiencies of Ad-TuD-122a and Ad-TuD-NC in Huh-7.5.1 1bFeo cells. The cells were transduced with Ad-TuD-122a or Ad-TuD-NC at multiplicities of infection (MOIs) of 25, 50, and 100 for 1.5 h. At 48 h after transduction, GFP expression was evaluated by flow cytometry. The data are expressed as the means ± S.D. (n = 3). The percentage of GFP-positive cells in the mock-transduced group was less than 0.2%. N.S.: not significant.

psiCheck-2 or psiCheck-122aT. The renilla and firefly luciferase expression was evaluated 48 h after transfection with the plasmid DNA. psiCheck-122aT, plasmid DNA containing the two copies of miR-122a complementary sequences in the 3′-UTR of the renilla luciferase gene, was constructed by ligation of NotI/XhoI-digested psiCheck-2 (Promega, Madison, WI) with the oligonucleotides 122aT-F and 122aT-R. The sequences of the oligonucleotides 122aT-F and 122aT-R are described in the Supplemental information. In mock-transduced cells, the relative renilla luciferase expression level by psiCheck-122aT was about 5-fold lower than that by the control plasmid psiCheck-2, which does not possess miR-122a target sequences, due to the endogenous expression of miR-122a in Huh-7 cells (Fig. 3). The renilla luciferase expression profiles following transfection with psiCheck-122aT were similar in the mock-transduced cells and Ad-TuD-NC-transduced cells, indicating that expression of the control TuD-RNA does not inhibit the miR-122a. Ad-TuD-122a did not alter the renilla luciferase expression level by psiCheck-2; on the other hand, psiCheck-122aT-mediated renilla luciferase expression was significantly restored by Ad-TuD-122a. The cells transduced with Ad-TuD-122a exhibited 2.8-fold and 3.5-fold higher renilla luciferase expression at MOIs of 25 and 100, respectively, than the mock-transduced cells following transfection with psiCheck-122aT. These results indicate that miR-122a is efficiently inhibited by Ad-TuD-122a. We also performed quantitative RT-PCR analysis for miR-122a following transduction with Ad-TuD-122a and Ad-TuD-NC in Huh-7 cells. No significant differences in the miR-122a expression levels were found in the cells transduced with Ad-TuD-122a and the cells transduced with Ad-TuD-NC (data not shown), probably because TuD-RNA does not induce degradation of miRNA, although TuD-RNA tightly binds to the target miRNA (Haraguchi et al., 2009).

Next, in order to examine whether TuD-122a-mediated inhibition of miR-122a suppresses the HCV replicon, Huh-7.5.1 1bFeo cells were transduced with Ad-TuD-122a and Ad-TuD-NC at the indicated MOIs. Huh-7.5.1 1bFeo cells express an mRNA consisting of the HCV 5′-UTR and the upstream part of the core region, connected in-frame with the firefly luciferase gene, which allows the simple evaluation of the HCV replicon levels by measuring the firefly luciferase activity in the cells (Yokota et al., 2003). Huh-7.5.1 1bFeo cells were transduced with the Ad vectors at MOIs of 25, 50, and 100 for 1.5 h. After a total 48-h incubation,
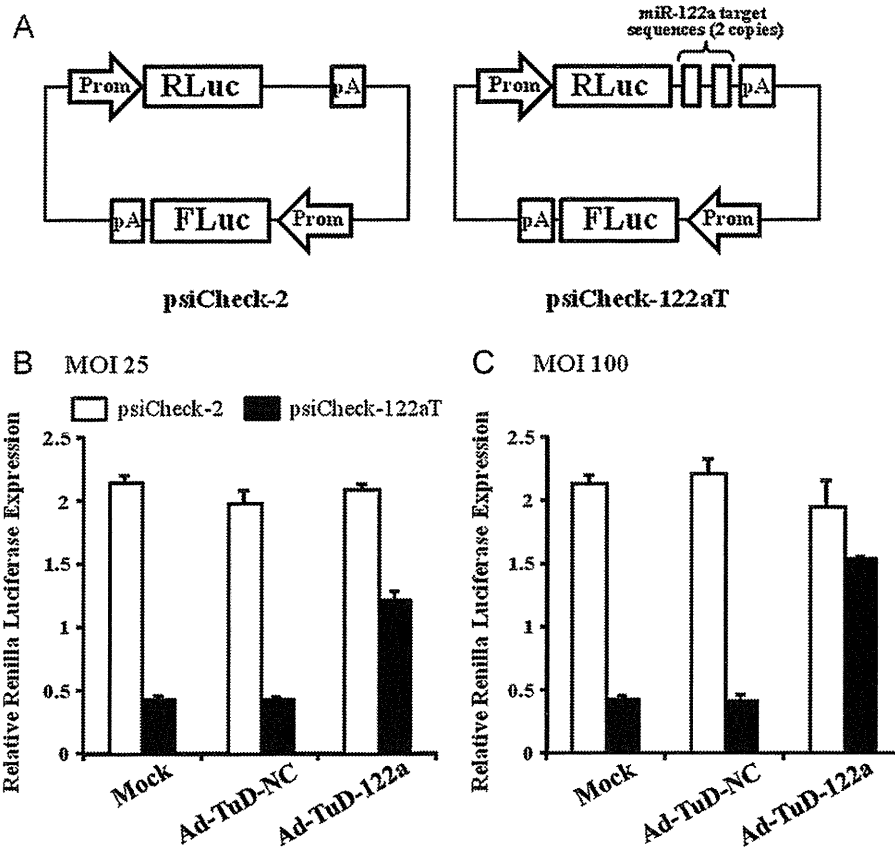
A

psiCheck-2

psiCheck-122aT

miR-122a target
sequences (2 copies)

B MOI 25

C MOI 100

Fig. 3. Inhibition of miR-122a by Ad vector-meditated TuD-122a expression. (A) Structure of the reporter gene-expressing plasmids psiCheck-2 and psiCheck-122aT. (B and C) Relative renilla luciferase expression levels following transduction with Ad-TuD-NC or Ad-TuD-122a at MOIs of 25 (B) and 100 (C). The data are expressed as the means $\pm$ S.D. (n = 4)..

firefly luciferase expression levels were determined. Ad-TuD-122a significantly reduced the firefly luciferase expression levels in a dose-dependent manner (Fig. 4a). The firefly luciferase expression level was reduced to 29% of that in the cells transduced with Ad-TuD-NC at MOI of 100 by transduction with Ad-TuD-122a at MOI of 100. In contrast, no significant changes in the firefly luciferase expression were found by transduction with Ad-TuD-NC.

To examine whether inhibition of miR-122a by Ad vector-mediated TuD-122a expression leads to a reduction in HCV replicon RNA levels, strand-specific real-time RT-PCR analysis was performed to determine the HCV replicon RNA levels. Briefly, Huh-7.5.1 1bFeo cells were transduced with the Ad vectors as described above, and the total RNA was isolated 48 h after transduction. Real-time RT-PCR analysis for the HCV positive-strand RNA genome was performed as follows. Briefly, 2 μg of total RNA was reverse-transcribed to cDNA using the primer specific for the HCV positive-strand genome (RC21; 5′-ctc ccg ggg cac tcg caa gc-3′). Real-time RT-PCR was performed using the primers (RC21 and RC1; 5′-gtc tag cca tgg cgt tag ta-3′) and SYBR Premix Ex Taq II (Takara Bio Inc., Kyoto, Japan). Similarly to the results for the firefly luciferase expression in Fig. 4A, HCV replicon RNA levels were significantly reduced by Ad-TuD-122a (Fig. 4B). There was an approximately 2.2-fold decline in the HCV replicon RNA level in the cells transduced with Ad-TuD-122a at an MOI of 100, compared with the HCV replicon RNA level in the cells transduced with Ad-TuD-NC at an MOI of 100. Ad-TuD-NC did not apparently decrease the HCV replicon RNA levels. These results indicate that the inhibition of miR-122a by Ad vector-mediated TuD-122a expression efficiently suppresses the replication of the HCV replicon.

The present study demonstrates that Ad vector-mediated TuD-122a expression significantly inhibits the function of miR-122a and

replication of the HCV replicon. Replication of the HCV genome is promoted by the direct interaction between miR-122a and the complementary sequences in the 5′-UTR of the HCV genome (Henke et al., 2008; Jangra et al., 2010), indicating that sequestration of miR-122a leads to suppression of the HCV replication. In order to suppress the HCV replicon by inhibiting miR-122a, TuD-RNA was selected as an inhibitor of miRNA in this study, because TuD-RNA potently inhibits miRNA by strongly binding to miRNA (Haraguchi et al., 2009). In addition, TuD-RNA can be expressed by conventional gene delivery vectors, including virus vectors. One drawback of TuD-RNA is that TuD-RNA does not discriminate miRNA members that belong to the same miRNA family (Haraguchi et al., 2009); however, miR-122a does not constitute a family of miRNA, suggesting that TuD-122a would not inhibit other miRNAs.

As described above, an Ad vector is suitable for liver-specific expression of TuD-RNA due to the strong hepatotropism. Previous studies demonstrated that Ad vectors expressing short-hairpin RNA (shRNA) or antisense RNA against the HCV genome successfully exhibited the suppressive effects on HCV infection in vivo (Gonzalez-Carmona et al., 2011; Sakamoto et al., 2008). Another advantage of using an Ad vector for treatment of HCV-related diseases is that in vivo administration of an Ad vector induces type I interferon (IFN) production via innate immune responses (Huarte et al., 2006; Zhu et al., 2007). Our group previously demonstrated that VA-RNA, which is a small non-coding RNA expressed from a replication-incompetent Ad vector as well as wild-type Ad, stimulates type I IFN production in an IFN-β promoter stimulator-1 (IPS-1)-dependent manner (Yamaguchi et al., 2010). Ad vector-induced type I IFN would contribute to suppression of HCV infection. The anti-HCV activity of Ad-TuD-122a can also be up-regulated by insertion of an expression cassette of an
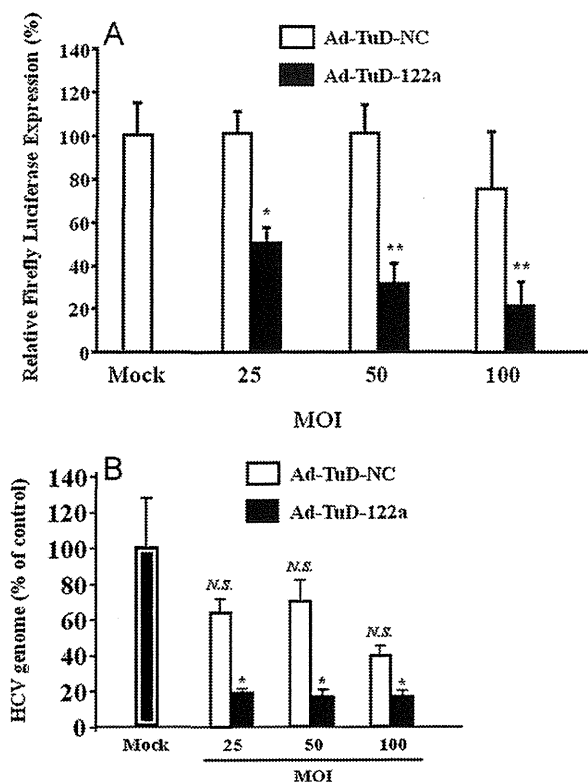
Fig. 4. Suppression of the HCV replicon by Ad vector-mediated TuD-122a expression. (A) Firefly luciferase expression levels and (B) HCV replicon RNA levels in Huh-7.5.1 1bFeo cells following transduction with the Ad vectors. All the data are shown as the means ± S.D. ($n = 3$). N.S.: not significant. *$P < 0.05$, **$P < 0.005$ between mock-transduced cells and cells transduced with Ad-TuD-122a.

anti-HCV gene, including type I IFN genes and short-hairpin RNA (shRNA) or antisense RNA against the HCV genome, into the Ad vector genome. Our group has developed various types of Ad vectors in which two or three transgene expression cassettes can be inserted into a single Ad vector genome (Mizuguchi et al., 2001, 2005, 2003).

Previous studies have demonstrated that lipid droplets, which are lipid-storage intracellular organelles, are crucial for the production of infectious HCV particles (Hinson and Cresswell, 2009; Miyanari et al., 2007). Miyanari et al. demonstrated that HCV capsid proteins recruit the non-structural proteins and the replication complex to the lipid droplet-associated membrane (Miyanari et al., 2007). miR-122a is an important factor that regulates cholesterol and fatty-acid metabolism in the hepatocytes (Esau et al., 2006; Iliopoulos et al., 2010). Intravenous administration of the antisense oligonucleotide against miR-122a resulted in a reduction in the plasma levels of cholesterol and triglycerides (Esau et al., 2006; Lanford et al., 2010). In addition to the enhancement of accumulation and translation of the HCV genome, miR-122a might up-regulate HCV infection by regulating lipid metabolism in the hepatocytes.

Almost similar levels of reduction in the HCV replicon RNA copy numbers were found for Ad-TuD-122a at MOIs of 25, 50, and 100, although there was dose-dependent reduction in the firefly luciferase expression following transduction with Ad-TuD-122a. It remains unclear why dose-dependent reduction in the HCV replicon RNA copy numbers was not found, however, miR-122a plays a crucial role in the enhancement of both translation and stability of HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007; Shimakami et al., 2012). Stability of HCV genome might be more susceptible to inhibition of miR-122a than translation. The averages of HCV replicon RNA levels were also reduced following transduction with Ad-TuD-NC, although

statistically significant differences were not found, compared with the mock-transduced cells. Replication-incompetent Ad vectors express non-coding small RNA (VA-RNA), which forms RNA-induced silencing complex (RISC) with argonaute 2 (Ago2) (Xu et al., 2007). Ago2 is an important factor for miRNA processing (Diederichs and Haber, 2007). Processing of miR-122a might be slightly disturbed by Ad vector-expressed VA-RNA, leading to the reduction in the HCV replicon RNA levels.

In summary, we efficiently suppressed the HCV replicon levels by Ad vector-mediated expression of TuD-122a, which blocks the function of miR-122a. This study indicates that Ad vector-mediated expression of TuD-122a in liver hepatocytes would offer an alternative approach for the treatment of HCV infection.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2012.02.003.

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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 (8.9–37.4×10^4) to 15.8×10^4 (10.2–40.6×10^4) /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.

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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 amino-transferase, *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 geno-types 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 deter-mined 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 Biosys-tems). 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 (denatur-ation 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 mono-therapy 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
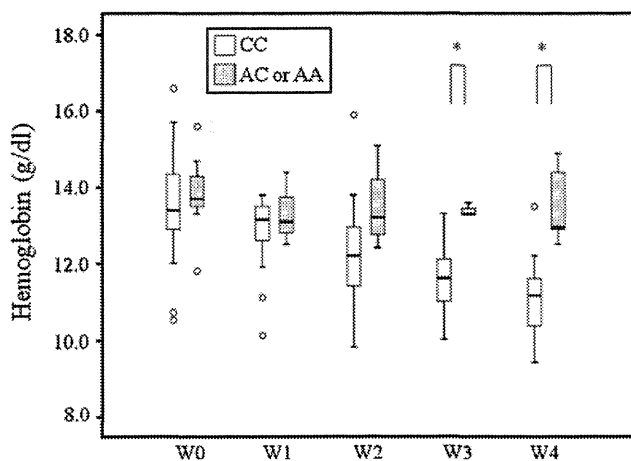

Fig. 1 Changes in hemoglobin according to inosine triphosphatase (*ITPA*) single-nucleotide polymorphism (SNP) genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum values for hemoglobin in patients divided into two groups based on *ITPA* SNP genotype. *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,
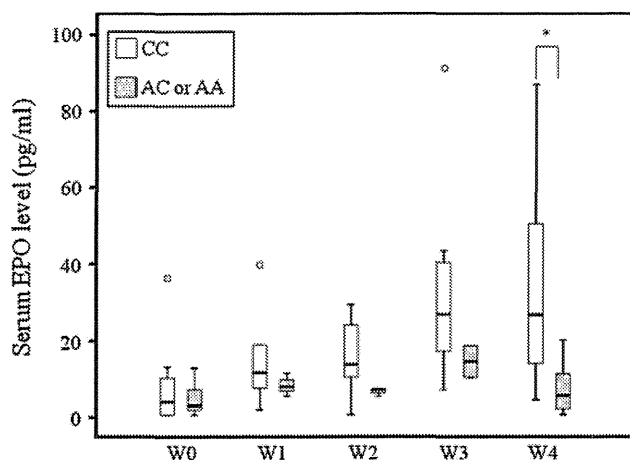
Fig. 2 Changes in serum erythropoietin (*EPO*) according to *ITPA* SNP genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum values for serum EPO in patients divided into two groups based on the *ITPA* SNP genotype. *P* values were calculated using 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. *$P < 0.05$
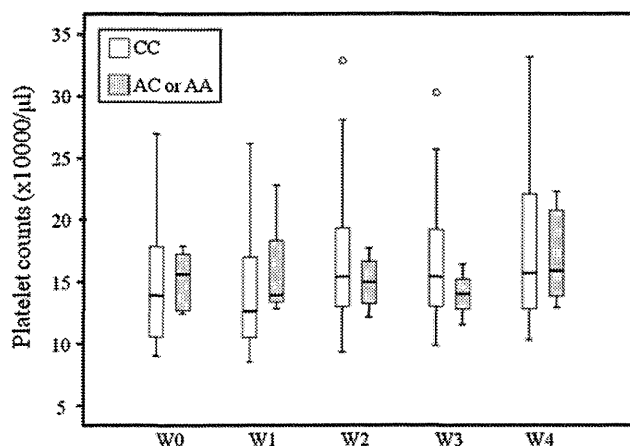

Fig. 3 Changes in platelet counts according to *ITPA* SNP genotype. *Box plots* display the minimum, the first quartile, the median, the third quartile, and the maximum value for platelet counts in patients divided into two groups based on *ITPA* SNP genotype. The *white boxes* represent patients with 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 (9.1–37.1×10^4) vs. 12.4×10^4 (8.9–15.5×10^4) /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
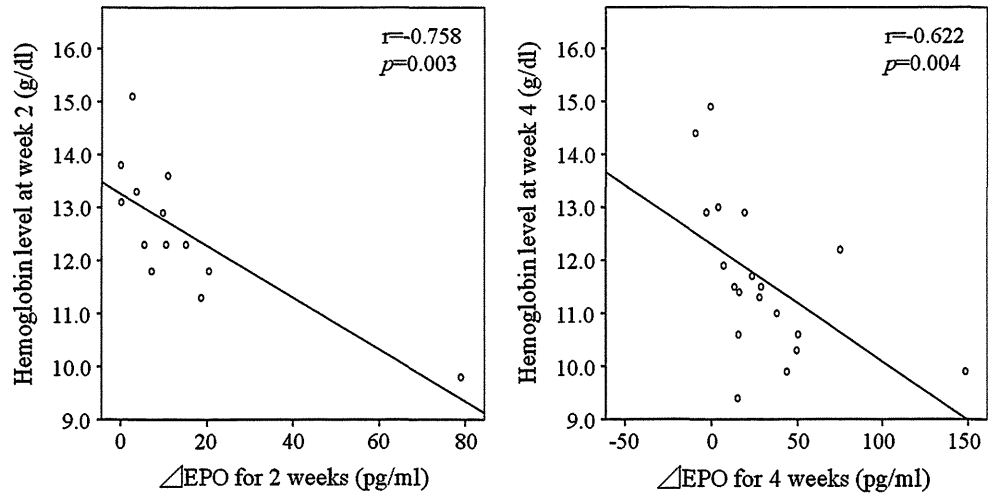
Fig. 5 Correlations between platelet counts and hematopoietic hormones. Correlation coefficients and P values were calculated using Spearman's rank correlation coefficient test. *TPO* thrombopoietin
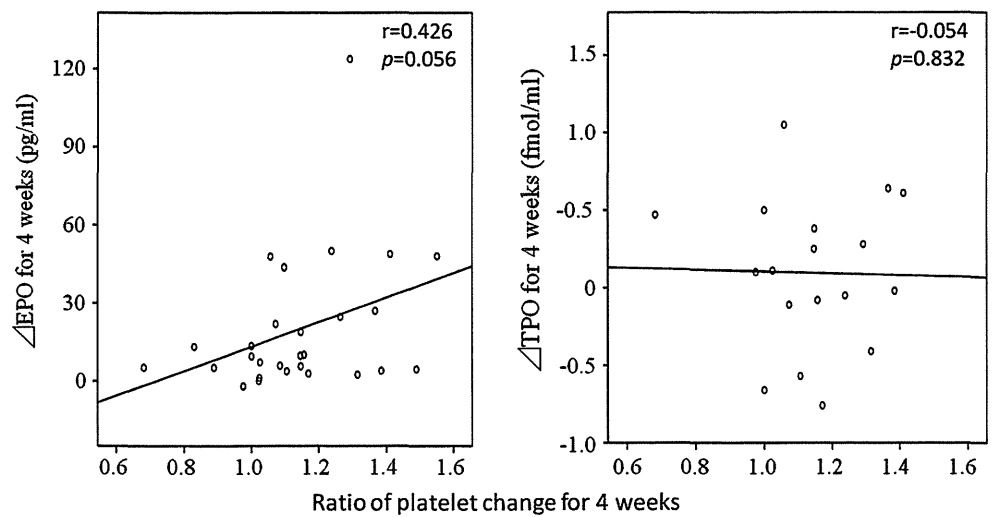
Fig. 6 Correlation between the platelet change ratio and EPO based on the presence/absence of treatment-induced anemia. The platelet change ratio was defined as the platelet count at week 4/platelet count at baseline. Correlation coefficients and P values were calculated using Spearman's rank correlation coefficient test. *Hb* hemoglobin
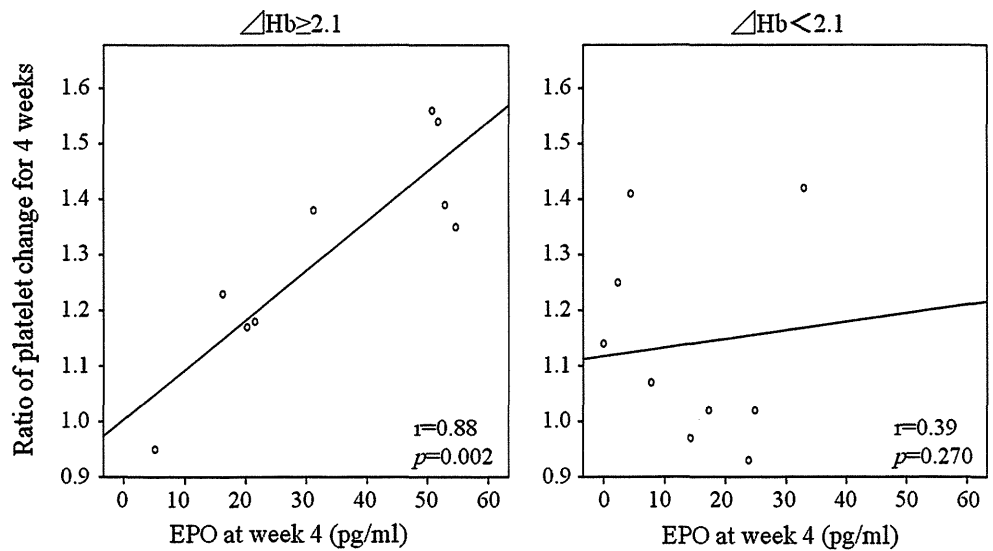
Table 4 Associations between hematological parameters and platelet counts

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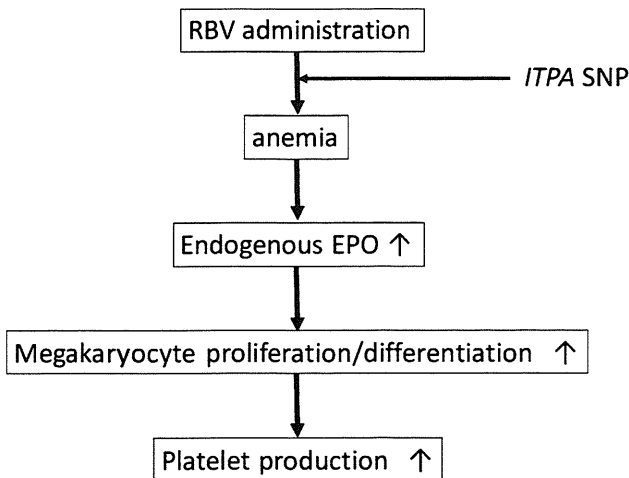
Fig. 7 Model of the mechanism leading to increases in platelet numbers during ribavirin (*RBV*) monotherapy

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