

Research Article

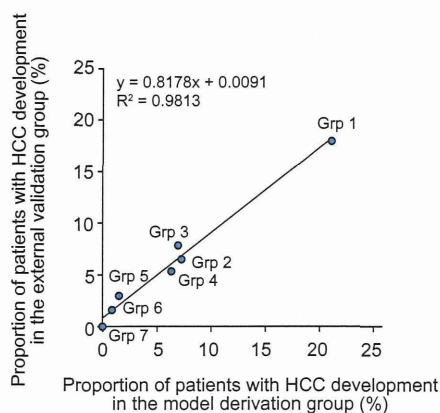


Fig. 2. External validation of the decision tree model with an independent cohort. Each patient in the external validation group was allocated to groups 1–7 following the flowchart of the decision tree. The HCC development rates were then calculated for each group and the graph plotted. The x-axis represents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

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

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

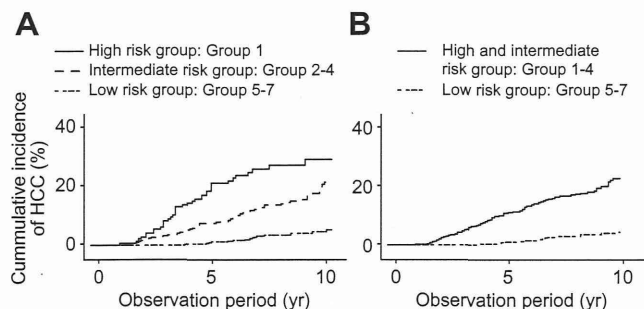


Fig. 3. Cumulative incidence of HCC development beyond 5 years in subgroups of patients defined by the decision tree model. Cumulative incidences of HCC in the groups classified by the decision tree model are compared. (A) The cumulative HCC development rate beyond 5 years is higher in the high- (group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

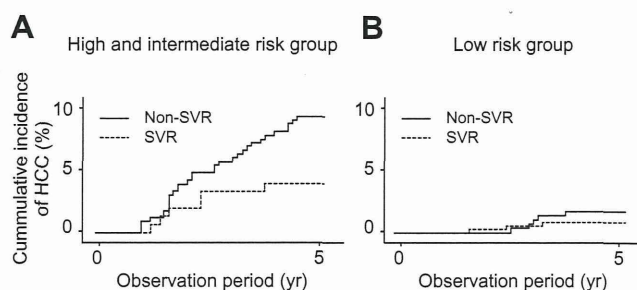


Fig. 4. Sustained virological response to PEG-IFN plus RBV therapy reduces the incidence of HCC development after stratification by the HCC risk. The 600 nonSVR patients and the 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and classified into the high and intermediate-risk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

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

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

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

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

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

Conflict of interest

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

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

Tomoe Kobayashi · Shuhei Hige · Katsumi Terashita · Masato Nakai · Hiromasa Horimoto · Takuya Sho · Mitsuru Nakanishi · Koji Ogawa · Makoto Chuma · Naoya Sakamoto · Masahiro Asaka

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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 ($8.9\text{--}37.4 \times 10^4$) to 15.8×10^4 ($10.2\text{--}40.6 \times 10^4$) /mm³ ($P = 0.003$). 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

<i>ITPA</i>	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
<i>DDRGK1</i>	DDRGK domain-containing protein 1

T. Kobayashi · S. Hige (✉) · K. Terashita · M. Nakai · H. Horimoto · T. Sho · M. Nakanishi · M. Chuma
Department of Gastroenterology,
Hokkaido University Hospital, North 17 Jo,
West 5 Cho-me, Kita-ku, Sapporo 060-8638, Japan
e-mail: shuhei-h@med.hokudai.ac.jp

T. Kobayashi
e-mail: tk990063@med.hokudai.ac.jp

K. Ogawa
Department of Gastroenterology, Hakodate Municipal Hospital,
10-1 Minato-cho, Hakodate 041-8680, Japan

N. Sakamoto
Department of Gastroenterology and Hepatology,
Tokyo Medical and Dental University, 1-5-45 Yushima,
Bunkyo-ku, Tokyo 113-8519, Japan

M. Asaka
Department of Cancer Preventive Medicine, Hokkaido
University Graduate School of Medicine, North 12 Jo,
West 7 Cho-me, Kita-ku, Sapporo 060-0812, Japan

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 triphosphate (*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 (RebetolTM; 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
<i>rs8099917</i> (TT/TG or GG)	25/5	
<i>rs1127354</i> (AA/AC or CC)	7/23	
<i>rs11697186</i> (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 μ l 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 (TaqManTM 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- μ l 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 TTTGTCACCTGTTCTCCTTTTG and the reverse primer was TGCTGGGCCCTAACTGATAC. The forward primer for *rs1127354* was ATGAGAAAGG CCGATGACAG 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 (QuantikineTM Human TPO; R&D Systems, Minneapolis, MN, USA) in patient blood samples taken at 0, 2, and 4 weeks. Both assays were performed according to the manufacturers' instructions.

Pathological findings

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

Measurement of serum ribavirin concentration

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

Statistical analyses

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

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

Results

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

Changes in values during RBV monotherapy are shown in Table 2. During 4 weeks of RBV monotherapy, the median hemoglobin level of the patients decreased significantly, from 13.6 (10.5–16.6) to 11.7 (9.4–14.9) g/dl ($P < 0.001$). The median platelet count increased significantly, from 14.0×10^4 ($8.9\text{--}37.4 \times 10^4$) to 15.8×10^4 ($10.2\text{--}40.6 \times 10^4$) /mm³ ($P = 0.003$). 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) %_o ($P = 0.002$). 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) log₁₀ 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) %_o ($P = 0.001$) 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) %_o; 4 weeks, 11.3 (9.0–20.5) %_o, 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	<i>P</i> 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 (% _o)	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

	<i>ITPA</i> (<i>rs1127354</i>)		<i>P</i> value
	CC allele (<i>n</i> = 23)	AA or AC allele (<i>n</i> = 7)	
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
<i>rs8099917</i> (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 (×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

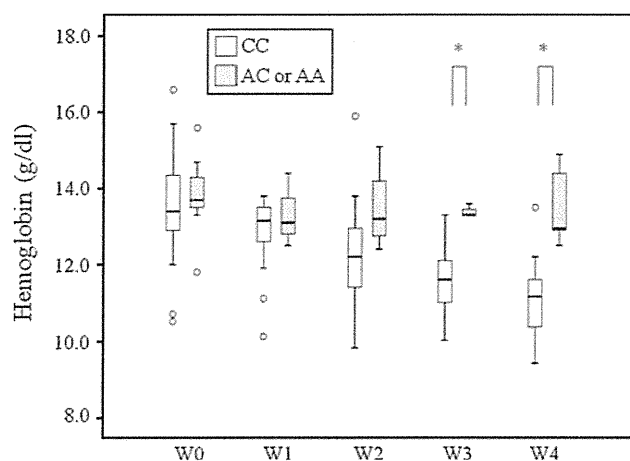


Fig. 1 Changes in hemoglobin according to inosine triphosphatase (*ITPA*) single-nucleotide polymorphism (SNP) genotype. Box plots display the minimum, the first quartile, the median, the third quartile, and the maximum values for hemoglobin in patients divided into two groups based on *ITPA* SNP genotype.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

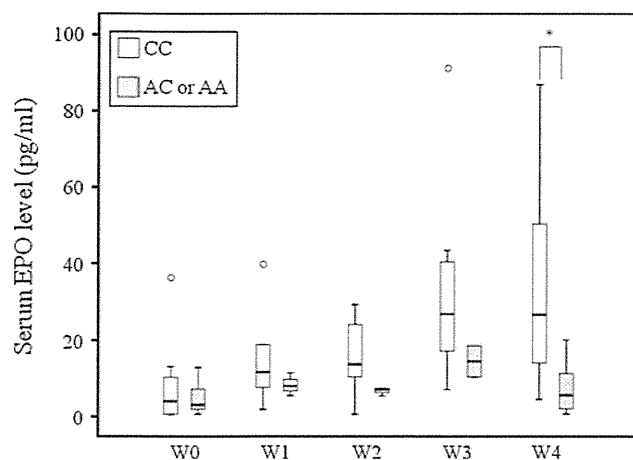


Fig. 2 Changes in serum erythropoietin (EPO) according to *ITPA* SNP genotype. Box plots display the minimum, the first quartile, the median, the third quartile, and the maximum values for serum EPO in patients divided into two groups based on the *ITPA* SNP genotype. *P* values were calculated using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

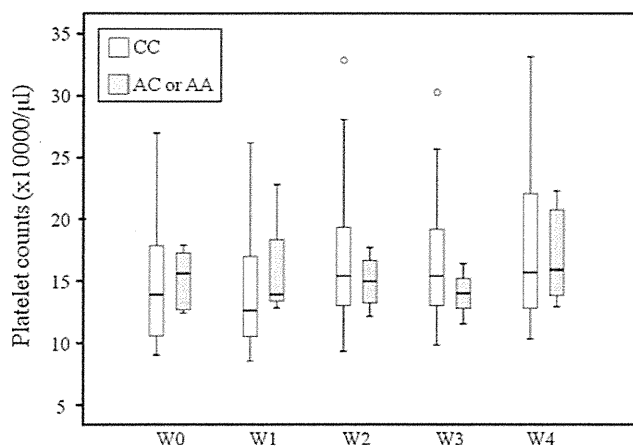


Fig. 3 Changes in platelet counts according to *ITPA* SNP genotype. Box plots display the minimum, the first quartile, the median, the third quartile, and the maximum value for platelet counts in patients divided into two groups based on *ITPA* SNP genotype. The white boxes represent patients with the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

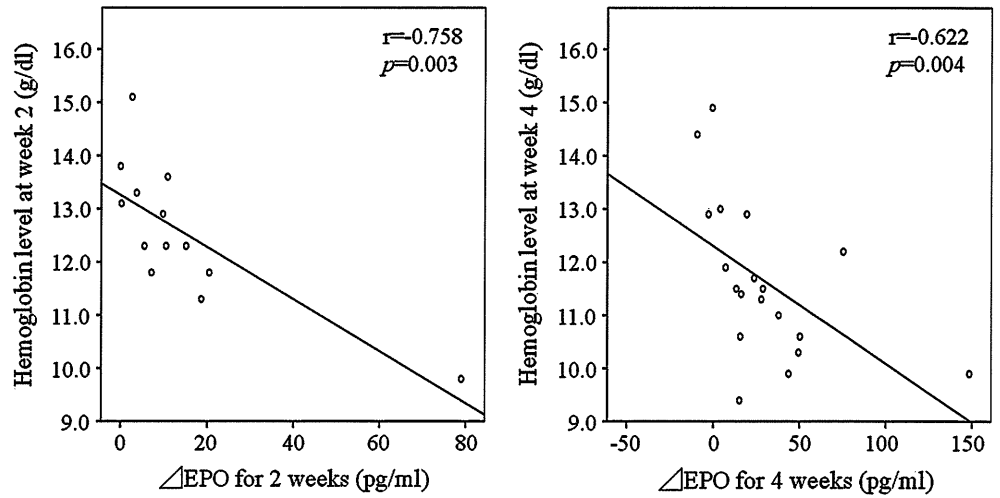


Fig. 5 Correlations between platelet counts and hematopoietic hormones. Correlation coefficients and *P* values were calculated using Spearman's rank correlation coefficient test. *TPO* thrombopoietin

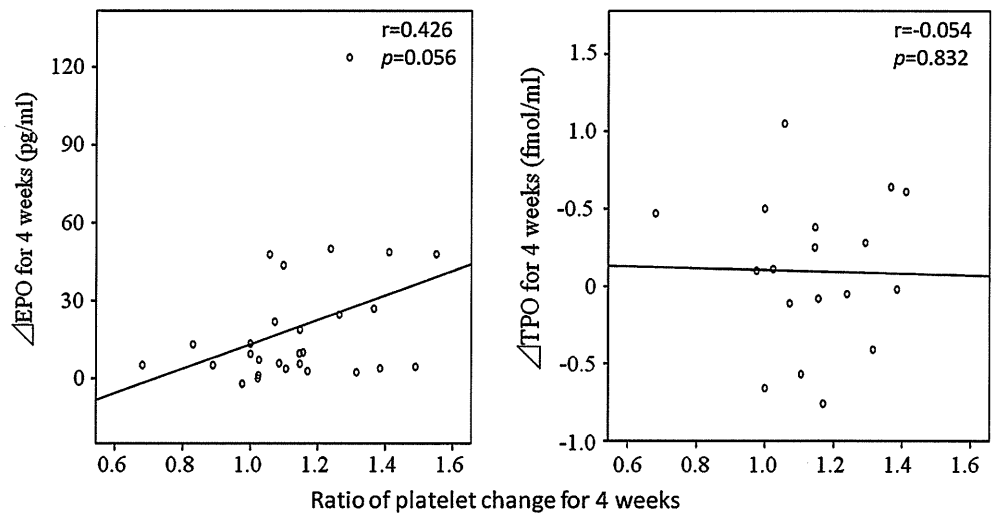


Fig. 6 Correlation between the platelet change ratio and EPO based on the presence/absence of treatment-induced anemia. The platelet change ratio was defined as the platelet count at week 4/platelet count at baseline. Correlation coefficients and *P* values were calculated using Spearman's rank correlation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

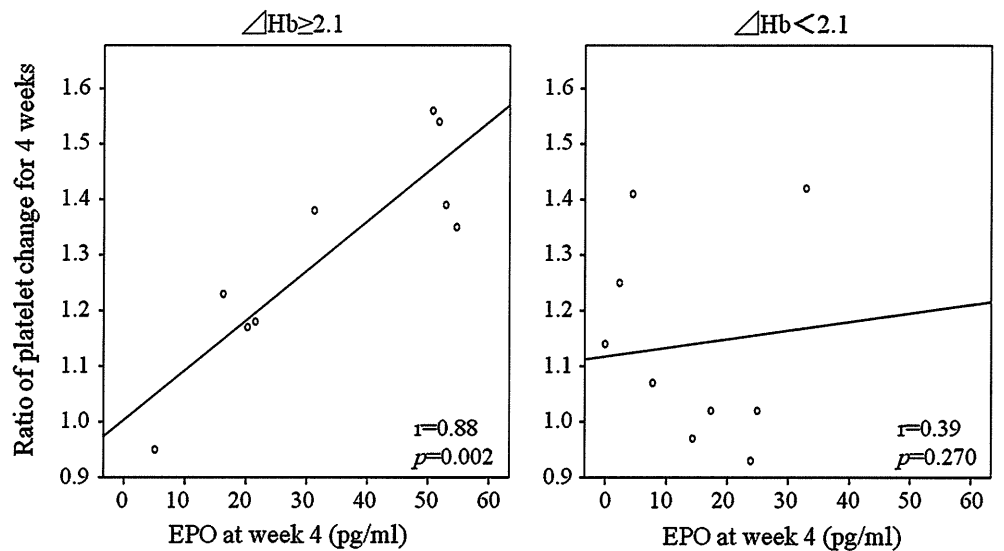


Table 4 Associations between hematological parameters and platelet counts

	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 (×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

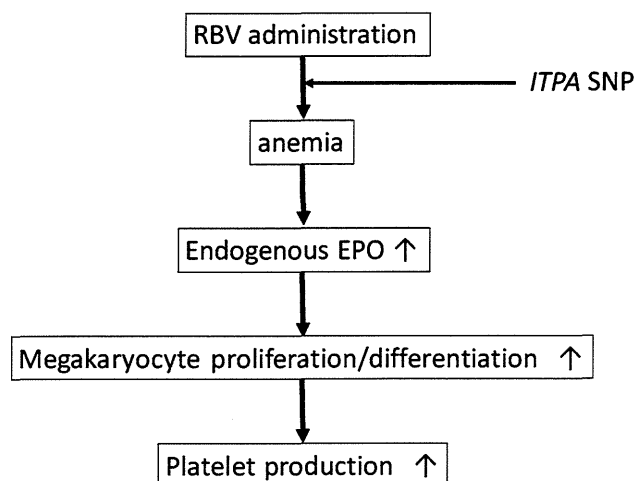


Fig. 7 Model of the mechanism leading to increases in platelet numbers during ribavirin (RBV) monotherapy

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 *DDRGKI* SNP near the *ITPA* gene, like the *ITPA* SNP, was associated with treatment-induced anemia; moreover, the *DDRGKI* SNP was associated with treatment-induced thrombocytopenia during PEG-IFN/RBV combination therapy. Because a protective *DDRGKI* allele showed linkage with a protective *ITPA* allele in all the patients enrolled in the present study, the association between *DDRGKI* 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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Inhibition of Both Protease and Helicase Activities of Hepatitis C Virus NS3 by an Ethyl Acetate Extract of Marine Sponge *Amphimedon* sp.

Yuusuke Fujimoto¹, Kazi Abdus Salam^{2,3}, Atsushi Furuta^{3,4,5}, Yasuyoshi Matsuda^{3,4}, Osamu Fujita^{3,4}, Hidenori Tani⁵, Masanori Ikeda⁶, Nobuyuki Kato⁶, Naoya Sakamoto⁷, Shinya Maekawa⁸, Nobuyuki Enomoto⁸, Nicole J. de Voogd⁹, Masamichi Nakakoshi¹⁰, Masayoshi Tsubuki¹⁰, Yuji Sekiguchi³, Satoshi Tsuneda⁴, Nobuyoshi Akimitsu², Naohiro Noda^{3,4}, Atsuya Yamashita^{1*}, Junichi Tanaka^{11*}, Kohji Moriishi^{1*}

1 Department of Microbiology, Division of Medicine, Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan, **2** Radioisotope Center, The University of Tokyo, Tokyo, Japan, **3** Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan, **4** Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan, **5** Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan, **6** Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, **7** Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, **8** First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan, **9** Netherlands Centre for Biodiversity Naturalis, Leiden, The Netherlands, **10** Institute of Medical Chemistry, Hoshi University, Tokyo, Japan, **11** Department of Chemistry, Biology and Marine Science, University of the Ryukyus, Okinawa, Japan

Abstract

Combination therapy with ribavirin, interferon, and viral protease inhibitors could be expected to elicit a high level of sustained virologic response in patients infected with hepatitis C virus (HCV). However, several severe side effects of this combination therapy have been encountered in clinical trials. In order to develop more effective and safer anti-HCV compounds, we employed the replicon systems derived from several strains of HCV to screen 84 extracts from 54 organisms that were gathered from the sea surrounding Okinawa Prefecture, Japan. The ethyl acetate-soluble extract that was prepared from marine sponge *Amphimedon* sp. showed the highest inhibitory effect on viral replication, with EC₅₀ values of 1.5 and 24.9 μg/ml in sub-genomic replicon cell lines derived from genotypes 1b and 2a, respectively. But the extract had no effect on interferon-inducing signaling or cytotoxicity. Treatment with the extract inhibited virus production by 30% relative to the control in the JFH1-Huh7 cell culture system. The *in vitro* enzymological assays revealed that treatment with the extract suppressed both helicase and protease activities of NS3 with IC₅₀ values of 18.9 and 10.9 μg/ml, respectively. Treatment with the extract of *Amphimedon* sp. inhibited RNA-binding ability but not ATPase activity. These results suggest that the novel compound(s) included in *Amphimedon* sp. can target the protease and helicase activities of HCV NS3.

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* E-mail: atsuyay@yamanashi.ac.jp (AY); jtanaka@sci.u-ryukyu.ac.jp (JT); kmoriishi@yamanashi.ac.jp (KM)

† These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) is an enveloped RNA virus of the genus *Hepacivirus* of the *Flaviviridae* family. More than 170 million patients persistently infected with HCV have been reported worldwide, leading to liver diseases including steatosis, cirrhosis, and hepatocellular carcinoma [1,2]. The genome of HCV is characterized as a single positive-strand RNA with a nucleotide length of 9.6 kb, flanked by 5' and 3'-untranslated regions (UTRs). The genomic RNA encodes a large polyprotein consisting of approximately 3,000 amino acids [3], which is translated under the control of an internal ribosome entry site (IRES) located within the 5'-UTR of the genomic RNA [4]. The translated polyprotein is cleaved by host and viral proteases, resulting in 10 mature viral

proteins [3]. The structural proteins, consisting of core, E1, and E2, are located in the N-terminal quarter of the polyprotein, followed by viroporin p7, which has not yet been classified into a structural or nonstructural protein. Further cleavage of the remaining portion by viral proteases produces six nonstructural proteins—NS2, NS3, NS4A, NS4B, NS5A, and NS5B—which form a viral replication complex with various host factors. The viral protease NS2 cleaves its own C-terminal between NS2 and NS3. After that, NS3 cleaves the C-terminal ends of NS3 and NS4A and then forms a complex with NS4A. The NS3/4A complex becomes a fully active form to cleave the C-terminal parts of the polyprotein, including nonstructural proteins. NS3 also possesses

RNA helicase activity to unwind the double-stranded RNA during the synthesis of genomic RNA [5,6].

Although the previous standard therapy, combining pegylated interferon with ribavirin, was effective in only about half of patients infected with genotype 1, the most common genotype worldwide [7–9], recent biotechnological advances have led to the development of a novel therapy using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication and have improved the sustained virologic response (SVR) [10–12]. Telaprevir and boceprevir, which are categorized as advanced NS3/4A protease inhibitors, were recently approved for the treatment of chronic hepatitis C patients infected with genotype 1 [13,14]. The triple combination therapy with pegylated interferon, ribavirin, and telaprevir improved SVR by 77% in patients infected with genotype 1 [15]. However, this therapy exhibits side effects including rash, severe cutaneous eruption, influenza-like symptoms, cytopenias, depression, and anemia [7,16,17]. Furthermore, the possibility of the emergence of drug-resistant viruses is a serious problem with therapies that use antiviral compounds [18,19].

Recent technical advances in the determination of molecular structures and the synthesis of chemical compounds have led to the development of various drugs based on natural products, especially drugs identified from terrestrial plants and microbes [20–22]. Marine organisms, including plants and animals, were recently established as representative of a natural resource library for drug development. Potent biological activity is often found in products isolated from marine organisms because of their novel molecular structures [23,24]. Trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven), which are known as antitumor drugs, were developed from compounds found in marine organisms [25].

In this study, we screened 84 extracts prepared from 54 marine organisms by using replicon cell lines derived from HCV genotype 1b and attempted to identify the extract that inhibits HCV RNA replication. A marine organism may produce anti-HCV agent(s) that could inhibit the protease and helicase activities of NS3.

Results

Effect of the Extract from Marine Sponge and Tunicate on HCV Replication

We prepared methanol (MeOH)- and ethyl acetate (EtOAc)-soluble extracts from 54 marine organisms in order to test which of these extracts could best suppress HCV replication. Each extract was added at 25 µg/ml to the culture supernatant of HCV replicon cell lines derived from O and Con1 strains of genotype 1b, which produce the luciferase/neomycin hybrid protein depending on RNA replication. Luciferase activity and cell viability were measured 72 h after treatment with the extracts (Table 1). The extracts exhibiting more than 85% cell viability and lower than 15% luciferase activity were selected as arbitrary candidates for the extract including anti-HCV compounds. The EtOAc-extract prepared from sample C-29 (C-29EA) was selected as a candidate in both cell lines. Thus, the anti-HCV activity of extract C-29EA was tested.

The EtOAc-soluble extract C-29EA was prepared from the marine sponge *Amphimedon* sp. (Fig. 1A), which inhabits the sea surrounding Okinawa Prefecture, Japan. HCV replication was inhibited in a dose-dependent manner but did not exhibit cytotoxicity when replicon cells were treated with C-29EA (Fig. 1B). The extract C-29EA exhibited EC₅₀ values of 1.5 µg/ml (Table 2). Furthermore, treatment with C-29EA suppressed the HCV replication derived from the genotype 2a strain JFH1 with an EC₅₀ of 24.9 µg/ml, irrespective of cell viability (Fig. 2A and

Table 2). Extract C-29EA also inhibited the production of infectious viral particles, viral RNA, and core protein from JFH1-infected cells in the supernatant (Fig. 2B and C). These results suggest that the marine sponge *Amphimedon* sp. possesses anti-HCV agents.

Effect of Extract C-29EA on IRES-dependent Translation

Extract C-29EA had the most potent inhibitory activity against HCV replication. The viral replication (Fig. 1B and 2A) and viral proteins (Fig. 3A and B) in replicon cell lines derived from genotype 1b strain Con1 and 2a strain JFH1 were decreased 72 h after treatment in a dose-dependent manner. HCV protein has been translated based on the positive-sense viral RNA in an IRES-dependent manner. The replicon RNA of HCV is composed of the 5'-UTR of HCV, indicator genes (a luciferase-fused drug-resistant gene), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3'-UTR of HCV, in that order [26]. The replicon RNA replicated autonomously in several HCV replication-permissive cell lines derived from several hepatoma cell lines. Nonstructural proteins in replicon cells were polycistronically translated through EMCV IRES. The cap-dependent translated mRNA, including *Renilla* luciferase, EMCV IRES, and the firefly luciferase/neomycin-resistant gene, in that order, was constructed to examine the effect of the extract on EMCV-IRES-dependent translation (Fig. 3C). When the mRNA expression was transcribed by an EF promoter of the transfected plasmid in the presence of C-29EA, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed (Fig. 3C). This suggested that treatment with C-29EA exhibited no effect on EMCV-IRES-dependent translation. Furthermore, treatment with C-29EA did not significantly affect the activity of HCV IRES that was used instead of EMCV IRES in the system described above (Fig. 3D). Thus, these results suggest that treatment with C-29EA exhibits no effect on EMCV- or HCV-IRES-dependent translation.

Effect of C-29EA on the Interferon Signaling Pathway

It has been well known that HCV replication in cultured cells is potently inhibited by interferon [27,28]. We examined whether or not treatment with C-29EA elicits an interferon-inducible gene from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 15 µg of C-29EA per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible gene 2', 5'-OAS, was induced with IFN-alpha 2b but not with a 10-times EC₅₀ concentration of C-29EA (Fig. 4). These results suggest that the inhibitory effect of C-29EA on the replication of the HCV replicon is independent of the IFN signaling pathway.

Effect of C-29EA on the NS3 Helicase Activity

We previously established an assay system for unwinding HCV activity based on photoinduced electron transfer (PET) [29,30]. The fluorescent dye (BODIPY FL) is attached to the cytosine at the 5'-end of the fluorescent strand and quenched by the guanine base at the 3'-end of the complementary strand via PET. When helicase unwinds the double-strand RNA substrate, the fluorescence of the dye emits a bright light upon the release of the dye from the guanine base. The capture strand, which is complementary to the complementary strand, prevents the reannealing of the unwound duplex. Treatment with C-29EA inhibited the helicase activity in a dose-dependent manner, with an IC₅₀ value of 18.9 µg/ml (Fig. 5A). We confirmed the effect of C-29EA on NS3 helicase unwinding activity by the RNA helicase assay using ³²P-labeled double-stranded RNA (dsRNA) as a substrate. Treatment

Table 1. Effect of marine organism extracts on HCV replication and cell viability.

No.	Sample	Luciferase activity (% of control)		Cell viability (% of control)		Phylum	Specimen	Extract	Site
		O	Con1	O	Con1				
1	A-1	10	111	105	104	Sponge	<i>Unidentified</i>	MeOH	A
2	A-2	82	209	91	132	Soft coral	<i>Briareum</i>	MeOH	A
3	A-3	87	177	54	110	Tunicate	<i>unidentified</i>	MeOH	A
4	A-4	82	186	84	100	Sponge	<i>Liosina</i>	MeOH	A
5	B-5	110	165	86	110	Sponge	<i>unidentified</i>	MeOH	B
6	B-6	70	149	103	119	Sponge	<i>Xestospongia</i>	MeOH	B
7	B-7	89	191	111	144	Sponge	<i>Epipolasis</i>	MeOH	B
8	B-8	89	182	115	132	Sponge	<i>unidentified</i>	MeOH	B
9	B-9	57	72	92	124	Sponge	<i>Strongylophora</i>	MeOH	B
10	B-10	106	182	73	96	Sponge	<i>Stylotella aurantium</i>	MeOH	B
11	C-12	96	162	114	98	Sponge	<i>Epipolasis</i>	MeOH	B
12	C-13	123	141	91	103	Sponge	<i>unidentified</i>	MeOH	B
13	C-14	89	175	77	100	Sponge	<i>Hippospongia</i>	MeOH	B
14	C-16	80	177	108	88	Sponge	<i>unidentified</i>	MeOH	B
15	C-18	119	170	93	94	Sponge	<i>unidentified</i>	MeOH	B
16	C-19	0	0	0	4	Sponge	<i>unidentified</i>	MeOH	B
17	C-20	101	158	61	106	Sponge	<i>Xestospongia testudinaria</i>	MeOH	B
18	C-21	85	161	83	102	Sponge	<i>unidentified</i>	MeOH	B
19	C-22	109	88	38	89	Sponge	<i>unidentified</i>	MeOH	B
20	C-23	94	156	32	90	Sponge	<i>unidentified</i>	MeOH	B
21	C-24	118	86	42	94	Sponge	<i>Theonella</i>	MeOH	B
22	C-25	82	111	91	106	Sponge	<i>unidentified</i>	MeOH	B
23	C-27	0	0	15	2	Sponge	<i>unidentified</i>	MeOH	B
24	C-28	90	166	30	90	Sponge	<i>Petrosia</i>	MeOH	B
25	C-29	65	151	29	101	Sponge	<i>Amphimedon</i>	MeOH	B
26	D-31	81	127	55	91	Tunicate	<i>unidentified</i>	MeOH	C
27	D-32	80	141	47	93	Sponge	<i>unidentified</i>	MeOH	C
28	D-33	88	153	72	90	Gorgonian	<i>Junceella fragilis</i>	MeOH	C
29	E-35	114	156	40	118	Sponge	<i>Phyllospongia sp.</i>	MeOH	C
30	E-36	80	125	69	116	Tunicate	<i>Didemnum molle</i>	MeOH	C
31	E-37	88	129	54	108	Sponge	<i>Xestospongia sp.</i>	MeOH	C
32	E-38	70	153	35	112	Sponge	<i>unidentified</i>	MeOH	C
33	F-40	119	170	38	104	Sponge	<i>unidentified</i>	MeOH	C
34	F-41	88	166	48	101	Soft coral	<i>unidentified</i>	MeOH	C
35	G-42	113	157	31	126	Sponge	<i>unidentified</i>	MeOH	D
36	H-43	83	0	39	5	Sponge	<i>unidentified</i>	MeOH	D
37	J-44	62	183	27	105	Sponge	<i>Cinachyra</i>	MeOH	D
38	J-45	96	140	47	103	Sponge	<i>Liosina</i>	MeOH	D
39	J-46	83	149	77	102	Sponge	<i>unidentified</i>	MeOH	D
40	J-47	94	37	40	111	Sponge	<i>unidentified</i>	MeOH	D
41	J-48	24	16	53	70	Sponge	<i>Stylotella</i>	MeOH	D
42	J-49	78	123	55	105	Sponge	<i>unidentified</i>	MeOH	D
43	J-50	93	138	51	108	Sponge	<i>unidentified</i>	MeOH	D
44	J-51	103	73	41	115	Sponge	<i>unidentified</i>	MeOH	D
45	J-52	162	237	113	131	Sponge	<i>unidentified</i>	MeOH	D
46	J-53	51	90	93	122	Tunicate	<i>Didemnum</i>	MeOH	D
47	J-54	42	90	113	124	Sponge	<i>unidentified</i>	MeOH	D

Table 1. Cont.

No.	Sample	Luciferase activity (% of control)		Cell viability (% of control)		Phylum	Specimen	Extract	Site
		O	Con1	O	Con1				
48	J-55	88	133	131	110	Jellyfish	<i>unidentified</i>	MeOH	D
49	J-56	28	51	113	103	Sponge	<i>unidentified</i>	MeOH	D
50	J-57	8	63	94	85	Tunicate	<i>Pseudodistoma kanoko</i>	MeOH	D
51	J-58	0	2	48	65	Sponge	<i>unidentified</i>	MeOH	D
52	J-59	0	2	45	71	Sponge	<i>unidentified</i>	MeOH	D
53	J-60	98	134	122	95	Annelid	<i>unidentified</i>	MeOH	D
54	A-2	0	1	6	15	Soft coral	<i>Briareum</i>	EtOAc	A
55	A-3	0	0	6	9	Tunicate	<i>unidentified</i>	EtOAc	A
56	A-4	22	36	74	76	Sponge	<i>Liosina</i>	EtOAc	A
57	B-5	33	107	69	93	Sponge	<i>unidentified</i>	EtOAc	B
58	B-6	0	0	5	8	Sponge	<i>Xestospongia</i>	EtOAc	B
59	B-7	0	0	5	9	Sponge	<i>Epipolasis</i>	EtOAc	B
60	B-8	0	0	2	46	Sponge	<i>unidentified</i>	EtOAc	B
61	B-9	0	0	8	14	Sponge	<i>Strongylophora</i>	EtOAc	B
62	B-10	0	0	3	8	Sponge	<i>Stylorella aurantium</i>	EtOAc	B
63	C-12	0	0	4	14	Sponge	<i>Epipolasis</i>	EtOAc	B
64	C-13	0	0	4	5	Sponge	<i>unidentified</i>	EtOAc	B
65	C-14	48	119	82	102	Sponge	<i>Hippospongia</i>	EtOAc	B
66	C-15	0	0	8	11	Sponge	<i>unidentified</i>	EtOAc	B
67	C-18	0	0	4	3	Sponge	<i>unidentified</i>	EtOAc	B
68	C-19	23	76	63	109	Sponge	<i>unidentified</i>	EtOAc	B
69	C-20	34	32	63	112	Sponge	<i>Xestospongia testudinaria</i>	EtOAc	B
70	C-21	1	0	52	12	Sponge	<i>unidentified</i>	EtOAc	B
71	C-22	76	34	74	110	Sponge	<i>unidentified</i>	EtOAc	B
72	C-24	0	0	20	7	Sponge	<i>Theonella</i>	EtOAc	B
73	C-26	41	43	80	110	Sponge	<i>unidentified</i>	EtOAc	B
74	C-27	1	0	35	40	Sponge	<i>unidentified</i>	EtOAc	B
75	C-28	68	62	82	115	Sponge	<i>Petrosia</i>	EtOAc	B
76	C-29	10	11	93	88	Sponge	<i>Amphimedon</i>	EtOAc	B
77	D-31	20	71	85	120	Tunicate	<i>Eudistoma</i>	EtOAc	C
78	D-33	0	0	5	7	Gorgonian	<i>Junceella fragilis</i>	EtOAc	C
79	E-35	0	0	4	5	Sponge	<i>Phyllospongia sp.</i>	EtOAc	C
80	E-36	71	83	75	100	Tunicate	<i>Didemnum molle</i>	EtOAc	C
81	F-40	72	110	87	130	Sponge	<i>unidentified</i>	EtOAc	C
82	F-41	8	33	73	104	Soft coral	<i>unidentified</i>	EtOAc	C
83	H-43	0	197	4	119	Sponge	<i>unidentified</i>	EtOAc	D
84	J-46	113	58	103	126	Sponge	<i>unidentified</i>	EtOAc	D

There are a total of 54 marine organisms, while 84 extracts were prepared from them with ethyl acetate and/or methanol. Aragusuku, Iriomote, Kohama, and Ishigaki islands are indicated by A, B, C, and D, respectively, in the collection-site column (right end). EtOAc: Ethyl acetate; MeOH: Methanol.
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with C-29EA inhibited dsRNA dissociation at a concentration of 16 µg/ml and above (Fig. 5B).

The unwinding ability of HCV helicase depends on ATP binding, ATP hydrolysis, and RNA binding [30,31]. We examined the effect of C-29EA on the ATPase activity of NS3. The ratio of free phosphate ($^{32}\text{P-Pi}$) to ATP ($^{32}\text{P-ATP}$) was determined in the presence of C-29EA. The reaction was carried out between 16 and 250 µg of C-29EA per milliliter. The ATPase activity of NS3 helicase was not inhibited (Fig. 6A), although the helicase activity

was decreased to less than 20% in the presence of 50 µg of C-29EA per milliliter (Fig. 5A). Next, we examined the effect of C-29EA on the binding of NS3 helicase to single-strand RNA (ssRNA). A gel-mobility shift assay was employed to estimate the binding activity of NS3 to the 21-mer of ssRNA. The binding of NS3 to ssRNA was inhibited by C-29EA in a dose-dependent manner (Fig. 6 B and C). These results suggest that treatment with C-29EA inhibits the helicase activity of NS3 by suppressing RNA binding.

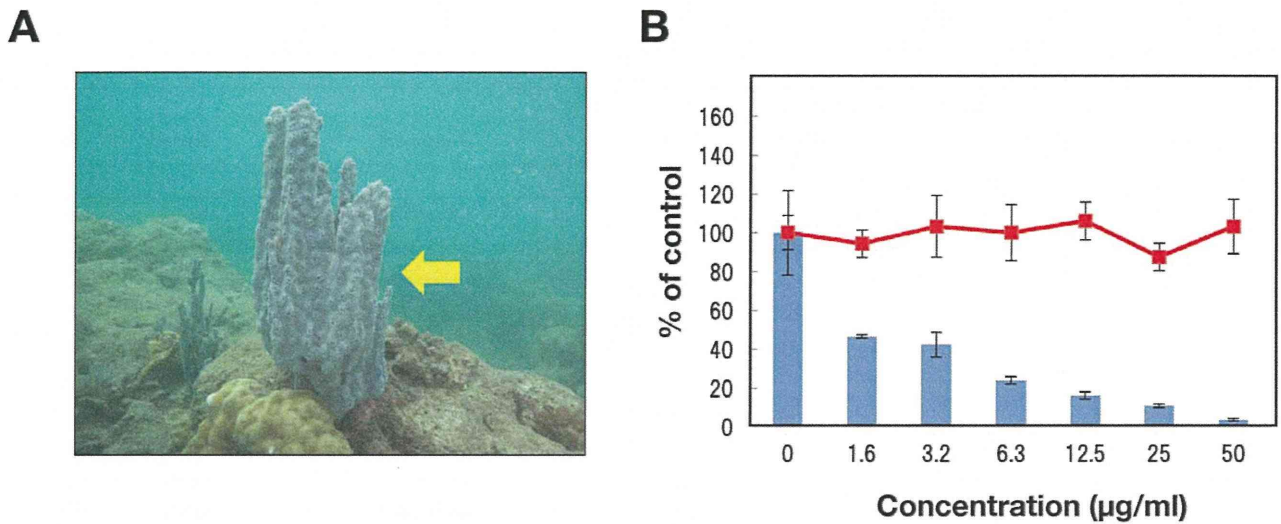


Figure 1. Effect of the extract prepared from a marine sponge on viral replication in the replicon cell line derived from viral genotype 1b. (A) *Amphimedon* sp. belongs to a marine sponge. The ethyl acetate fraction prepared from the marine organism was designated C-29EA in this study. (B) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g001

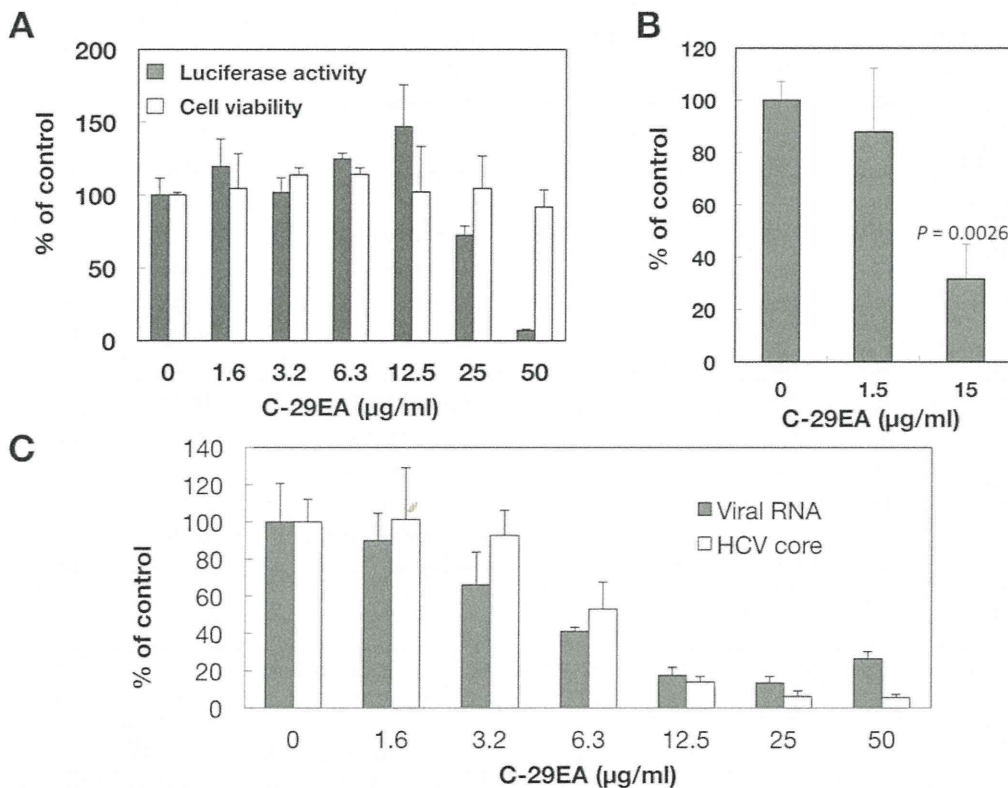


Figure 2. Effect of C-29EA extract on viral replication in the replicon cell line derived from viral genotype 2a. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 2a strain JFH1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. (B) The Huh7 OK1 cell line infected with HCVcc JFH1 was incubated with various concentrations of C-29EA or DMSO (0). The virus titers were determined by a focus-forming assay. The significance of differences in the means was determined by Student's *t*-test. (C) Amounts of viral RNA and core protein were estimated by qRT-PCR and ELISA, respectively. Error bars indicate standard deviation. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g002

Table 2. Effect of C29EA on HCV replication.

HCV strain (genotype)	EC ₅₀ (μg/ml) ^a	CC ₅₀ (μg/ml) ^b	SI ^c
Con 1 (1b)	1.5	>50	>33.3
JFH1 (2a)	24.9	>50	>2.3

^a: Fifty percent effective concentration based on the inhibition of HCV replication.

^b: Fifty percent cytotoxicity concentration based on the reduction of cell viability.

^c: SI, selectivity index (CC₅₀/EC₅₀).

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Effect of C-29EA on NS3 Protease Activity

Serine protease and helicase domains are respectively located on the N-terminal and C-terminal portions of NS3 [32]. Thus, we examined the effect of C-29EA on NS3 protease activity by using

an NS3 protease assay based on FRET. NS3/4A serine protease was mixed with various concentrations of C-29EA. The initial velocity at each concentration of C-29EA was calculated during a 120 min reaction. The initial velocity in the absence of C-29EA represented 100% of relative protease activity. C-29EA decreased the serine protease activity in a dose-dependent manner (Fig. 7). The IC₅₀ of C-29EA was 10.9 μg/ml, which is similar to the value estimated by helicase assay. These results suggest that C-29EA includes the compound(s) inhibiting the protease activity of NS3 in addition to the helicase activity.

Combination Antiviral Activity of C-29EA and Interferon-alpha

Treatment with C-29EA may potentiate inhibitory action of interferon-alpha, since it inhibited the protease and helicase activities of NS3 but not induce the interferon response as described above. Then, we examined effect of treatment using both interferon and C-29EA on HCV replication. The replication

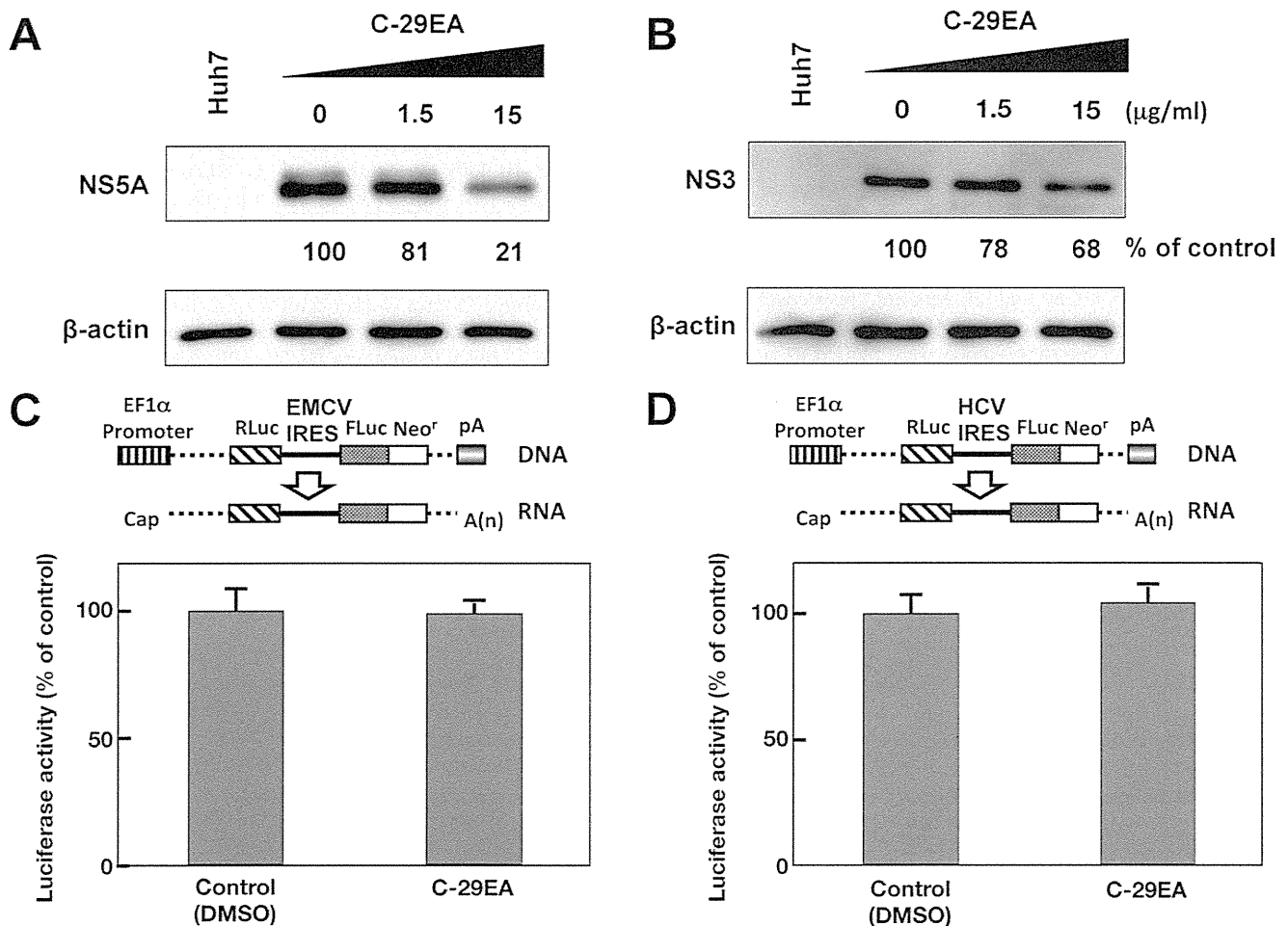


Figure 3. Effect of C-29EA on expression of viral proteins in replicon cell lines. The Huh7 replicon cell lines derived from genotype 1b (A) and 2a (B) were incubated with C-29EA at 37°C for 72 h. The treated cells were harvested and then subjected to Western blotting. Treatment with DMSO corresponds to '0'. The bicistronic gene is transcribed under the control of the elongation factor 1α (EF1α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r), and is translated under the control of the EMCV IRES (C) or HCV IRES (D). The Huh7 cell line transfected with the plasmid (each above the panel in C and D) was established in the presence of G418. The cells were incubated for 72 h without (control) and with 15 μg/ml of C-29EA. Firefly or *Renilla* luciferase activity was measured by the method described in Materials and Methods and was normalized by the protein concentration. F/R: relative ratio of firefly luciferase activity to *Renilla* luciferase activity. F/R is presented as a percentage of the control condition. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g003

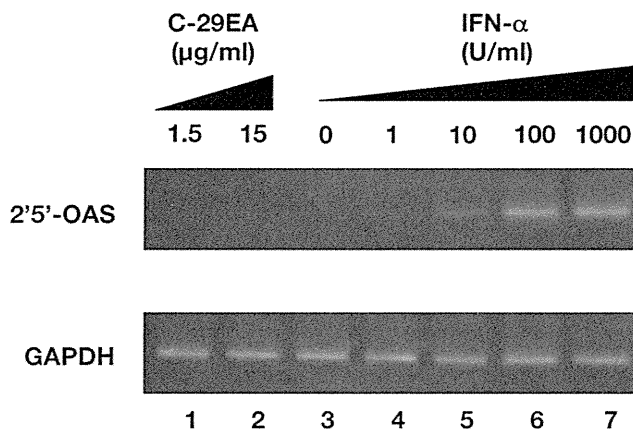


Figure 4. Effect of C-29EA on interferon signaling pathway. The Huh7 replicon cell line of genotype 1b was treated without (lane 3) or with 1, 10, 100, or 1000 U/mL interferon-alpha 2b (lanes 4–7), and 1.5 or 15 μg/ml C-29EA (lanes 1–2) for 48 h. Treatment with DMSO corresponds to '0'. The mRNAs of 2', 5'-OAS, and GAPDH as an internal control were detected by RT-PCR. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g004

of replicon was decreased in the presence of C-29EA or interferon-alpha and further decreased by combination treatment using interferon-alpha and C-29EA (Fig. 8A). Furthermore, we employed the isobologram method [33] to determine whether antiviral effect of the combination treatment exhibits additive or synergistic. EC_{90} values of interferon-alpha and C-29EA were estimated at 10.7 U/ml and 26.4 μg/ml, respectively, in the absence of each other. EC_{90} values of C-29EA in the presence of 0, 2.5 and 5 U/ml interferon-alpha were plotted to generate an isobole. Figure 8B shows that the isobole exhibits concave

curvilinear, representing synergy but not additivity. These results suggest that combination treatment of interferon-alpha and C-29EA exhibits synergistic inhibition of HCV replication.

Discussion

Several natural products have been reported as anti-viral agents against HCV replication. Silbinin, epigallocatechin 3-gallate, and proanthocyanidins, which were prepared from milk thistle, green tea, and blueberry leaves, respectively, have exhibited inhibitory activity against HCV replication in cultured cells [34–37]. In our previous report, we identified manoalide as an anti-HCV agent from a marine sponge extract by high-throughput screening targeting NS3 helicase activity [38]. Manoalide inhibited ATPase, RNA binding, and NS3 helicase activity in enzymological assays. The EtOAc extract of the marine feather star also suppressed HCV replication in HCV replicon cell lines derived from genotype 1b, and it inhibited the RNA-binding activity but not the ATPase activity of NS3 helicase [30]. In this study, we screened 84 extracts of marine organisms for their ability to inhibit HCV replication in replicon cell lines and HCV cell culture system. Among these extracts, C-29EA, which was extracted from *Amphimedon* sp., most strongly inhibited HCV replication regardless of cytotoxicity. We previously reported that the EtOAc extract (SG1-23-1) of the feather star *Alloecomatella polycladia* inhibited HCV replication with an EC_{50} of 22.9 to 44.2 μg/ml in HCV replicon cells derived from genotype 1b [30]. Treatment with C-29EA potently inhibited HCV replication with an EC_{50} of 1.5 μg/ml and with an SI of more than 33.3 in the replicon cell line derived from genotype 1b, regardless of cytotoxicity (Fig. 1B and Table 2). However, C-29EA exhibited an EC_{50} of 24.9 μg/ml in a replicon cell line derived from genotype 2a at a weaker level than in the replicon cell line derived from genotype 1b (Figs. 1 and 2), suggesting that the ability of C-29EA to suppress HCV replication is dependent on the viral genotype or strain.

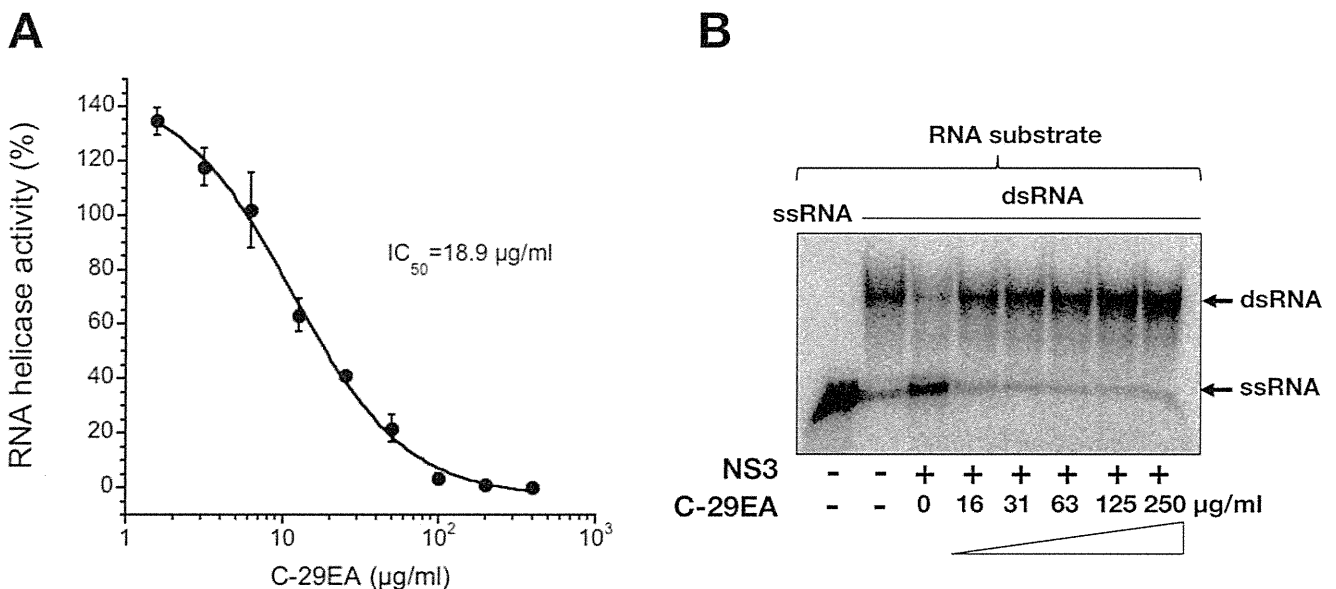


Figure 5. Effect of C-29EA on unwinding activity of NS3 helicase. (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of C-29EA. Helicase activity in the absence of C-29EA was defined as 100% helicase activity. Treatment with DMSO corresponds to '0'. The data are presented as the mean \pm standard deviation for three replicates. (B) The unwinding activity of NS3 helicase was measured by an RNA unwinding assay using radioisotope-labeled RNA. The heat-denatured single-strand RNA (26-mer) and the partial duplex RNA substrate were applied to lanes 1 and 2, respectively. The duplex RNA was reacted with NS3 (300 nM) in the presence of C-29EA (lanes 4–9, 16–250 μg/ml). The resulting samples were subjected to native polyacrylamide gel electrophoresis. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g005