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

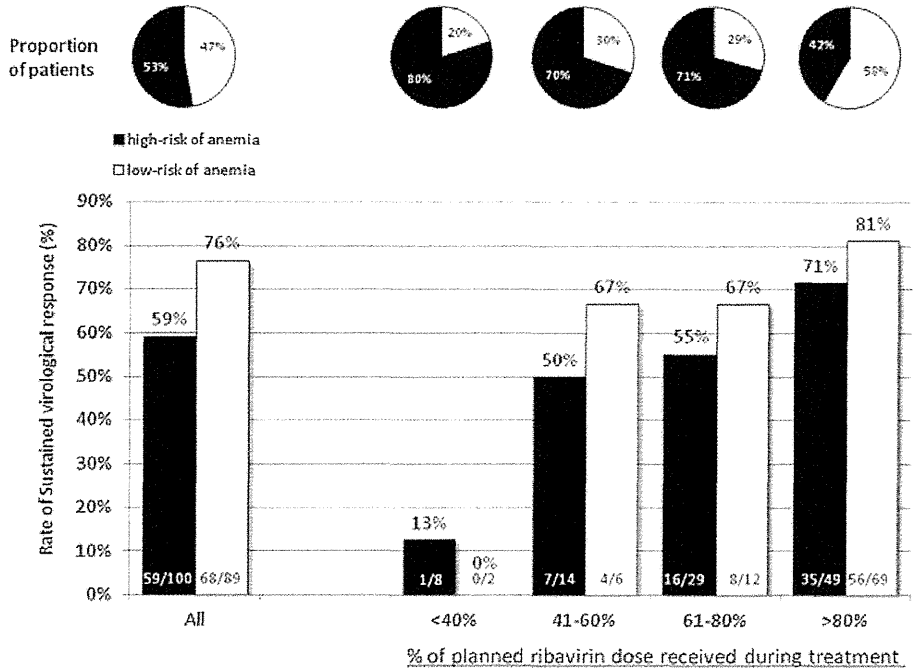

Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of 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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Original Article

Serum RANTES level influences the response to pegylated interferon and ribavirin therapy in chronic hepatitis C

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Aim: Prediction of treatment responses to pegylated interferon (PEG IFN) plus ribavirin (RBV) therapy is uncertain for genotype 1b chronic hepatitis C.

Methods: In this study, 96 patients were investigated for the correlation between 36 pretreatment serum chemokine/cytokine levels and PEG IFN/RBV treatment efficacy by a sandwich enzyme-linked immunoassay (ELISA) and a bead array.

Results: First, chemokines/cytokines were measured semi-quantitatively by sandwich ELISA in 31 randomly-selected patients and the serum regulated on activation normal T-cell expressed and secreted (RANTES) level was found to be significantly higher in the sustained virological response (SVR) group than the non-SVR group ($P = 0.048$). Precise RANTES measurement in all 96 patients using a bead array confirmed this correlation ($P = 0.002$). However, the genetic RANTES haplotype was not significantly related to the serum level. The serum RANTES level was extracted by multivariate analysis (odds ratio = 4.09, 95% confidence interval = 1.02–16.5, $P = 0.048$) as an independent variable contributing to SVR.

Conclusion: The serum RANTES level is an important determinant influencing the virological response to PEG IFN/RBV therapy in chronic hepatitis C.

Key words: hepatitis C virus, pegylated interferon plus ribavirin therapy, RANTES

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a major cause of chronic liver disease worldwide and persistent infection may lead to liver cirrhosis and hepatocellular carcinoma.[1] Therapy leading to HCV eradication is the only treatment with proven efficacy in decreasing the occurrence of hepatocellular carcinoma.[2] Recently, treatment with telaprevir, a non-structural (NS)3/4A protease inhibitor, combined with pegylated interferon (PEG IFN) and ribavirin (RBV), increased the rates of sustained viral response (SVR) up to 64–75%[3,4] compared to the SVR rate of approximately 50% for the previous PEG IFN/RBV therapy. However, it has become evident that genotype 1-infected patients with a null response to previous PEG IFN/RBV therapy have poor responses to PEG IFN/RBV/telaprevir,[5] with an SVR rate as low as approximately 30%, illustrating the difficulty in treating patients infected with genotype 1 HCV. Therefore, precise and accurate prediction of the viral response to PEG IFN/RBV therapy remains an important issue.

Treatment resistance is attributed to various factors associated with the virus and host. Viral factors, such as amino acid (a.a.) sequence variation in the core and NS5A regions, have been investigated extensively for their contribution to the outcome of IFN-based therapy,[6,7] including PEG IFN/RBV therapy. On the other hand, host factors such as African-American race, older age, being obese, the presence of cirrhosis and

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steatosis, and insulin resistance have been reported to be associated with treatment resistance.[8–11] Especially, single nucleotide polymorphisms (SNP) near the interleukin (*IL*)-28B gene, including rs12979860 and rs8099917, have been reported to have a significant correlation with the response to IFN-based therapy.[12,13] However, even with inclusion of these factors, prediction of the treatment response in chronic HCV infection remains uncertain at present.

Chemokines are a group of small, exogenously secreted cytokines that modulate the migration of leukocytes to sites of tissue damage and inflammation in a variety of infectious and autoimmune diseases.[14] In chronic HCV infection, chemokines such as *RANTES* (regulated on activation normal T-cell expressed and secreted), macrophage inflammatory protein (*MIP*)-1α, *MIP-1β* and interferon-γ inducible protein 10 kDa (*IP-10*) are elevated and considered to play crucial roles in inflammatory processes and viral elimination, as well as the transition from innate to adaptive immunity.[14,15] Upregulation of several serum chemokines, such as eotaxin, *IP-10* and *RANTES* also has been reported in HCV infection, possibly reflecting hepatic inflammation.[16] Considering the roles of chemokines/cytokines in establishing chronic hepatitis, it is possible that these chemokines also affect the response to antiviral therapy, and actually several chemokines as interleukin (*IL*)-8, *IL-10*, *MIP-1β*, *RANTES* or *IP-10* have been investigated previously for their association with the treatment response.[16–20] However, the importance of those chemokines has not been established yet and, moreover, these studies did not characterize in detail these chemokines in association with other factors, including *IL-28B* influencing the response to therapy.

In this study, we explored extensively the association of 36 serum cytokines/chemokines and the treatment response, with detailed information of host and virus, to predict better the treatment response to PEG IFN and RBV therapy in genotype 1b HCV infection. Because the pretreatment serum *RANTES* level was found to be correlated significantly with the response, we analyzed further the association between the serum level of *RANTES* and the genomic SNP.

METHODS

Patients

NINETY-SIX CONSECUTIVE PATIENTS with genotype 1b HCV and receiving PEG IFN/RBV therapy between 2004 and 2010 at Yamanashi University Hospital were recruited retrospectively into the study. All patients received the standard therapy according to the treatment protocol of PEG IFN/RBV therapy for Japanese patients, established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan (PEG IFN-α-2b 1.5 μg/kg bodyweight, once weekly s.c., and RBV 600–800 mg daily p.o. for 48 weeks).[21] All patients enrolled fulfilled the following criteria: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease or alcoholic liver disease; (iii) not co-infected with HIV; and (iv) a signed consent was obtained for the study protocol that had been approved by the Human Ethics Review Committee of Yamanashi University Hospital. The study was approved by the ethics committees of University of Yamanashi, and the study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

Definition of treatment outcome

An SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Relapse was defined as reappearance of detectable HCV RNA levels following discontinuation of treatment. Null response was defined as less than 2 log decrease of the baseline HCV RNA levels after 12 weeks of treatment. Based on this definition, when patients were classified according to the achievement of SVR, patients with relapse or null response were classified as non-SVR.

Serum cytokine measurement

Sandwich enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected before initiation of treatment and were stored at −80°C until use. Semiquantitation of serum cytokines was performed using the Proteome Profiler Human Cytokine Array Kit Panel A (R&D Systems, Minneapolis, CA, USA) according to the manufacturer's instructions. The kit consists of a nitrocellulose membrane containing 36 different anti-cytokine antibodies (anti-C5a, anti-CD154, anti-G-CSF, anti-GM-CSF, anti-CXCL1, anti-CCL1, anti-sICAM-1, anti-IFN-γ, anti-IL-1α, anti-IL-1β, anti-IL-1ra, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-8, anti-IL-10, anti-IL-12p70, anti-IL-13, anti-IL-16, anti-IL-17, anti-IL-17E, anti-IL-23, anti-IL-27, anti-IL-32α, anti-IP-10, anti-CXCL11, anti-CCL2, anti-MIF, anti-CCL3, anti-CCL4, anti-PAI-1, anti-RANTES, anti-CXCL12, anti-TNF-α, anti-sTREM-1), spotted in duplicate. Serum samples were diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture

was then incubated with the membrane. Any cytokine/detection antibody complex present was bound to the membrane by its cognate immobilized capture antibody. Following washing to remove unbound material, streptavidin-horseradish peroxidase and chemiluminescent detection reagents (ECL Western Blotting Analysis System; GE Healthcare, Buckinghamshire, UK) were added sequentially. Arrays were scanned using a LAS-3000 mini-luminescent image analyzer (Fujifilm, Tokyo, Japan) and were quantified for the densities using Multi Gauge ver. 3.0 software (Fujifilm). Concentrations of cytokines and chemokines were expressed as their signal intensity ratios relative to that of the positive control spotted on the same membrane.

Bead array

Precise serum concentrations of regulated on *RANTES* were measured using the Luminex Bio-Plex system (Bio-Rad, Hercules, CA, USA) and the Procarta Cytokine Assay Kit (Panomics, Fremont, CA, USA) in a 96-well plate ELISA-based format according to the manufacturers' recommendations. The sensitivity of the assays is greater than 10 pg/mL cytokine. Serum and standards were incubated with a mixture of the Luminex antibody-conjugated beads for 30 min with constant shaking. After washing, the detection antibodies and substrates were added and incubated for another 30 min. Fluorescent signals were collected and data expressed, using internal standards, in pg/mL as the mean of two individual experiments carried out in duplicate.

Viral core and interferon sensitivity-determining region (ISDR) sequence determination by direct sequencing

Hepatitis C virus RNA extraction from serum samples, complementary DNA synthesis and amplification by two-step nested polymerase chain reaction (PCR) were carried out using specific primers for the HCV core and ISDR. PCR amplicons were sequenced directly by Big Dye Terminator ver. 3.1 (ABI, Tokyo, Japan) with universal M13 forward and reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

SNP typing of the *RANTES* and *IL-28B* genes

Genomic DNA of the patients was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR with a model 7500 (ABI) using FAM-labeled SNP primers for the loci rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 (ABI) for *RANTES* and the locus rs8099917 (ABI) for IL-28B.

Statistical analysis

Student's *t*-test and Mann–Whitney *U*-test were used to analyze continuous variables, as appropriate. Fisher's exact test was used for the analysis of categorical variables. Receiver–operator curve (ROC) analyses were performed to establish cut-off values for serum cytokine concentration. The optimum cut-off was defined as the value that maximized the area under the ROC. Spearman's correlation coefficient (R) was calculated to clarify the strength of relationship between the pretreatment serum cytokine concentrations and clinical parameters. Variables that achieved statistical significance ($P < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The odds ratios and 95% confidence intervals also were calculated. Data were analyzed using Ekuseru-Toukei 2008 (SSRI, Tokyo, Japan). The haplotype block among rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 variants was analyzed using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan). $P < 0.05$ was considered significant.

RESULTS

Semiquantitative measurement of pretreatment serum cytokines in 31 randomly-selected patients

AT FIRST, TO identify cytokines/chemokines related to the treatment responses to PEG IFN/RBV therapy, semiquantitative measurement of the serum concentrations of 36 comprehensive cytokines/chemokines was performed by sandwich ELISA method by randomly selected patients. Next, to further confirm the result, cytokines showing the associations with the response were measured more precisely by bead array method in all patients.

In the first analysis, 31 patients were randomly selected from the 96 patients. The clinical characteristics of these 31 patients at the start of the therapy are shown in Table 1. Significant differences in the clinical backgrounds between those who did and those who did not

Table 1 Baseline characteristics of the 31 patients analyzed using the sandwich ELISA method

Factor	SVR ($n = 20$)	Non-SVR ($n = 11$)	*P*-value
Age (years)	52 ± 11†	57 ± 10	0.25‡
Sex (male : female)	11:9	6:5	0.64§
Bodyweight (kg)	60.9 ± 9.6†	61.9 ± 13.9	0.81‡
Body mass index (kg/m^2)	22.6 (18.9–31.3)¶	22.7 (17.5–26.8)	0.87††
History of IFN therapy (%)	30	36	0.78§
ALT (IU/L)	130 ± 100†	75 ± 35	0.09‡
AST (IU/L)	76 (22–331)¶	64 (24–178)	0.73††
γ-GTP (IU/L)	40 (12–289)	52 (24–137)	0.17††
Albumin (g/dL)	4.1 (3.7–4.5)	4.0 (3.0–4.7)	0.46††
Total cholesterol (mg/dL)	170 ± 24†	149 ± 33	0.06‡
HbA1c (%)	5.3 ± 0.5	5.3 ± 0.6	0.95‡
Creatinine (mg/dL)	0.71 ± 0.15	0.68 ± 0.15	0.54‡
WBC count (/μL)	4561 ± 1631	4056 ± 1277	0.38‡
Neutrophil count (/μL)	2130 (820–4200)¶	1500 (800–2700)	0.02††
Hemoglobin (g/dL)	14.5 ± 1.0†	13.8 ± 1.6	0.15‡
Platelet count ($\times 10^{-4}$/μL)	16.4 ± 5.4	12.2 ± 3.9	0.03‡
α-Fetoprotein (ng/mL)	4.6 (1.4–28.9)¶	22.3 (11.4–79.7)	0.00005††
HCV RNA (KIU/mL)	1520 ± 1079†	2146 ± 899	0.11‡
Fibrosis (F1/F2/F3/F4)‡‡	14/1/1/2	3/2/2/3	0.02††
Activity (A1/A2/A3)‡‡	12/5/1	3/5/2	0.06††

†Mean ± standard deviation.
‡Student's *t*-test.
§Fisher's exact probability test.
¶Median (range).
††Mann–Whitney *U*-test.
‡‡SVR, $n = 18$; non-SVR, $n = 10$.
Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunoassay; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

achieve SVR were neutrophil counts, platelet counts, serum α-fetoprotein levels and the score of fibrosis in liver biopsies. Table 2 shows the difference in the cytokine/chemokine expression between the SVR and the non-SVR group. Because some cytokines/chemokines were below the measurement limit of the ELISA kit, as shown in Table 1, those cytokine/chemokines were not studied further. As shown here, the *RANTES* level was significantly higher in the SVR group than the non-SVR group ($P = 0.048$).

Precise measurement of serum *RANTES* in all 96 patients

Because the semiquantitative measurement of pretreatment serum *RANTES* levels in 31 randomly selected patients demonstrated their significant correlation with the SVR, we determined the precise serum RANTES levels in all 96 patients using the bead array method and investigated the correlation between those concentrations and the treatment outcome. The clinical characteristics of the 96 patients are shown in Table 3. Significant differences were seen between those with and without SVR in platelet count, viral loads and the liver fibrosis score, but there was no apparent difference in the total doses of PEG IFN and RBV. As shown in Figure 1, the distribution of serum *RANTES* levels in each treatment response differed significantly; the median serum *RANTES* level in the SVR group was significantly higher than that in the non-SVR group. Successive ROC analysis confirmed a significant association of the serum *RANTES* level with SVR, and the cut-off value of 3400 pg/mL to be most appropriate (Table 4). Using the cut-off value of 3400 pg/mL, 50.9% sensitivity, 79.5% specificity, 78.4% positive predictive value and 52.5% negative predictive value (area under the ROC, 0.643) were obtained for the prediction of SVR by serum *RANTES* level.

Table 2 Difference in cytokine and chemokine expression between the SVR group and the non-SVR group in the 31 patients

Cytokine/chemokine	SVR ($n = 20$)	Non-SVR ($n = 11$)	*P*-value
RANTES	4.99 (0.25–8.32)†	1.24 (0.17–8.01)	0.048‡
MIF	1.31 (0.06–3.31)†	0.45 (0.08–2.67)	0.0630
IL-1ra	0.09 (0.00–3.30)†	0.07 (0.00–2.05)	0.2300
PAI-1	3.10 (0.35–7.34)†	2.73 (0.46–8.42)	0.3900
sICAM-1	3.18 (0.37–8.33)†	2.78 (0.74–10.3)	0.4800
IL-23	0.08 (0.01–0.78)†	0.07 (0.00–0.38)	0.5900
IL-27	0.05 (0.02–0.18)†	0.05 (0.00–0.23)	0.6500
IL-6	0.08 (0.01–3.22)†	0.10 (0.00–1.36)	0.7100
C5a	0.21 (0.01–2.72)†	0.12 (0.00–1.67)	0.7700
IFN-γ	0.07 (0.02–0.31)†	0.08 (0.00–0.40)	0.8000
CCL4	0.04 (0.01–3.08)†	0.05 (0.00–0.69)	0.8400
IL-32α	0.04 (0.00–0.71)†	0.07 (0.00–0.20)	0.9000
IL-8	0.16 (0.05–2.61)†	0.17 (0.03–2.21)	0.9300
IL-1α			N.A.
IL-1β			N.A.
IL-2			N.A.
IL-4			N.A.
IL-5			N.A.
IL-10			N.A.
IL-12 p70			N.A.
IL-13			N.A.
IL-16			N.A.
IL-17			N.A.
IL-17E			N.A.
CCL1			N.A.
CCL2			N.A.
CCL3			N.A.
CXCL1			N.A.
CXCL11			N.A.
CXCL12			N.A.
CD154			N.A.
G-CSF			N.A.
GM-CSF			N.A.
IP-10			N.A.
TNF-α			N.A.
sTREM-1			N.A.

†Median (range).
‡Mann–Whitney *U*-test.
N.A., not available; SVR, sustained virological response.

Correlation between serum *RANTES* level and clinical parameters

Spearman's correlation coefficients between the pre-treatment serum *RANTES* level and clinical parameters in all 96 patients are shown in Table 5. As a result, a significant negative correlation with aspartate aminotransferase level and a significant positive correlation with platelet count were found, while no significant correlation was observed in other clinical parameters.

Univariate and multivariate analysis of factors related to SVR

Univariate and multivariate analyses were performed successively in order to clarify the factors related to SVR. The viral factors included in the analysis were the ISDR and core a.a. 70 and 91, along with the host factor, IL-28B SNP. Those factors, conventional clinical background factors and serum *RANTES* levels were subjected to univariate and multivariate analysis. In the univariate

Table 3 Baseline characteristics of all patients analyzed using the bead array method ($n = 96$)

Factor	SVR ($n = 57$)	Non SVR ($n = 39$)	*P*-value
Age (years)	53 ± 10†	57 ± 8	0.08‡
Sex (male : female)	34:23	23:16	0.56§
Bodyweight (kg)	60.6 ± 10.5†	57.8 ± 7.8	0.17‡
Body- mass index (kg/m^2)	22.9 ± 2.8	22.1 ± 2.2	0.15‡
History of IFN therapy (%)	25	28	0.74§
ALT (IU/L)	68 (19–413)¶	64 (20–215)	0.25††
AST (IU/L)	58 (21–331)	62 (21–178)	0.80††
γ-GTP (IU/L)	37 (11–289)	50 (13–167)	0.12††
Albumin (g/dL)	4.1 ± 0.3†	4.1 ± 0.4	0.93‡
Total cholesterol (mg/dL)	166 ± 30	158 ± 31	0.25‡
HbA1c (%)	5.2 (4.7–6.6)¶	5.3 (4.5–7.4)	0.47††
Creatinine (mg/dL)	0.72 ± 0.15†	0.69 ± 0.16	0.39††
WBC count (/μL)	4497 ± 1247	4501 ± 1281	0.99‡
Neutrophil count (/μL)	2243 ± 857	2144 ± 825	0.57‡
Hemoglobin (g/dL)	14.1 ± 1.2	14.2 ± 1.2	0.87‡
Platelet count (×10^{-4}/μL)	15.1 (7–29)¶	13.2 (6.9–19.7)	0.03††
α-Fetoprotein (ng/mL)	4.8 (1.3–137.1)	9.0 (1.4–79.7)	0.05††
HCV RNA (KIU/mL)	1300 (100–5000)	2400 (620–5000)	0.0002‡
Fibrosis (F1/F2/F3/F4)‡‡	35/6/5/6	11/13/5/6	0.006††
Activity (A1/A2/A3)‡‡	27/18/7	12/20/3	0.26††
PEG IFN dose (%)	92 (40–113)¶	73 (27–147)	0.23††
RBV dose (%)	97 (44–147)	100 (33–135)	0.38††

†Mean ± standard deviation.
‡Student's *t*-test.
§Fisher's exact probability test.
¶Median (range).
††Mann–Whitney's *U*-test.
‡‡SVR, $n = 52$; non-SVR, $n = 35$.
Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

analysis, significant differences were observed for the ISDR mutation, core a.a. 70, viral loads, platelet counts, IL-28B SNP and serum *RANTES* levels. When multivariate analysis was carried out with these factors, the serum RANTES level was extracted as an independent factor related to SVR (Table 6).

RANTES haplotyping and serum *RANTES* level

Because a high serum *RANTES* level was an independent factor predicting SVR, we sought to examine further the role of the RANTES gene and tried to clarify the association of the SNP of the gene with the serum levels. First, we determined how many and which SNP in the *RANTES* gene should be investigated to represent all *RANTES* haplotypes found in the Japanese population. Reference to the HapMap project database (http://snp.cshl.org) made it clear that the information from five unique SNP was required to determine the majority of haplotypes found in the Japanese population. Therefore, to determine the *RANTES* haplotype of each patient, we investigated these five SNP in the 65 of the 96 patients available for the haplotype analysis. The *RANTES* haplotypes were finally divided into three types (named R1, R2 and R3 for convenience), as shown in Figure 2(a). However, the *RANTES* gene haplotype and serum RANTES level did not show any clear correlation (Fig. 2b).

DISCUSSION

FROM THE ANALYSIS of 36 cytokine and chemokine species, we discovered that a high pretreatment serum *RANTES* level was significantly related to SVR

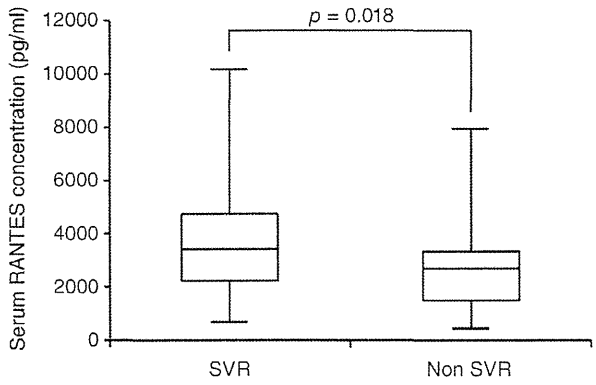


Figure 1 Difference in serum *RANTES* levels between the sustained virological response (SVR) group and the non-SVR group. Ninety-six patients who underwent the standard therapy for 48 weeks were analyzed for serum *RANTES* level using the bead array method. They were divided into the SVR ($n = 57$) and non-SVR groups ($n = 39$) and their serum *RANTES* levels compared. Box and whisker plots show the distributions of serum *RANTES* levels for the SVR and non-SVR groups. The boxes represent the 25th to 75th percentile and horizontal lines within the box show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann–Whitney's *U*-test.

following PEG IFN/RBV combination therapy of patients infected with genotype 1b HCV. In particular, a high serum *RANTES* level was an independent factor contributing to SVR in the multivariate analysis, even among other treatment-restricting factors as the HCV ISDR, core a.a. 70, viral loads, platelets or IL-28B SNP. On the other hand, a systematic haplotyping study did not reveal any correlation between the *RANTES* haplotype and serum *RANTES* level.

RANTES, also known as CC-chemokine ligand (CCL)5, is classified as a chemotactic T-helper (Th)1-type chemokine.[23] In chronic hepatitis C, *RANTES* is significantly upregulated in the infected liver, and considered to play a role in recruiting T cells to portal and periportal regions, regulating liver inflammation and innate and adaptive immunity through interactions with CC-chemokine receptor (*CCR*)5, *CCR1* and *CCR3* expressed on activated T cells.[24] The serum *RANTES* level is significantly upregulated in the early stages of fibrosis in chronic hepatitis and its upregulation becomes weaker in advanced chronic disease.[14] HCV-encoded proteins are considered to affect *RANTES* production, for example, exposure of peripheral blood mononuclear cells to the HCV envelope 2 (E2) protein induces the release of *RANTES*,[25] the HCV NS3/4A proteins suppress *RANTES* promoter activity[26] and the HCV core protein may either induce or inhibit the expression of *RANTES* in various cell types.[27] A recent *in vitro* study has shown that human hepatoma cells secrete *RANTES* via the Toll-like receptor (*TLR*)3-mediated recognition of HCV dsRNA and activation of the nuclear factor (*NF*)-*kB* pathway, suggesting that the hepatocytes themselves may serve as the source of *RANTES*.[15]

In this study, we showed the close association between the serum *RANTES* level and SVR in the PEG IFN/RBV combination therapy by analyzing 31 randomly selected, primary test patients and then all 96 patients. In addition to the association with SVR, we also searched the association between *RANTES* and the initial viral response because SVR could be influenced by the initial viral dynamics, and revealed that complete early viral response (HCV RNA negative at 12 weeks

Table 4 Association between the serum *RANTES* level and SVR rate in all 96 patients analyzed using the bead array method

Cytokine/ chemokine	Serum concentration	SVR rate	*P*-value
RANTES	≥3400 pg/mL†	78% (29/37)	0.002‡
	<3400 pg/mL†	47% (28/59)	

†A cut-off value of 3400 pg/mL was determined by receiver-operator curve analysis in all 96 patients.
‡Fisher's exact probability test.
SVR, sustained virological response.

Table 5 Spearman's correlation coefficient (*R*) between the pretreatment serum *RANTES* level and clinical parameters ($n = 96$)

Clinical parameters	Serum *RANTES* level	
	R	*P*-value
Platelet count	−0.30	0.0025
Aspartate aminotransferase	−0.24	0.0200
White blood cell	−0.15	0.1600
Total cholesterol	−0.11	0.2700
Alanine aminotransferase	−0.088	0.3900
α-Fetoprotein	−0.088	0.4100
Neutrophil count	−0.064	0.5400
Hemoglobin A1c	−0.056	0.6300
γ-Glutamyl transpeptidase	−0.047	0.6500
Albumin	−0.021	0.7900
Hemoglobin	−0.025	0.8000
Creatinine	−0.00098	0.9900

Table 6 Factors associated with SVR analyzed by univariate and multivariate analysis

Characteristic	Subcategory	Univariate analysis			Multivariate analysis		
		Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Platelet count		1.13	1.03–1.25	0.012	1.20	1.00–1.41	0.042
IL-28B SNP	T/T or not	16.0	3.37–76.2	0.0005	9.48	1.40–64.3	0.02
RANTES	≥3400†	4.01	1.58–10.2	0.0036	4.09	1.02–16.5	0.048
Viral loads		0.99	0.99–0.99	0.0012	0.99	0.99–1.00	0.51
ISDR mutation	≥2	21.7	2.76–170	0.0034	28.2	2.05–388	0.013
Core a.a. 70	R or not	2.52	1.03–6.20	0.044	3.19	0.73–13.9	0.12

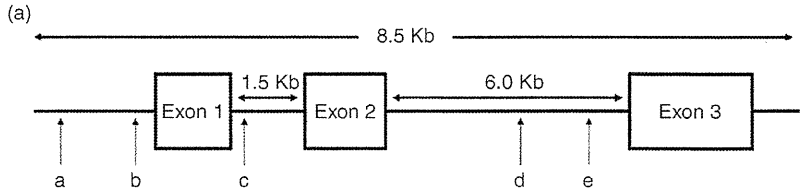
†The cut-off value of 3400 pg/mL was determined by receiver–operator curve analysis in all 96 patients.
a.a., amino acids; CI, confidence interval; IL, interleukin; ISDR, interferon sensitivity-determining region; R, arginine; SNP, single nucleotide polymorphisms.

after commencement of therapy) was also significantly correlated with high pretreatment serum *RANTES* level ($P = 0.015$, data not shown). Moreover, we could also show that high serum *RANTES* levels correlated with the clinical background factors low alanine aminotransferase values and high platelet counts, suggesting that the patients with high *RANTES* levels have less severe hepatitis. A previous study also showed a tendency for correlation between the serum *RANTES* level and SVR in PEG IFN/RBV therapy, but this correlation did not reach significance.[16] Although the reason for this discrepancy is not known, we speculate that a difference in drug dosage may have contributed. In our study, most of the patients received a sufficient dose of both PEG IFN and RBV, as shown in Table 3. However, the previous study lacks information regarding drug dosage, suggesting that the study group comprised a heterogeneous population.

Then, what is the mechanism of the association between high serum *RANTES* levels and high SVR? Because *RANTES* is a chemotactic Th1-type chemokine, it may be speculated that a high serum *RANTES* level reflects activation and preservation of the Th1-type immune responses needed to suppress viral replication and so enhances viral elimination by PEG IFN/RBV therapy. Although it is also possible that a high *RANTES* level could be simply a reflection of early stages of the disease, we suggest that it could have a more direct role in achieving SVR, because multivariate analysis extracted a high serum *RANTES* level as a variable contributing to SVR independently of the platelet count, which reflects the stage of disease. Importantly, our result also demonstrated that the serum *RANTES* level was a factor contributing to SVR independently of other treatment-restricting factors, including the *IL-28B* SNP and the viral factors of NS5A and core. This independent contribution of a high serum *RANTES* level among other variables indicates its importance and potency in improving the prediction of the treatment efficacy.

Concerning the association between the serum *RANTES* level and *RANTES* haplotype, we could not find a significant correlation in the HCV-infected patients, although there was a tendency that patients with the R3 haplotype had higher serum *RANTES* levels. In patients with coronary artery disease and type 1 diabetes mellitus, and in healthy volunteers, the serum *RANTES* level has been reported to correlate with the *RANTES* gene SNP. Specifically, those patients and healthy volunteers with the A allele in the *RANTES* promoter polymorphism at position −403 (rs2107538) had lower serum *RANTES* levels than those with the G allele.[28,29] On the other hand, in previous reports of chronic hepatitis C, no evident correlation was reported between the *RANTES* SNP at position −403 (rs2107538) and serum *RANTES* level.[30] In this study, through more systematic haplotyping analysis based upon the HapMap Database, we tried to determine the correlation between the serum *RANTES* level and the *RANTES* gene SNP in chronic hepatitis C in more detail. However, we could not find any association and the result shows that the serum *RANTES* level is not primarily determined by the *RANTES* haplotype in chronic hepatitis C. The result seems strange at first, however, it is understandable considering that *RANTES* expression is modulated by multiple factors in chronic hepatitis C, including viral components and the stage of liver disease, as described before.

However, there are some limitations in our study. Namely, the number of investigated patients was rather small, and included patients for the analysis were limited to those with genotype 1b HCV infection. Therefore, it is considered that additional independent studies including the analysis of other genotypes would



RANTES haplotype	a	b	c	d	e	Haplotype frequency
	rs2107538	rs2280788	rs2280789	rs4796120	rs3817655	
R1	G	C	T	A	A	0.599
R2	A	C	C	G	T	0.197
R3	A	G	C	A	T	0.189

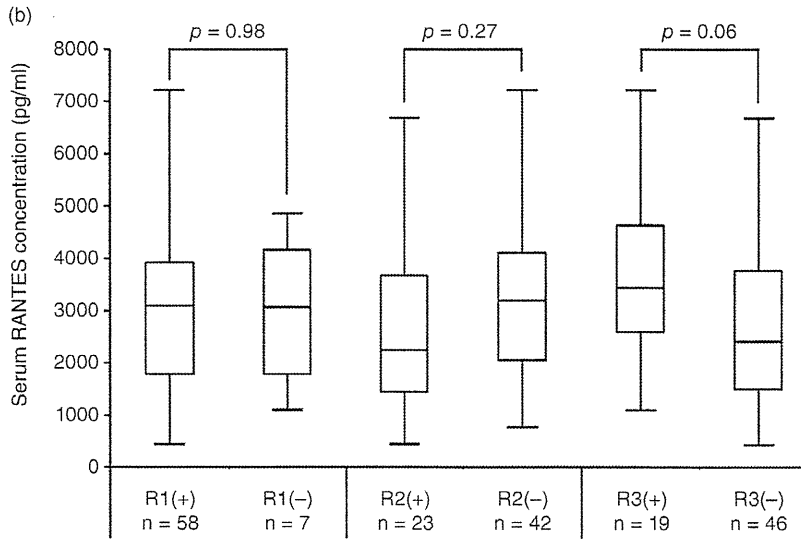


Figure 2 *RANTES* haplotypes and serum *RANTES* level. (a) *RANTES* haplotypes in the patients studied. The human *RANTES* gene spans 8.5 kb on chromosome 17q11-q12 and has the characteristic three exon and two intron organization of the CC chemokine family.[22] Exons are shown as open boxes while introns are shown as solid lines. Five single nucleotide polymorphisms (SNP) (rs2107538/rs2280788/rs2280789/rs4796120/rs3817655) were selected on the basis of data from the HapMap project (http://snp.cshl.org) to obtain complete coverage of the *RANTES* gene in the Japanese population. The locations of SNP variants are indicated by arrows. After the analysis of five *RANTES* SNP in 65 hepatitis C virus patients, haplotypes were determined using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan) and divided into three groups on the basis of linkage disequilibrium. These were designated R1, R2 and R3 on the basis of haplotype frequency. (b) Serum *RANTES* level and *RANTES* haplotype. The correlation between serum the *RANTES* level and *RANTES* haplotype was investigated. Box and whisker plots shows distributions of serum *RANTES* levels for the haplotypes R1(+), R1(−), R2(+), R2(−), R3(+) and R3(−). The boxes represent the 25th to 75th percentile and horizontal lines within the boxes show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann–Whitney's *U*-test. R1(+), the patients with the R1 haplotype; R1(−), the patients with a non-R1 haplotype; R2(+), the patients with the R2 haplotype; R2(−), the patients with a non-R2 haplotype; R3(+), the patients with the R3 haplotype; R3(−), the patients with a non-R3 haplotype.

further clarify the correlation. On the other hand, we could not show an association of pretreatment cytokines/chemokine concentrations with the treatment response to PEG IFN/RBV therapy for the other 35 cytokine and chemokine species investigated in this study. Recently, the serum level of *IP-10* was reported to be strongly associated with the response to PEG IFN/RBV therapy and baseline *IP-10* levels were elevated in patients infected with HCV genotype 1 or 4 who did not achieve an SVR after completion of interferon therapy.[19,20] In our study, however, *IP-10* was not extracted as a molecule associated with treatment responses. Actually, due to the measurement limit of the ELISA kit used, several cytokines and chemokines, including *IP-10*, were undetectable in this study, as shown in Table 2, raising the possibility that some cytokines and chemokines associated with SVR were not extracted. Therefore, our study cannot exclude the possibility of other cytokine/chemokines making a contribution to treatment efficacy.

In conclusion, we found that a high pretreatment serum *RANTES* level was related to the efficacy of PEG IFN/RBV therapy in genotype 1b HCV, independent of other treatment-restricting factors, and prediction of treatment outcome could be improved with the measurement of the pretreatment serum *RANTES* level.

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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.

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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 EC_{50} 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 IC_{50} 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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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. 1 B). The extract C-29EA exhibited EC_{50} 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 EC_{50} 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. 1 B and 2 A) and viral proteins (Fig. 3 A 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 EC_{50} 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 IC_{50} 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 ^{32}P-labeled double-stranded RNA (dsRNA) as a substrate. Treatment