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Comprehensive Analysis for Viral Elements and Interleukin-28B Polymorphisms in Response to Pegylated Interferon Plus Ribavirin Therapy in Hepatitis C Virus 1B Infection

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To comprehensively characterize the contribution of virological factors as well as interleukin-28B (IL28B) single-nucleotide polymorphisms (SNPs) in determining treatment responses in pegylated-interferon plus ribavirin (Peg-IFN/RBV) therapy for chronic hepatitis C virus (HCV)-1b infection, we undertook a retrospective cohort analysis for the pretreatment dominant complete HCV open reading frame (ORF) amino-acid (aa) sequence study in 103 consecutive HCV-1b Japanese patients. The dominant HCV sequences classified by the response were subjected to systematic sliding-window comparison analysis to characterize response-specific viral sequences, along with IL28B SNP analyses (rs8099917). In each comparison of the patients between with and without rapid viral response (RVR), nonearly viral response (nEVR), sustained virological response (SVR), or relapse, the following regions were extracted as most significantly associated with the different responses respectively: nonstructural protein 5A (NS5A) aa.2224-2248 (P = 1.2E-07); core aa.70 (P = 4E-04); NS5A aa.2340-2382 (P = 7.0E-08); and NS5A aa.2360-2377 (P = 1.1E-05). Those NS5A regions nearly coincided with the interferon (IFN) sensitivity-determining region (NS5A aa.2209-2248) and the IFN/RBV resistance-determining region (NS5A aa.2339-2379). In a multivariate analysis, the IL28B SNP (odds ratio [OR] = 16.8; P = 0.009) and NS5A aa.2340-2382 (OR = 13.8; P = 0.0003) were extracted as the two most-significant independent variables contributing to the final outcome. *Conclusion:* In Peg-IFN/RBV therapy, polymorphisms in IL28B, NS5A aa.2224-2248, core aa.70, and, most important, NS5A aa.2340-2382 have a tremendous influence on treatment response in association with viral kinetics, resulting in significantly different outcomes in chronic HCV-1b infection. (HEPATOLOGY 2012;56:1611-1621)

Hepatitis C vrus (HCV) is a major cause of chronic liver disease (CLD) worldwide, causing CLD that may progress to hepatocellular carcinoma (HCC).[1] Treatment response of the conventional pegylated interferon (Peg-IFN) plus ribavirin (RBV) therapy is highly variable, and half of the patients cannot eradicate the virus (i.e., sustained virological response; SVR).[2] Recently, direct-acting

Abbreviations: aa, amino acid; AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; cEVR, complete early viral response; cEVR-8w, HCV RNA <50 IU/mL at between weeks 5 and 8; cEVR-12w, HCV RNA <50 IU/mL at between weeks 9 and 12; CI, confidence interval; CLD, chronic liver disease; DAAs, direct-acting antiviral agents; ETR, end-of-treatment response; EVR, early viral response; Hb, hemoglobin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IL28B, interleukin-28B; IRRDR, IFN/RBV resistance-determining region; ISDR, IFN sensitivity-determining region; nEVR, nonearly viral response; NS5A, nonstructural protein 5A; OR, odds ratio; ORF, open reading frame; PCR, polymerase chain reaction; Peg-IFN, pegylated IFN; PePHD, PKR-eIF2 phosphorylation homology domain; pEVR, partial early viral response; PKR-BD, PKR-binding domain; PLT, platelet count; RBV, ribavirin; RVR, rapid viral response; SNPs, single-nucleotide polymorphisms; SVR, sustained viral response; T-Cho, total cholesterol.

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antiviral agents (DAAs) have been under development, and telaprevir and boceprevir have now been included in HCV treatment regimens in the Unites States. However, it has gradually became learned that HCVs showing resistance to Peg-IFN/RBV therapy might demonstrate higher resistance to these new regimens of Peg-IFN/RBV plus DAAs.[3] In this background, it is urgent to clarify a comprehensive characterization of viral and host determinants for Peg-IFN/RBV therapy and to determine the most appropriate candidates for the new therapies.

In interferon (IFN)-based therapy, treatment response is influenced by multiple host and viral factors. Among the host factors, younger age, milder fibrosis stage, being nonobese,[4] being Asian or Caucasian rather than African,[5] and, recently, the interleukin-28B (IL28B) major allele type[6-8] are associated with favorable responses. Among the viral factors, low baseline viral load and genotype 2/3, rather than genotype 1/4, show favorable responses.[9] On the other hand, the contribution of other viral factors, such as polymorphisms in several restricted viral genetic regions, has long been debated in terms of their association with treatment responses. HCV genetic elements, including the IFN sensitivity-determining region (ISDR) in nonstructural protein 5A (NS5A),[10,11] PKR-binding domain (PKR-BD) in NS5A,[12,13] the V3 region in NS5A,[14] the IFN/RBV resistance-determining region (IRRDR) in NS5A,[15] the PKR-eIF2 phosphorylation homology domain (PePHD) of E2,[16] the C-terminal region of NS5A (G404S and E442G),[17] F415Y in NS5B,[18] polymerase motif in NS5B,[19] and amino acid (aa).70 and 91 in core,[20] have been investigated for their correlation with the clinical outcome of IFN-based therapy or RBV in genotype 1 infection. Complete open reading frame (ORF) analyses in Peg-IFN/RBV therapy also revealed the link between treatment response at day 28 or treatment outcome with viral diversities in several viral genomic regions in genotype 1 infection.[21,22] Importantly, most recent studies reported the strong contribution of core aa.70, ISDR, and IL28B polymorphisms in the response of Peg-IFN/RBV therapy in genotype 1b infection.[11,23]

Nevertheless, a comprehensive analysis of how these viral elements affect treatment response has not been presented clearly yet, especially along with IL28B single-nucleotide polymorphisms (SNPs). Moreover, inconsistent results that have been reported on for some of those regions made the association with the response obscure. Under these circumstances, the previous studies had limitations regarding the following points: (1) Viral regions selected for analysis were partial; (2) associations among different viral regions were not evaluated; (3) most studies investigated the associations only with the final SVR rate, although this is influenced by multiple factors, other than a simple virological response; (4) some studies have included patients with different racial backgrounds; and (5) most studies lacked analysis with IL28B polymorphisms.

To overcome these limitations, we have recently determined complete HCV ORF sequences of 88 patients receiving Peg-IFN/RBV, and confirmed that the NS5A-ISDR and core 70 were specifically extracted as regions most significantly correlated to rapid viral response (RVR) and nonearly viral response (nEVR), respectively.[24] In the present study, we undertook more comprehensive, detailed analysis to disclose the effect of HCV ORF on determining early viral response (EVR), final outcome, and relapse by extending the previous result through adding the information of IL28B polymorphisms in Japanese patients given Peg-IFN/RBV therapy for genotype 1b HCV.

Patients and Methods

Study Patients. We retrospectively analyzed consecutive patients with chronic HCV-1b infection treated with combination therapy of Peg-IFN/RBV at the Yamanashi University Hospital (Yamanashi, Japan) between December 2004 and July 2008. Eligible patients were 18-75 years of age, seronegative for hepatitis B surface antigen and antibodies against human immunodeficiency virus, and had an absolute neutrophil count $\geq$1,500/mm^3, a normal hemoglobin (Hb) level, and available pretreatment serum sample conserved for HCV-sequence analysis. Patients were excluded if they had decompensated liver cirrhosis or HCC. Consequently, 103 patients were eligible for this study. In addition to those 103 patients, 30

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Additional Supporting Information may be found in the online version of this article.

consecutive patients who received the standard length of Peg-IFN/RBV at the Yamanashi University Hospital from August 2008 to April 2011 and were meeting the above-mentioned criteria were also included in the study to perform uni- and multivariate analysis for SVR and relapse. The study was approved by the ethics committees of the University of Yamanashi, and the study protocol conformed to the ethical guidelines of the 2000 Declaration of Helsinki.

Doses and treatment periods were determined according to a standard treatment protocol for Japanese patients, established by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Patients were treated with Peg-IFN-α-2b (1.5 μg/kg, once-weekly, subcutaneously) and RBV (600-800 mg daily, per os) for 48 weeks. When patients failed to achieve a 2-log reduction of HCV RNA at week 12 (nEVR), or failed to achieve HCV RNA clearance (HCV RNA, <50 IU/mL) at week 24 (null viral response), the therapy was discontinued if they did not desire to continue. For patients without viral clearance by week 13, the therapy period was extended up to 72 weeks if they agreed. For patients having achieved viral clearance (HCV RNA, <50 IU/mL) within 4 weeks (RVR), the therapy could be reduced to 24 weeks if they agreed.

Analytic Methods. The following patient characteristics were analyzed: age; sex; stage of fibrosis on liver biopsy; body mass index (BMI); alanine aminotransferase (ALT); Hb; gamma-glutamyl transpeptidase (γ-GTP); total cholesterol (T-Cho); albumin (ALB); platelet counts (PLTs); alpha-fetoprotein (AFP); serum HCV RNA; Peg-IFN dose; and RBV dose. Liver-biopsy specimens were evaluated blindly by an independent interpreter. HCV RNA was determined by polymerase chain reaction (PCR) (Amplicor HCV RNA kit, version 2.0; Roche Diagnostics Corp., Indianapolis, IN).

Viral Response. Patients were subdivided into four groups according to the initial response at week 12. Each group was defined as follows: RVR (<50 IU/mL at week 4); complete early viral response (cEVR; HCV RNA <50 IU/mL at between weeks 5 and 12); partial EVR (pEVR; HCV RNA ≥2-log reduction, but still detectable [≥50 IU/mL] at week 12); and nEVR (HCV RNA <2-log drop at week 12). SVR was defined as undetectable HCV RNA 24 weeks after completion of therapy. Viral relapse after the achievement of end-of-treatment response (ETR) were also evaluated. In some analysis, cEVR was further divided into two groups of cEVR-8w (HCV RNA <50 IU/mL at between weeks 5 and 8) and cEVR-12w (HCV RNA <50 IU/mL at between weeks 9 and 12).

Complete HCV ORF Sequencing. Extraction of RNA, complementary DNA synthesis, and nested PCR were performed using patient serum collected before starting therapy, as described previously.[25] The full-length HCV genome was amplified by nested PCR with 20 partially overlapping primer sets. Both strands of PCR products were cycle-sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer's instructions, using an M13 forward as well as reverse primers. Products were sequenced by an automated DNA sequencer (3130 series; Applied Biosystems). Nucleotide and predicted aa sequences of 20 HCV genomic fragments were determined and assembled using vector NTI software (Invitrogen, Tokyo, Japan).

Sliding-Window Analysis. A sliding-window analysis was introduced to search for HCV polypeptide regions related to treatment response. Briefly, the total number of aa substitutions, compared to the consensus sequence, within a given number of consecutive aas (window) was counted at each aa position in each HCV sequence. The distribution of aa substitutions in the HCV ORF was scanned, applying these windows from aa.1 to aa.3010. The substitution numbers in each window and the treatment response was compared statistically between the two groups, showing different treatment response by Mann-Whitney's U test for each aa window. In each comparison, the length of peptide window was changed from 1 to 100 aas to search for those regions. Consequently, approximately 300,000 windows (100 width × 3,010 aas) were analyzed for each HCV aa sequence. To visualize the result, windows showing significantly low P values were colored in red and nonsignificant P values were colored in green to generate a "heat map" appearance using Microsoft Excel (Microsoft Corp., Redmond, WA), whereas the window with the lowest P value was colored in white to be distinguished clearly.

IL28B SNP Analysis. Human genomic DNA 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 (model 7500; Applied Biosystems) using fluorescein-amidite–labeled SNP primer for the locus rs8099917 (purchased from Applied Biosystems).

Statistical Analysis. Statistical differences in parameters, including all available patient demographic, biochemical, hematological, and virological data, was determined between patients in various groups by the Student t test or Mann-Whitney's U test for numerical variables and Fisher's exact probability test for categorical variables.

Table 1. Baseline Characteristics of 103 Patients and SVR Rate

Variables	Initial 103 Patients
Age, years	56 (31-70)
Gender, male (%)	64 (62)
Fibrosis, F2-F4 (%)	46 (44)
HCV RNA, kIU/mL	1,500 (28-8,392)
BMI	22.7 (17.5-31.7)
ALB, g/dL)	4.1 (3.0-4.9)
γ-GTP, IU/mL	43 (11-289)
ALT, IU/mL	68 (20-413)
T-Cho, mg/dL	165 (104-240)
WBCs, per μL	4,450 (2,520-7,850)
Hb, g/dL	14.2 (11.2-17.9)
PLT, $\times 10^4/\mu L$	14.5 (6.5-27.3)
AFP, ng/mL	5.8 (0.7-468.4)
IL28B TT (%)	65 (73)*
Peg-IFN dose (%)	89 (43-147)
RBV dose (%)	98 (49-133)
SVR rate (n, %)	
All (n = 103)	55 (53)
Standard therapy (n = 76)	
RVR (n = 10)	10 (100)
cEVR (n = 35)	28 (80)
pEVR (n = 15)	3 (20)
nEVR (n = 16)	0 (0)
Extended therapy (n = 27)	
RVR (n = 0)	–
cEVR (n = 5)	3 (60)
pEVR (n = 18)	11 (61)
nEVR (n = 4)	0 (0)

Abbreviation: WBCs, white blood cells.
*n = 89.

Variables with $P < 0.05$ in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors with the odds ratios (ORs) as well as 95% confidence intervals (CIs). All P values of <0.05 by the two-tailed test were considered significant.

Results

Patient Characteristics. Clinical background factors of the 103 patients are shown in Table 1. Responses at 12 weeks were closely related to the final outcome of therapy. In the standard therapy up to 48 weeks, the SVR rate was 100%, 80%, 20%, and 0% for the RVR, the cEVR, the pEVR, and the nEVR, respectively. Among 103 patients, 27 patients from three groups received extended therapy (5 from cEVR-12w, 18 from pEVR, and 4 from nEVR). Although improvement of SVR was observed in the pEVR (from 20% to 61%), there was no improvement in cEVR or nEVR.

Clinical background factors of the 30 patients who were additionally included for uni- and multivariate analysis for SVR and relapse receiving the standard period of Peg-IFN/RBV therapy are also shown (Supporting Table 1).

IL28B SNPs and Their Relationship to Viral Diversity. To evaluate the contribution of the IL28B polymorphism in the 103-patient study group, we investigated the rs8099917 SNPs in 89 patients available for analysis. The polymorphism was closely related to the viral response at week 12 (Table 2). To clarify the relationship between viral diversity and IL28B SNPs, we compared viral sequences between the major allele groups showing favorable initial response (TT) and the minor allele groups showing poor initial responses (TG or GG). IL28B SNP was significantly correlated with the aa residue at core aa.70 in full HCV ORF analysis ($P = 3.4E-06$); non-arginine at core aa.70 was closely related to minor IL28B alleles and vice versa (Supporting Fig. 1).

HCV Sequences Related to RVR and nEVR. To characterize the HCV sequences related to RVR and nEVR, we determined the full dominant HCV ORF sequences by direct sequencing and searched for polymorphic aa positions specifically related to the different responses. Though aa.2240 was extracted as the most-different single position between the RVR and the remainder (data not shown), successive sliding-window analysis revealed that aa.2224 to aa.2248 of the NS5A region, being completely included in the ISDR (aa.2209 to aa.2248), was the region most significantly related to the RVR ($P = 0.00037$; Fig. 1A). On the other hand, when the nEVR and the remainder were compared, core aa.70 was extracted as the most-significant single aa position discriminating the two groups ($P = 7.0E-8$; Fig. 1B). In this comparison of the nEVR versus the remainder, a sliding-window analysis also extracted regions around aa.70 to be the most significantly different (data not shown).

HCV Sequences Related to Final Outcome. We also compared the viral sequence between SVR and non-SVR patients. In comparing complete HCV ORFs, we confined this analysis to HCV sequences obtained from the standard therapy (n = 76) to exclude the influence of therapy duration. In the analysis of each single aa, various differences were observed

Table 2. IL28B SNPs at rs8099917 and the Initial Viral Responses*

	RVR (%) (n = 8)	cEVR-8w (%) (n = 17)	cEVR-12w (%) (n = 15)	pEVR (%) (n = 31)	nEVR (%) (n = 18)
TT	8 (100)	16 (94)	13 (87)	24 (77)	4 (22)
TG	0 (0)	1 (6)	1 (7)	7 (23)	12 (67)
GG	0 (0)	0 (0)	1 (7)	0 (0)	2 (11)

*n = 89.

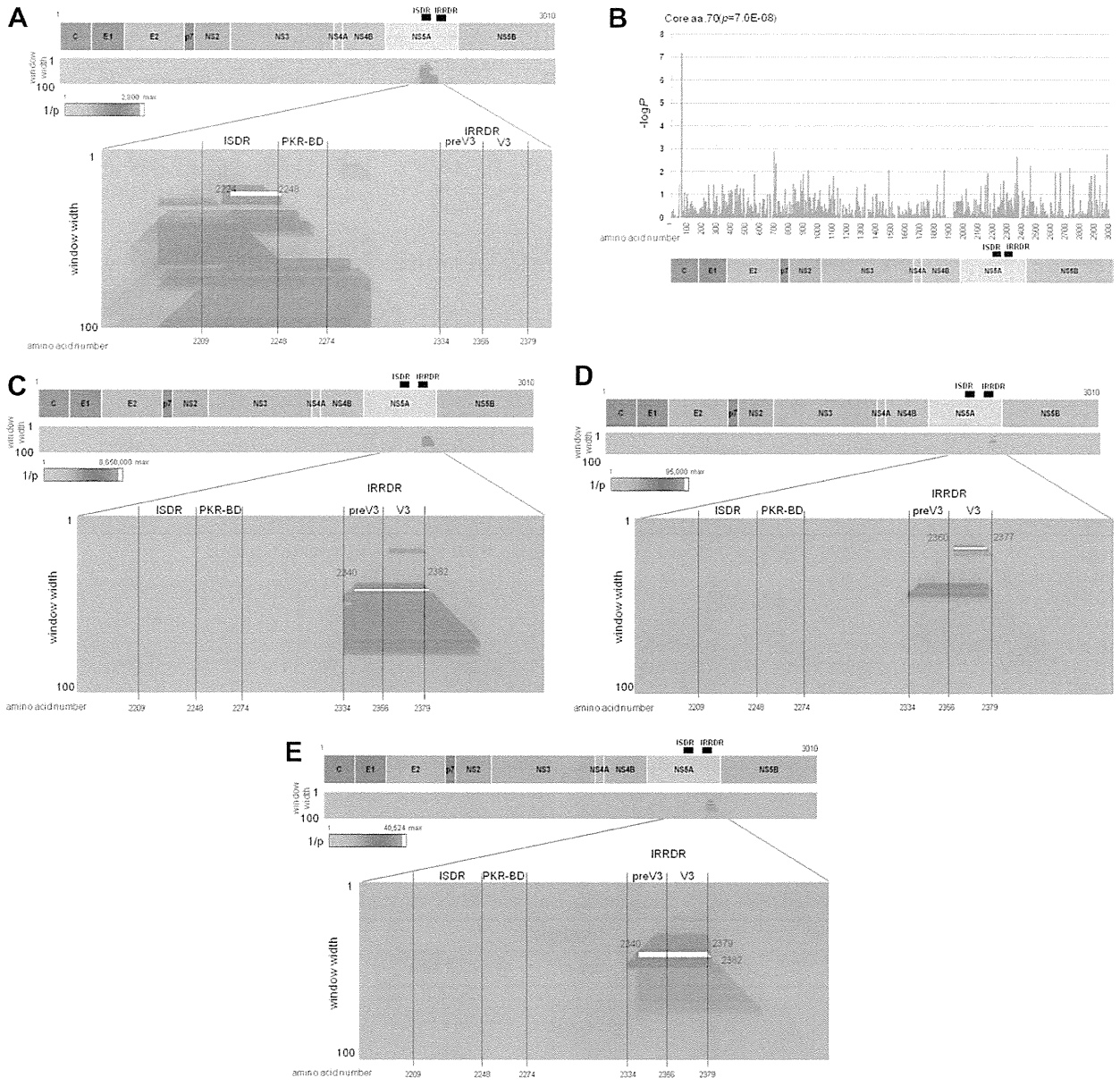

Fig. 1. The contribution of viral sequences and IL28B SNPs in the treatment response to Peg-IFN/RBV was studied. (A) Sliding-window analysis for RVR versus the remainder (n = 103). (B) Single aa analysis for nEVR versus the remainder (n = 103). (C) Sliding-window analysis for SVR versus non-SVR (n = 76). (D) Sliding-window analysis for relapsers versus nonrelapsers among ETR (n = 57). (E) Sliding-window analysis for SVR versus non-SVR in IL28B TT patients with standard therapy (n = 47).

in the HCV ORF, including core aa.70 and NS5B (data not shown). However, a sliding-window analysis disclosed that NS5A region aa.2340 to aa.2382, the region almost coinciding with IRRDR, was extracted as the most clearly related to the final outcome (P = 1.2E-07; Fig. 1C).

HCV Sequences Related to Relapse. To identify the viral regions related to relapse, we compared SVR patients and non-SVR patients among 57 patients with standard therapy achieving ETR (40 nonrelapsers and 17 relapsers). A sliding-window analysis disclosed that the NS5A region aa.2360 to aa.2377, the region almost coinciding with the V3 region in the IRRDR, could be extracted as the most strongly related to relapse (P = 1.1E-05; Fig. 1D).

Uni- and Multivariate Analyses. We performed further analyses to extract the factors associated with RVR, nEVR, SVR, and relapse by univariate, as well as multivariate, analyses. For achieving RVR, ISDR aa.2224-2248 and HCV-RNA were extracted as independent variables (Table 3). Because all the RVR patients possessed IL28B TT alleles and OR

Table 3. Factors Associated With RVR Analyzed by Uni- and Multivariate Logistic Regression Analysis*

		Univariate			Multivariate		
		OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age, years	60≤	0.7	0.18-2.59	0.57			
Gender	Male	1.5	0.36-6.07	0.59			
ISDR 2224-2248	1≤	24.6	4.70-129	8.5E-07†	14.7	1.10-198	0.04‡
IRRDR 2340-2382	4≤	6.2	0.76-51.1	0.06			
Core 70	Arg	0.7	0.18-3.07	0.68			
Fibrosis	<2	3.6	0.72-17.8	0.10			
HCV RNA	<600 k/Ul/mL	74.7	8.55-653	8.3E-10†	51.2	3.97-662	0.003†
BMI	<23	1.3	0.34-4.87	0.71			
ALB	4.1 g/dL≤	1.1	0.30-4.28	0.85			
γ-GTP	50 IU/mL≤	0.9	0.24-3.49	0.91			
ALT	60 IU/mL<	0.9	0.25-3.59	0.94			
T-Cho	<170 mg/dL	1.2	0.33-4.67	0.76			
WBC	4,700/μL≤	1.9	0.47-7.89	0.36			
Hb	14 g/dL≤	1.5	0.37-6.35	0.55			
PLT	150,000/μL≤	1.8	0.48-6.88	0.37			
AFP	10 ng/mL≤	0.3	0.03-2.37	0.22			
Peg-IFN dose (%)	80≤	1.3	0.33-5.55	0.68			
RBV dose (%)	80≤	3.0	0.79-11.4	0.09			

Because all RVR patients possessed IL28B TT alleles and OR calculation was impossible, IL28B SNPs were secluded from analysis.
Abbreviation: WBC, white blood cell count.
*n = 103.
†$P < 0.01$.
‡$P < 0.05$.

calculation was impossible, IL28B SNPs were excluded from the analysis. Likewise, core aa.70 and IL28B were extracted as independent variables associated with nEVR (Table 4). In performing the analysis for SVR and relapse, we excluded patients with extended length of therapy to standardize the treatment periods. Because this restriction reduced the number of available patients for the analysis, we included 30 additional patients (Supporting Table 1) with available clinical information, including HCV core, NS5A, and

Table 4. Factors Associated with nEVR Analyzed by Uni- and Multivariate Logistic Regression Analysis*

		Univariate			Multivariate		
		OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age, years	60≤	1.18	0.42-3.30	0.75			
Gender	Male	0.86	0.31-2.38	0.77			
ISDR 2224-2248	1≤	0.97	0.29-3.28	0.96			
IRRDR 2340-2382	4≤	0.25	0.09-0.69	5.0E-03‡	0.21	0.03-1.33	0.1
Core 70	Arg	0.03	0.01-0.16	2.0E-08‡	0.04	0.00-0.04	0.008‡
IL28B†	Major allele	0.05	0.01-0.17	5.4E-08‡	0.1	0.01-0.57	0.011§
Fibrosis	<2	0.28	0.08-1.0	0.04§	0.5	0.03-0.57	0.55
HCV RNA	<600 k/Ul/mL	0.19	0.02-1.5	0.08			
BMI	<23	0.97	0.36-2.58	0.95			
ALB	4.1 g/dL≤	0.69	0.26-1.85	0.46			
γ-GTP	50 IU/mL≤	1.95	0.73-5.22	0.18			
ALT	60 IU/mL<	0.38	0.14-1.03	0.05			
T-Cho	<170 mg/dL	0.34	0.11-1.03	0.06			
WBC	4,700/μL≤	0.64	0.23-1.76	0.38			
Hb	14 g/dL≤	0.82	0.29-2.26	0.70			
PLT	150,000/μL≤	0.42	0.15-1.19	0.10			
AFP	10 ng/mL≤	5.12	1.82-14.4	0.001‡	3.5	0.52-23.2	0.20
Peg-IFN dose (%)	80≤	0.37	0.14-1.01	0.048§	0.9	0.13-5.93	0.89
RBV dose (%)	80≤	0.38	0.12-1.23	0.10			

Abbreviation: WBC, white blood cell count.
*n = 103.
†n = 89.
‡$P < 0.01$.
§$P < 0.05$.

Table 5. Factors Associated With SVR Analyzed by Uni- and Multivariate Logistic Regression Analysis*

		Univariate			Multivariate		
		OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age, years	60≤	0.8	0.34-1.78	0.55			
Gender	Male	1.4	0.61-3.22	0.43			
ISDR 2224-2248	1≤	6.3	1.98-20.26	0.001†	13.4	1.86-96.5	0.010†
IRRDR 2340-2382	4≤	11.1	4.07-30.54	4.08E-07‡	13.8	3.31-57.4	0.0003‡
Core 70	Arg	3.2	1.37-7.59	0.007‡	2.2	0.43-11.7	0.34
IL28B	Major allele	9.6	2.92-31.34	0.00003‡	16.8	2.04-139	0.009‡
Fibrosis	<2	3.1	1.33-7.23	0.008‡	1.4	0.31-6.64	0.65
HCV RNA	<600 k/UI/mL	3.5	1.39-9.02	0.007‡	3.5	0.72-17.3	0.12
BMI	<23	1.0	0.44-2.20	0.97			
ALB	4.1 g/dL≤	0.9	0.39-1.96	0.75			
γ-GTP	<50 IU/mL	2.6	1.13-5.88	0.02†	3.5	0.90-13.47	0.07
ALT	≤60 IU/mL	0.8	0.35-1.77	0.57			
T-Cho	<170 mg/dL	1.7	0.71-3.94	0.24			
WBC	<4,700/μL	0.8	0.36-1.87	0.64			
Hb	<14 g/dL	0.9	0.35-2.13	0.75			
PLT	150,000/μL≤	2.6	1.06-6.56	0.03†	3.5	0.71-16.8	0.20
AFP	<10 ng/mL	3.7	1.49-9.29	0.004‡	3.4	0.54-21.2	0.20
Peg-IFN dose (%)	80≤	2.2	0.96-5.13	0.06			
RBV dose (%)	80≤	0.8	0.37-1.92	0.68			

Abbreviation: WBC, white blood cell count.
*n = 97.
†$P < 0.05$.
‡$P < 0.01$.

IL28B SNPs. Those 30 patients were consecutively introduced the Peg-IFN/RBV therapy at Yamanashi University Hospital in succession to the initial 103 patients. As a result, 97 patients were available for SVR analysis, and 78 patients were available for relapse analysis. ISDR aa.2224-2248, IRRDR aa.2340-2382, and IL28B SNPs were extracted as the independent variables affecting SVR (Table 5). On the other hand, IRRDR-V3 aa.2360-2377 was extracted as an independent factor for relapse (Supporting Table 2).

Contribution of IL28B SNPs and NS5A aa.2340-2382 in Determining Treatment Response. Because multivariate analysis finally extracted IL28B SNPs and IRRDR aa.2340-2382 as the two most-significant variables determining final outcome, the correlation of IL28B SNPs and IRRDR aa.2340-2382 in association with final outcome was further investigated. Alignment of IRRDR aa.2340-2382 in association with SVR was demonstrated (Fig. 2). By this analysis, it was evident that three or more mutations in IRRDR aa.2340-2382 were significantly associated with SVR. Last, to disclose the viral sequence contribution in the determination of final outcome in IL28B TT haplotype patients with the standard therapy (n = 47), sliding-window analysis was performed (Fig. 1E). As demonstrated here, NS5A IRRDR aa.2340-2379 (~2382) was finally extracted as the most-significant viral region contributing to final outcome ($P = 2.47E-05$).

The contribution of these three viral regions in the phase-specific treatment responses is schematically illustrated (Fig. 3).

Discussion

In this study, we determined 103 complete HCV ORF sequences in consecutive Japanese patients, infected with genotype 1b HCV and given PEG-IFN/RBV therapy, and systematically searched and investigated the contribution of viral regions associated with the phase-specific treatment responses with IL28B SNP haplotypes. To our knowledge, this study is most comprehensive in the following aspects: (1) complete HCV ORF studied with the largest analyzed number of patients; (2) analyzed according to viral kinetics closely related to outcome; (3) unified to a single genotype (1b); (4) unified background of patients; (5) introduction of a sliding-window method to screen the responsible viral regions systematically; and (6) analysis of IL28B SNPs.

In a recent randomized, controlled study of Peg-IFN/RBV combination therapy, the status of patients according to response to Peg-IFN/RBV therapy at 12 weeks showed a marked correlation with final outcome, and viral response at week 12 has been considered as a useful predictor in early-response–guided therapy.[26] In agreement with the previous study, virological responses to Peg-IFN/RBV at week 12 had a

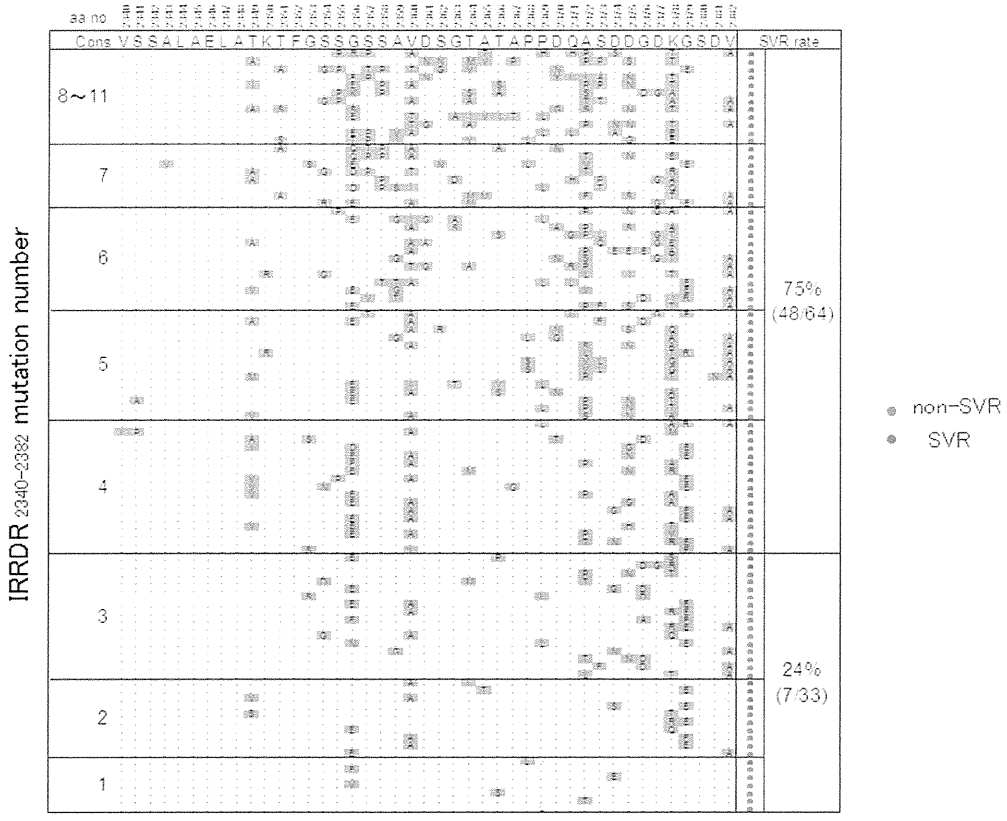

Fig. 2. Alignment of NS5A region around IRRDR aa.2340-2382, along with SVR.

distinct correlation with the final outcomes in our study group (SVR rate: 100%, 80%, 20%, and 0% for RVR, cEVR, pEVR, and nEVR in standard therapy). These results demonstrated that classification by viral response at week 12 provides distinct groups with different characteristics.

We first tried to identify regions of the HCV ORF by showing a distinct linkage to RVR and nEVR. We found that HCV substitutions around the ISDR (aa.2224-2248 in RVR) were most significantly correlated with early viral clearance in Peg-IFN/RBV therapy. In contrast, core aa.70 substitution was most significantly correlated with nEVR, demonstrating the association with treatment resistance. According to the results shown here, early HCV dynamics in Peg-IFN/RBV therapy are significantly regulated by the specific viral sequences in core and NS5A (Fig. 1A,B).

Next, we determined that HCV genomic region correlated with SVR of patients with standard therapy. We excluded patients with extended therapy to unify treatment duration. Considering the length of treatment, we first suspected that multiple factors might affect the final outcome of 48 weeks of standard therapy, and that determining viral regions reflecting pure

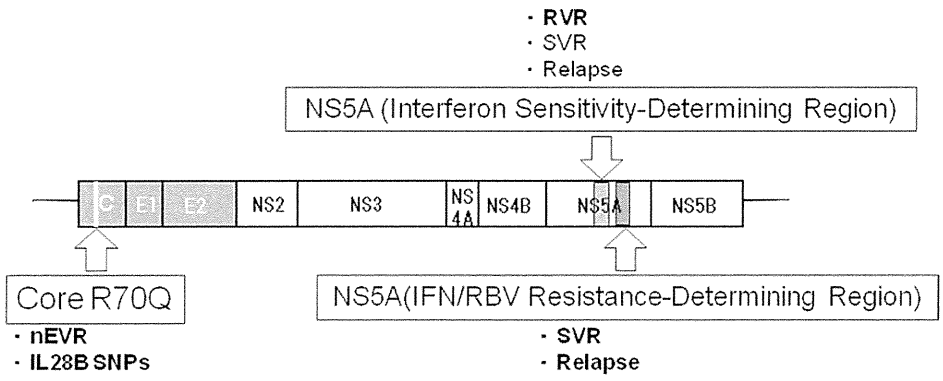

Fig. 3. Roles of three HCV-1b viral regions in the determination of time-dependent treatment response to Peg-IFN/RBV therapy.

biological response would be difficult. Contrary to our prediction, a region almost identical to the IRRDR (aa.2340-2382) was extracted by systematic sliding analysis as correlated with outcome, with a significantly high P value, demonstrating the remarkable influence of the IRRDR aa.2340-2382 in determining final outcome (Fig. 1C). Importantly, in addition to final outcome, when relapser and nonrelapser in the ETR were compared, aa.2360-2377, the region almost coinciding with the V3 region of the IRRDR, was extracted as the region discriminating these two groups (Fig. 1D).

In the analysis of IL28B SNPs (rs8099917), we observed a significant correlation between IL28B SNP and viral dynamics at week 12; patients with minor/minor or minor/major alleles showed significantly poor responses, as demonstrated in Table 2. On the other hand, because poor response was significantly associated with the substitution of the core aa.70 (as shown in Fig. 1B) in our study, we next tried to unveil the correlation between HCV ORF and IL28B SNPs. The significant link with the single core aa.70 substitution was observed through searching for the complete HCV ORFs (Supporting Fig. 1). The result coincides with recent studies[27-29] and, moreover, confirms that this single spot is extraordinarily linked to the initial poor response among the complete 3,010 HCV aa residues. Though the underlying mechanism for the association of IL28B and core aa.70 is unclear, the association would be a reflection of an interaction between the IL28B SNPs and HCV sequences in the development of chronic HCV infection, as discussed by Kurosaki et al.[29] Namely, it is possible that HCV sequences within the patient might have been selected during the course of chronic infection, depending on the IL28B SNPs, by selective pressures of unknown mechanism.

By multivariate analysis, IL28B SNP, IRRDR aa.2340-2382, and ISDR aa.2224-2248 were extracted as independent variables related to final outcome in patients with standard length of therapy with the inclusion of an additional 30 patients (Table 5). Among these, IL28B SNPs and IRRDR aa.2340-2382 were the two most-significant variables determining final outcome. Moreover, NS5A IRRDR aa.2340-2379 (~2382) was the most-significant viral region contributing to final outcome in patients with IL28B TT haplotype (P = 2.47E-05), demonstrating that combined information of the IL28B and IRRDR is significantly important in predicting viral kinetics and treatment outcome (Fig. 1D).

Most of the viral genomic regions identified in this study have already been reported on in previous, independent studies. However, the importance of our study is shown in the result that these specific viral regions of core, ISDR, and IRRDR were extracted all at once through systematic full HCV ORF sequence screening. What is unique in our study is the introduction of the sliding-window analysis; through this analysis, we could effectively confine viral regions of ISDR and IRRDR that were not identified in other previous HCV ORF studies.[21,22] Furthermore, our study also disclosed that the importance of these viral regions was different according to each treatment-phase; RVR, nEVR, SVR, and relapse were mostly related to the ISDR, core aa.70, the IRRDR, and IRRDR, respectively. The ISDR was the first region identified as being related to SVR in the era of IFN monotherapy in Japanese patients, such that multiple mutations in the ISDR were associated with favorable IFN responses.[10,30] The contribution of the core region in treatment response in IFN/RBV therapy was first reported on by Akuta et al., in that the polymorphisms of core aa.70 and 91 were closely related to final outcome.[20] The further significance of core polymorphism was reported on in hepatocarcinogenesis as well.[31,32] Our analysis also confirmed the recent studies reporting on the close correlation between viral core and IL28B SNPs.[11,29,32] The present finding that the core aa.70 is correlated with nEVR independently of IL28B seems to reflect the recent report that core aa.70 is an independent determinant of poor response to the triple therapy of Peg-IFN/RBV and telaprevir in patients with the IL28B minor allele.[27] On the other hand, the IRRDR was originally reported on by El-Shamy et al. as being related to the result of Peg-IFN/RBV therapy.[15] Importantly, our study revealed that final SVR and relapse were significantly correlated with mutations around the IRRDR. The result indicates its significant role in late-phase viral responses in Peg-IFN/RBV therapy.

Core is a main-component protein of viral nucleocapsid, and it has recently been found that the core located on the surface of lipid droplets associates with NS5A to facilitate virion formation.[33] HCV-JFH1 with core R70Q/H and L91M was reported to impair virion formation resulting in the accumulation of intracellular core protein, which causes endoplasmic reticulum stress leading to IFN resistance through suppressor of cytokine signaling 3 up-regulation induced by IL-6.[34] NS5A is a phosphoprotein and is considered to play a pivotal role both in viral replication and virion production, depending on its phosphorylation state.[35-37] Mutations in centrally located serine residues required for NS5A hyperphosphorylation as well as in

its adjacently located ISDR work as adaptive mutations in the HCV replicon, possibly through decreasing the hyperphosphorylated form of NS5A,[37-40] which seems to control HCV replication. The conservation of c-terminal serine residual cluster of NS5A, downstream to IRRDR, is required for NS5A basal phosphorylation, interaction with the core protein on the lipid droplet, and thus virion formation.[41,42] Taken together, it can be speculated that the structural changes in core and NS5A protein can coordinately modify HCV replication, especially through virion formation around lipid droplets. However, the precise mechanism through which these modulations of viral proteins lead to the different treatment response should be further investigated.

In conclusion, we have found that polymorphic viral sequences in core aa.70, NS5A-ISDR aa.2224-2248, and NS5A-IRRDR aa.2340-2382 in genotype 1b HCV infection are correlated significantly with the treatment phase-specific viral responses to Peg-IFN/RBV therapy. In addition, these viral responses were also significantly correlated with the polymorphism in IL28B SNP, and this polymorphism was significantly correlated with the polymorphism in the core. More important, combined information of IL28B and IRRDR aa.2340-2382 is significantly important in predicting viral kinetics and treatment outcome. We consider that our comprehensive study provides a new basis for introducing Peg-IFN/RBV therapy as well as a new generation of anti-HCV therapies.

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Article

Inhibition of Hepatitis C Virus Replication and Viral Helicase by Ethyl Acetate Extract of the Marine Feather Star *Alloeocomatella polycladia*

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Abstract: Hepatitis C virus (HCV) is a causative agent of acute and chronic hepatitis, leading to the development of hepatic cirrhosis and hepatocellular carcinoma. We prepared extracts from 61 marine organisms and screened them by an *in vitro* fluorescence assay targeting the viral helicase (NS3), which plays an important role in HCV replication, to identify effective candidates for anti-HCV agents. An ethyl acetate-soluble fraction of the feather star *Alloeocomatella polycladia* exhibited the strongest inhibition of NS3 helicase activity, with an IC_{50} of 11.7 μg/mL. The extract of *A. polycladia* inhibited interaction between NS3 and RNA but not ATPase of NS3. Furthermore, the replication of the replicons derived from three HCV strains of genotype 1b in cultured cells was suppressed by the extract with an EC_{50} value of 23 to 44 μg/mL, which is similar to the IC_{50} value of the NS3 helicase assay. The extract did not induce interferon or inhibit cell growth. These results suggest that the unknown compound(s) included in *A. polycladia* can inhibit HCV replication by suppressing the helicase activity of HCV NS3. This study may present a new approach toward the development of a novel therapy for chronic hepatitis C.

Keywords: marine organism; *Alloeocomatella polycladia*; hepatitis C virus; NS3 helicase

1. Introduction

Hepatitis C virus (HCV) is an etiological agent of liver disease including steatosis, cirrhosis, and hepatocellular carcinoma, and has infected over 170 million individuals worldwide [1,2]. HCV belongs to the genus *Hepacivirus* of the *Flaviviridae* family. The genome of HCV is a single positive-strand RNA composed of 9.6 kb flanked by 5' and 3'-untranscribed regions (UTRs) and encodes a polyprotein consisting of approximately 3000 amino acids [3]. The polyprotein is translated from a viral genome by an internal ribosome entry site (IRES), which is localized in 5'-UTR [4]. The translated polyprotein is cleaved by host and viral proteases into 10 proteins. The structural proteins consisting of core, E1, and E2 and a viroporin p7, which has not yet been classified as either a structural or nonstructural protein, are located in the *N*-terminal quarter of the polyprotein. The nonstructural proteins including

NS2, NS3, NS4A, NS4B, NS5A, and NS5B occupy the remaining portion of the polyprotein and form a replication complex with several host factors.

HCV NS3 is well known to play a crucial role in viral replication because it possesses helicase and protease activities [5,6]. The *N*-terminal third of NS3 forms a complex with the NS4A protein and exhibits serine protease activity (NS3-4A protease) to cleave the viral polyprotein for the maturation of viral proteins [7]. The remaining portion of NS3 occupies the RNA helicase domain, characterized by the activities of ATPase and RNA binding, both of which contribute to the unwinding of duplex RNA [8,9]. The helicase activity is needed to separate duplex RNA during viral RNA replication [10]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after the uncoating of a viral particle in the infected cells and then is itself used as a template to synthesize a positive-strand RNA packaged into the viral particle. Thus, helicase as well as protease activities of NS3 can be targeted for use in the development of antiviral agents against HCV.

The current therapy, which combines pegylated interferon with ribavirin, is effective in only about half of patients infected with the most common genotype worldwide, genotype 1 [11–13]. However, this therapy has side effects including influenza-like symptoms, cytopenias, and depression [11]. Furthermore, no effective vaccines for HCV have been developed yet. Biotechnological advances of the past decade have led to the development of novel therapies using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication. This approach has been named either "specifically targeted antiviral therapy for hepatitis C" (STAT-C) or "directed-acting antiviral agents" (DAA) [14–16]. Several compounds of STAT-C or DAA have proceeded to clinical trials. 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 in the US, EU, Canada, and Japan [17,18]. However, the emergence of drug-resistant viruses is the major problem for therapies using antiviral compounds [19,20]. Accordingly, several kinds of drugs targeting various molecules or positions will be required for the complete eradication of the virus from hepatitis C patients.

The helicase activity of NS3 could be targeted by development of anti-HCV compound in addition to its protease activity. Belon *et al.* reported that 1-*N*,4-*N*-*bis*[4-(1*H*-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamine, designated as $(BIP)_2B$, is a potent and selective inhibitor of HCV NS3 helicase [21]. $(BIP)_2B$ could not affect ATP hydrolysis without RNA or at a saturated concentration of RNA. QU663 inhibits the unwinding activity of NS3 helicase by binding to the RNA-binding groove irrespective of its own ATPase activity [22]. Compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity.

Various drugs have been generated from natural products, especially those from terrestrial plants and microbes. The development of drugs from natural products has declined in the past two decades by the emergence of high-throughput screening of synthetic chemical libraries. However, recent technical advances in the determination of molecular structures and in the synthesis of chemical compounds have raised awareness about natural products as a resource for drug development [23–25]. Several groups recently reported natural products that inhibit HCV replication *in vitro*. For instance, silbinin, which is identified from the milk thistle [26,27], epigallocatechin 3-gallate, which is from green tea [28], and proanthocyanidins, which are from blueberry leaves [29], can inhibit HCV replication in cultured cells. Marine organisms including plants and animals were recently established as a representative natural resource library for drug development, since there are estimated to be more than 300,000

species of marine organisms. The products isolated from the marine organisms often possess potent biological activities corresponding to the organisms' own novel molecular structures. Thus, marine natural products are considered to include highly significant lead compounds for drug development [30,31]. For example, trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven) are approved anticancer drugs developed from marine organisms [32]. However, marine organisms have not yet been screened for development into anti-HCV agents.

In this study, we screened extracts of marine organisms by using an *in vitro* fluorescence NS3 helicase assay and HCV replicon system to find candidates for safe and effective anti-HCV agents. The marine feather star *Alloeocomatella polycladia* may produce anti-HCV helicase agents that suppress HCV replication.

2. Results and Discussion

2.1. Primary Screening of Marine Organism Extracts on HCV NS3 Helicase Activity

We employed high-throughput screening using a photoinduced electron transfer (PET) assay to identify inhibitors of HCV NS3 helicase activity from extracts of marine organisms (Figure 1). The EtOAc- and MeOH-soluble extracts were prepared from marine organisms obtained from the sea around Okinawa Prefecture, Japan. We identified 16 extracts possessing an arbitrary level of inhibitory activity, which is defined as below 60% of the control in this study (Table 1). Five extracts exhibited high inhibition levels (<30%), and eleven extracts exhibited intermediate inhibition levels (30% to 60%). The EtOAc extract prepared from the feather star *Alloeocomatella polycladia* (Figure 2) exhibited the strongest inhibitory activity among them, and was designated SG1-23-1 in this study. Treatment with SG1-23-1 inhibited the helicase activity in a dose-dependent manner (Figure 3A). The value of IC_{50} is calculated as 11.7 ± 0.7 μg/mL. We confirmed the effect of SG1-23-1 on NS3 helicase unwinding activity by the RNA helicase assay using ^{32}P-labeled double-stranded RNA (dsRNA) as a substrate. Treatment with SG1-23-1 inhibited dsRNA dissociation at concentrations of 16 μg/mL and above (Figure 3B). These results suggest that treatment with SG1-23-1 inhibits the unwinding ability of HCV NS3 helicase.

Table 1. Inhibitory effects of marine organism extracts on hepatitis C virus (HCV) NS3 helicase activity.

Sample	Helicase Activity (% of control)	Specimen	Phylum	Extract	Collection Site
OK-99-2	78	*Agelas* sp.	Porifera	EtOAc	Shimoji Island
OK-99-3	73	*Plakortis* sp.	Porifera	EtOAc	Shimoji Island
OK-99-4	60	*Dysidea arenaria*	Porifera	EtOAc	Shimoji Island
OK-99-5	96	*Theonella cupola*	Porifera	EtOAc	Shimoji Island
OK-99-6	52	*Theonella conica*	Porifera	EtOAc	Shimoji Island
OK-99-7	85	*Epipolasis kushimotoensis*	Porifera	EtOAc	Shimoji Island
OK-99-9	51	*Hyrtios* sp.	Porifera	EtOAc	Shimoji Island

Table 1. *Cont.*

OK-99-10	75	*Theonella* sp.	Porifera	EtOAc	Shimoji Island
OK-99-12	53	*Isis hippuris*	Cnidaria	EtOAc	Shimoji Island
OK-99-13	68	*Acanthella* sp.	Porifera	EtOAc	Shimoji Island
OK-99-15	64	*Phyllospongia* sp.	Porifera	EtOAc	Shimoji Island
OK-99-17	59	*Petrosia* sp.	Porifera	EtOAc	Shimoji Island
OK-99-18	80	*Fasciospongia rimosa*	Porifera	EtOAc	Shimoji Island
OK-99-20	77	*Echinoclathria* sp.	Porifera	EtOAc	Shimoji Island
OK-99-21	68	*Strongylophora* sp.	Porifera	EtOAc	Shimoji Island
OK-99-23	74	*Dysidea herbacea*	Porifera	EtOAc	Shimoji Island
OK-99-26	55	*Dysidea* cf. *arenaria*	Porifera	EtOAc	Shimoji Island
OK-99-28	123	*Plakortis* sp.	Porifera	EtOAc	Shimoji Island
OK-99-31	118	*Spongia* sp.	Porifera	EtOAc	Okinawa Island
OK-99-34	119	*Theonella swinhoei*	Porifera	EtOAc	Okinawa Island
OK-99-35	108	*Petrosia* sp.	Porifera	EtOAc	Okinawa Island
OK-99-36	90	*Acanthella* sp.	Porifera	EtOAc	Okinawa Island
OK-99-37	102	*Luffariella* sp.	Porifera	EtOAc	Okinawa Island
OK-99-41	62	*Dysidea* cf. *arenaria*	Porifera	EtOAc	Okinawa Island
OK-99-43	85	*Xestospongia* sp.	Porifera	EtOAc	Okinawa Island
OK-99-44	61	*Dysidea arenaria*	Porifera	EtOAc	Okinawa Island
OK-99-47	108	*Dysidea* cf. *arenaria*	Porifera	EtOAc	Okinawa Island
OK-99-49	90	*Petrosia* sp.	Porifera	EtOAc	Chibishi
OK-99-51	69	*Isis hippuris*	Cnidaria	EtOAc	Chibishi
OK-99-52	78	*Petrosia* sp.	Porifera	EtOAc	Kuro Island
OK-99-55	65	*Acanthella* sp.	Porifera	EtOAc	Kuro Island
OK-99-57	84	*Theonella swinhoei*	Porifera	EtOAc	Kuro Island
OK-99-63	117	*Epipolasis kushimotoensis*	Porifera	EtOAc	Kuro Island
OK-99-64	98	*Xestospongia* sp.	Porifera	EtOAc	Kuro Island
SG1-1-2	77	*Comanthus gisleni*	Echinodermata	MeOH	Kume Island
SG1-2-2	112	*Stephanometra indica*	Echinodermata	MeOH	Kume Island
SG1-5-2	33	*Comantella* sp. cf. *maculata*	Echinodermata	MeOH	Kume Island
SG1-9-2	57	*Phanogenia gracilis*	Echinodermata	MeOH	Kume Island
SG1-12-2	39	*Comanthus parvicirrus*	Echinodermata	MeOH	Kume Island
SG1-14-2	117	*Comaster schlegelii*	Echinodermata	MeOH	Kume Island
SG1-15-2	26	Colobometridae sp.	Echinodermata	MeOH	Kume Island
SG1-16-2	66	*Cenometra bella*	Echinodermata	MeOH	Kume Island
SG1-19-2	78	*Comaster nobilis*	Echinodermata	MeOH	Kume Island
SG1-21-2	32	*Oxycomanthus* sp.	Echinodermata	MeOH	Kume Island
SG1-23-1	-3	*Alloeocomatella polycladia*	Echinodermata	EtOAc	Kume Island

Table 1. *Cont.*

SG1-24-1	24	*Comanthus* sp.	Echinodermata	EtOAc	Kume Island
SG1-26-2	51	*Oxycomanthus benetti*	Echinodermata	MeOH	Kume Island
SG1-28-2	38	*Lamprometra palmata*	Echinodermata	MeOH	Kume Island
SG1-30-1	25	*Colobometra perspinosa*	Echinodermata	EtOAc	Kume Island
SG1-31-1	26	*Comanthus* sp.	Echinodermata	EtOAc	Kume Island
SG1-33-1	32	*Basilometra boschmai*	Echinodermata	EtOAc	Kume Island
SG3-1	82	*Stereonephthya* sp.	Cnidaria	EtOAc	Tokashiki Island
SG3-4	73	*Dysidea* cf. *arenaria*	Porifera	EtOAc	Tokashiki Island
SG3-6	74	*Stylotella* sp.	Porifera	EtOAc	Tokashiki Island
SG3-10	139	*Epipolasis* sp.	Porifera	EtOAc	Tokashiki Island
SG3-11	97	*Nephthea* sp.	Cnidaria	EtOAc	Tokashiki Island
SG3-21	106	*Myrmekioderma* sp.	Porifera	EtOAc	Tokashiki Island
SG3-25	111	*Pseudoceratina purpurea*	Porifera	EtOAc	Tokashiki Island
SG3-26	95	*Leucetta* sp.	Porifera	EtOAc	Tokashiki Island
SG3-28	65	*Lyngbya* sp.	Cyanobacteria	EtOAc	Tokashiki Island
SG3-29	61	*Dysidea* sp.	Porifera	EtOAc	Tokashiki Island

Total number of marine organisms: 61; Marine organisms that strongly inhibit NS3 helicase activity (<30%) (boldface and underlined): 5; Extracts of organisms that exhibit intermediate inhibition of NS3 helicase activity (30%–60%) (underlined): 11; EtOAc: Ethyl acetate; MeOH: Methanol.

Figure 1. Schematic representation of the PET assay system for unwinding activity of HCV NS3 helicase. 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 photoinduced electron transfer. When the helicase unwinds the double-strand RNA substrate, the fluorescence of the dye emits 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.

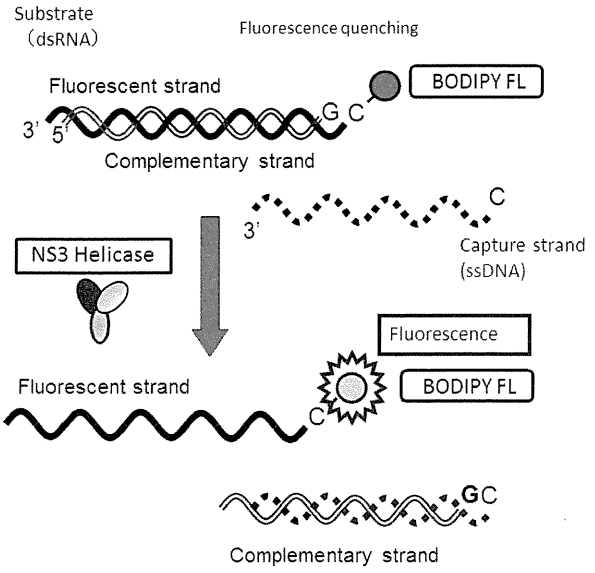


Figure 2. *Alloeocomatella polycladia* belongs to a class of feather star (Echinodermata, Crinoidea). The ethyl acetate fraction prepared from the marine organism was designated SG1-23-1 in this study.

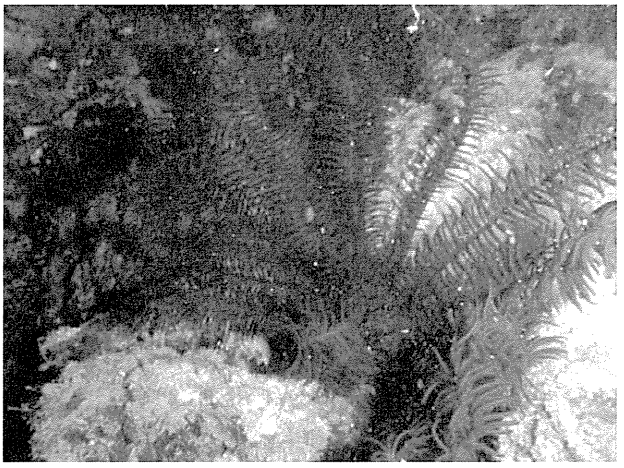
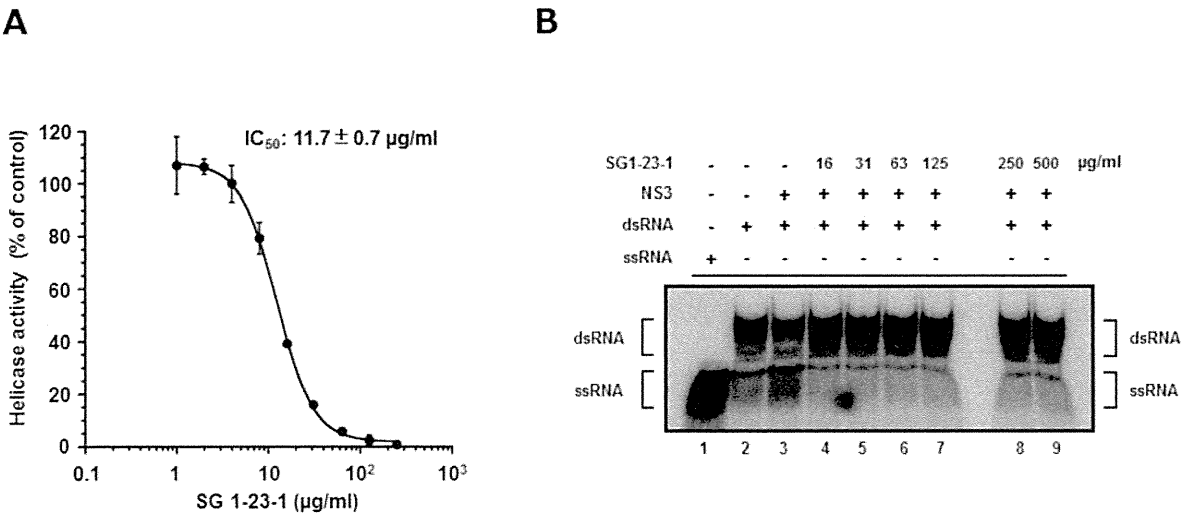


Figure 3. Effect of SG1-23-1 on the unwinding activity of NS3 helicase. (**A**) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of SG1-23-1. Helicase activity in the absence of SG1-23-1 was defined as 100% helicase activity. Each value represents the mean of three independent reactions. Error bars indicate standard deviation. The data represent three independent experiments. (**B**) The unwinding activity of NS3 helicase was measured by 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 SG1-23-1 (lanes 4 to 9, 16 to 500 μg/mL). The resulting samples were subjected to native polyacrylamide gel electrophoresis.

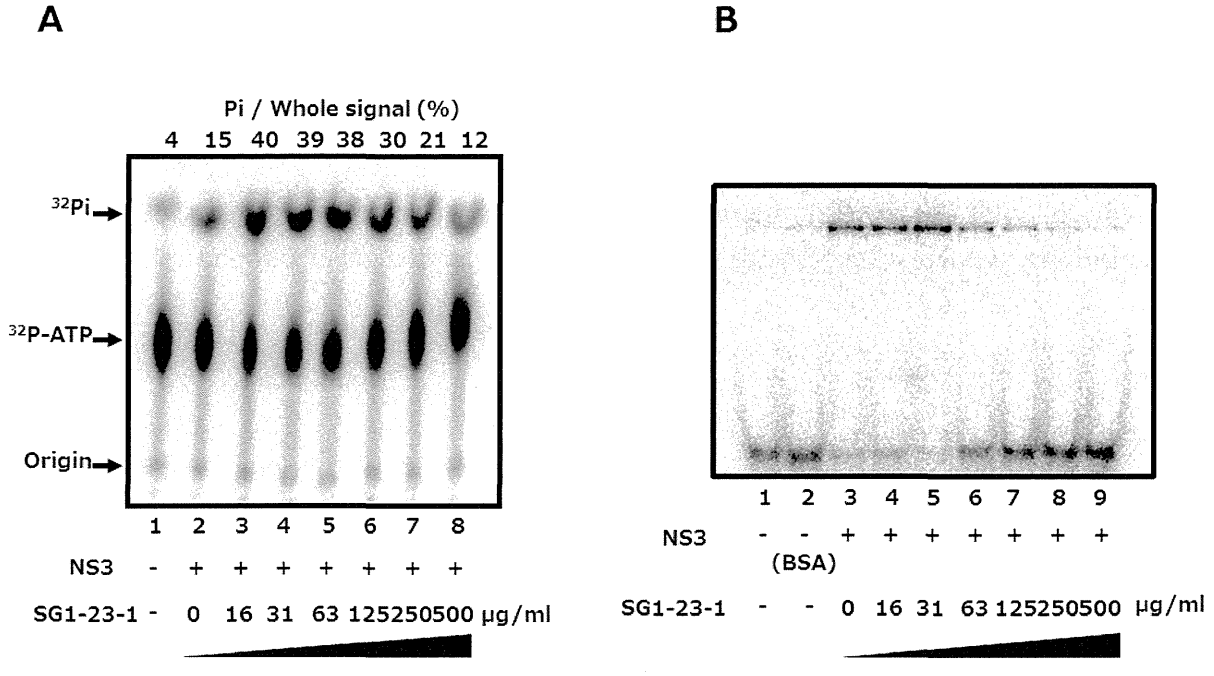


2.2. Effect of SG1-23-1 on HCV NS3 ATPase and RNA Binding Activities

The unwinding ability of HCV helicase is dependent on ATP binding, ATP hydrolysis, and RNA binding [8,9]. We examined the effect of SG1-23-1 on the ATPase activity of NS3 helicase. The ratio of free phosphate (^{32}P-Pi) in ATP (^{32}P-ATP) was measured in the presence of SG1-23-1. The reaction

was carried out between 16 and 500 μg of SG1-23-1 per milliliter. ATPase activity was slightly increased at 16 μg SG1-23-1 per milliliter and slightly decreased at 500 μg SG1-23-1 per milliliter (Figure 4A). However, the helicase activity was decreased to less than 10% in the presence of 50 μg of SG1-23-1 per milliliter (Figure 3A,B). Next, we examined the effect of SG1-23-1 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 21 mer of ssRNA. The binding of NS3 to ssRNA was inhibited with SG1-23-1 in a dose-dependent manner (Figure 4B). These results suggest that SG1-23-1 contains the compound that inhibits RNA binding to NS3 helicase.

Figure 4. Effect of SG1-23-1 on ATPase and RNA-binding activities of NS3 helicase. (**A**) The reaction mixtures were incubated with [γ-^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as "Origin", "^{32}P-ATP", and "^{32}P-Pi", respectively, on the left side of this figure. The data represent three independent experiments. (**B**) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out at the indicated concentration of SG1-23-1. The reaction mixture was subjected to gel mobility shift assay. The data represent three independent experiments.



2.3. Effect of SG1-23-1 on HCV RNA Replication in HCV 1b Replicon Cells

We investigated the effect of SG1-23-1 on both viral replication and growth of the replicon cell lines. The cell lines possess viral subgenomic RNAs derived from three genotype 1b strains (strains N [33], Con1 [34], and O [35]) or a full genomic RNA derived from the O strain [35]. Each cell line was treated with various concentrations of SG1-23-1. The treated cells were harvested 72 h post-treatment. Treatment with SG1-23-1 suppressed HCV RNA replications of all cell lines in a dose-dependent manner irrespectively of full- and sub-genome replicons; it exhibited no effect below 25 μg/mL and

little effect on cellular viability at the highest concentration, 50 μg/mL (Figure 5C,D). Both HCV NS3 and NS5A were decreased at the protein level in a dose-dependent manner, corresponding to the viral replication, but beta-actin was not changed in the cell line harboring subgenome replicon RNA of the Con1 strain (Figure 5E).

The inhibitory effect of SG1-23-1 on HCV replication is summarized in Table 2. The inhibitory effects on the HCV replication of the subgenome replicon derived from Con1, O, and N strains were 22.9 ± 0.4, 19.9 ± 1.8, and 44.2 ± 1.5 μg/mL, respectively, as EC_{50}; and 48.1 ± 1.5, 48.5 ± 0.3, and >50 μg/mL, respectively, as EC_{90}. Treatment with SG1-23-1 inhibited the replication of the subgenome replicon of the O strain (EC_{50}: 19.9 ± 1.8 μg/mL; EC_{90}: 48.5 ± 0.3 μg/mL) at a more potent level than the replication of the full genomic replicon of the O strain (EC_{50}: 39.5 ± 0.8 μg/mL; EC_{90}: >50 μg/mL). When luciferase of firefly or *Renilla* was expressed under the control of the EF promoter, neither showed a significant change in activity in the presence of SG1-23-1 (Figure 5F). The replicon RNA of HCV is composed of the 5'-UTR of HCV, indicator genes (luciferase and drug-resistant genes), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3'-UTR of HCV in that order [33–35]. 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 (Figure 5G). When the expression of the mRNA was transcribed by an EF promoter of the transfected plasmid in the presence of SG1-23-1, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed, suggesting that treatment with SG1-23-1 exhibited no effect on EMCV-IRES-dependent translation (Figure 5H). Thus, the inhibitory effect of SG1-23-1 on the luciferase activity must correspond to the replication efficiency of the replicon RNA but not to the inhibition of luciferase activity or the inhibition of EMCV-IRES-dependent translation. The inhibitory effect of the extract on the viral replication is similar to that of the extract on the helicase activity with respect to the values of IC_{50} and EC_{50} (Figure 3A and Table 2). These results suggest that treatment with SG1-23-1 inhibits HCV replication in a manner similar to that of the inhibitory effect on NS3 helicase activity.

Figure 5. Effect of SG1-23-1 on viral replication in replicon cell lines. (**A**–**D**) Huh7 Lunet/Con1 LUN Sb #26 (**A**), Huh7 rep Feo (**B**), Huh7#94/ORN3-5B#24 (**C**), and OR6 (**D**) cell lines were incubated in medium containing various concentrations of SG1-23-1. Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (**E**) Protein extract was prepared from Huh7 Lunet/Con1 LUN Sb #26 cells treated for 72 h with an indicated concentration of SG1-23-1 and then was subjected to Western blotting using antibodies to NS3, NS5A, and beta-actin. (**F**) Huh7 cell line transfected with pEF Fluc IN vector or pEF Rluc IN was established in the presence of G418. Both cell lines were incubated without (control) and with 50 μg/mL SG1-23-1. Firefly or *Renilla* luciferase activity was measured 72 h post-treatment. Luciferase activity was normalized with protein concentration. Error bars indicate standard deviation. The data represent three