independent experiments. (**G**) Schematic structure of the plasmid, pEF Rluc EMCV IRES Feo. The bicistronic gene is transcribed under the control of elongation factor 1α (EF1α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r), and is translated under the control of the EMCV IRES. (**H**) Huh7 cell line transfected with pEF Rluc EMCV IRES Feo was established in the presence of G418. The cells were incubated for 72 h without (control) and with 50 μg/mL of SG1-23-1. Firefly or *Renilla* luciferase activity was measured by the method described in Materials and Methods and was normalized by the protein concentration. F/R: Relative ratio of Firefly luciferase activity to *Renilla* luciferase activity. F/R is presented as a percentage of the control condition. Error bars indicate standard deviation. The data represent three independent experiments.

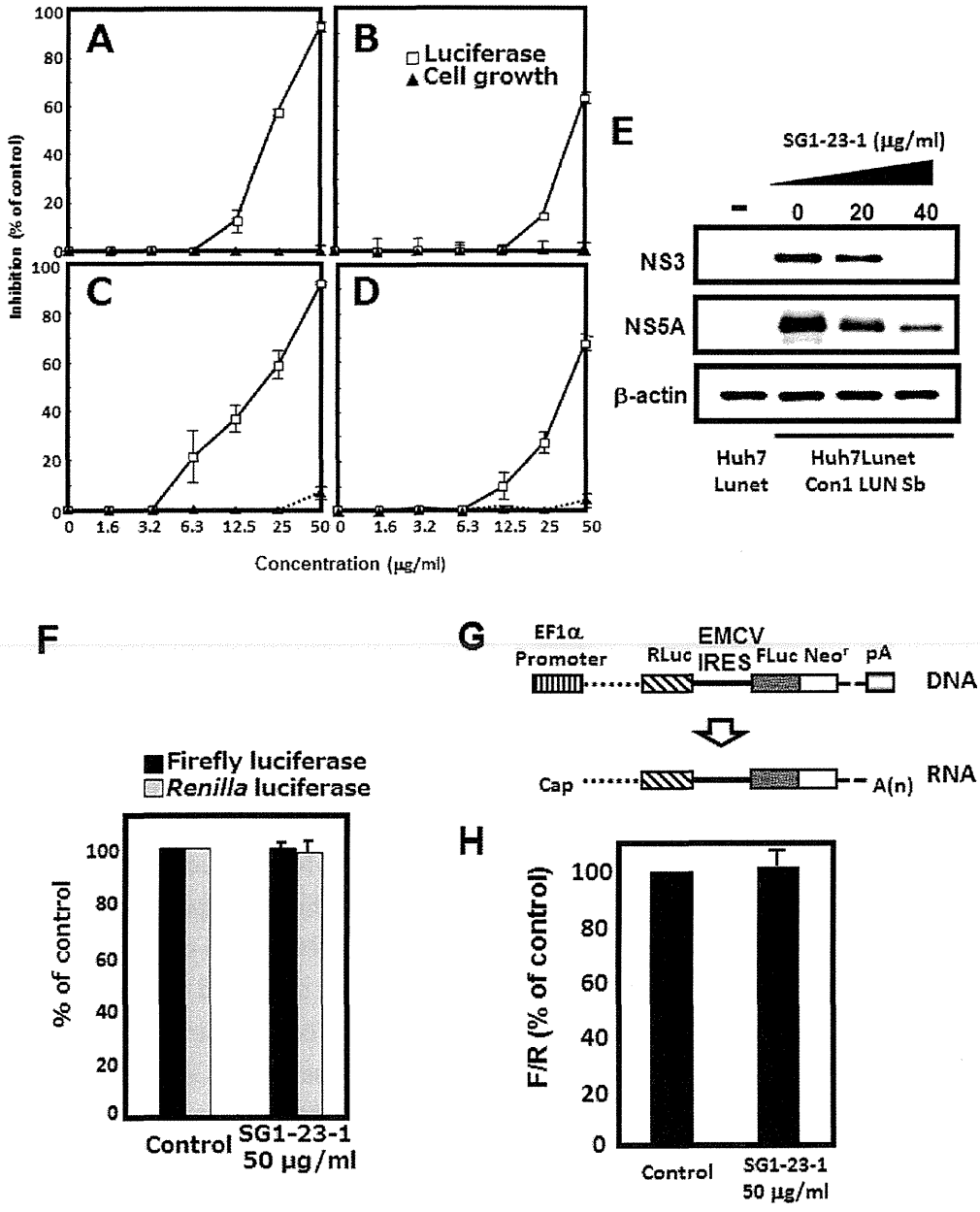


Table 2. Anti-HCV activity of SG1-23-1 in different replicon cell lines of genotype 1b.

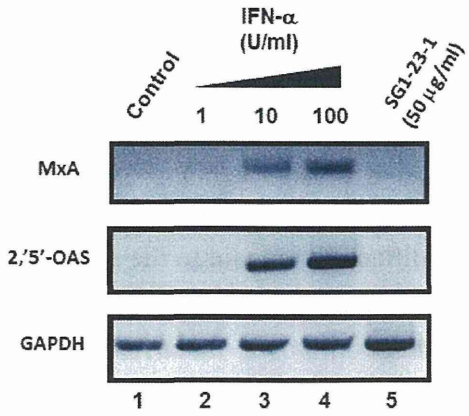
Replicon Cell Line	Virus Strain (Genotype 1b)	EC_{50} [a] (μg/mL)	EC_{90} [b] (μg/mL)	CC_{50} [c] (μg/mL)
Subgenome				
Huh7 Lunet/ Con1 LUN Sb #26	Con1	22.9 ± 0.4	48.1 ± 1.5	>50
Huh7 rep Feo	N	44.2 ± 1.5	>50	>50
Hu7#94/ORN3-5B#24	O	19.9 ± 1.8	48.8 ± 0.3	>50
Full genome				
OR6	O	39.5 ± 0.8	>50	>50

All data represent means ± standard deviation for three independent experiments; [a] Fifty percent effective concentration based on the inhibition of HCV replication; [b] Ninety percent effective concentration based on the inhibition of HCV replication; [c] Fifty percent cytotoxicity concentration based on the reduction of cell viability.

2.4. Effect of SG1-23-1 on the Interferon (IFN) Signaling Pathway

It has been reported that the HCV replication in cultured cells is potently inhibited by interferon (IFN) [36,37]. We examined whether or not treatment with SG1-23-1 induces interferon from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 50 μg of SG1-23-1 per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible genes, MxA and 2',5'-OAS, were induced with IFN-alpha 2b but not with SG1-23-1 (Figure 6). These results suggest that the inhibitory effect of SG1-23-1 on the replication of the HCV replicon is independent of the IFN signaling pathway.

Figure 6. Effect of SG1-23-1 on interferon signaling pathway. Huh7 Lunet/Con1 LUN Sb #26 cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNa-2b (lanes 2–4), and 50 μg/mL SG1-23-1 (lane 5) for 48 h. The mRNAs of MxA, 2',5'-OAS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse-transcription polymerase chain reaction (RT-PCR). Error bars indicate standard deviation. The data represent three independent experiments.



Treatment with SG1-23-1 suppressed the helicase activity of NS3 in a dose-dependent manner and exhibited an IC_{50} of 11.7 μg/mL. Interestingly, treatment with SG1-23-1 inhibited the RNA binding activity of the helicase but not the ATPase activity of NS3. Treatment with SG1-23-1 inhibited the luciferase activity corresponding to the HCV replication in the replicon cell lines, but not the enzymatic activity of luciferase or the translational activity of EMCV IRES, suggesting that treatment with SG1-23-1 decreases HCV replication. Figure 4 shows that the viral proteins NS3 and NS5A in replicon cells were decreased by treatment with SG1-23-1, supporting the notion that SG1-23-1 inhibits HCV replication but not the enzymatic activity of luciferase. The inhibition of cell growth would not contribute to the inhibition of HCV replication by SG1-23-1 (Figure 3 and Table 2). Treatment with SG1-23-1 did not induce the interferon-stimulated genes in the replicon cell lines (Figure 6), suggesting that inhibition of HCV replication by treatment with SG1-23-1 is not due to interferon induction or interferon signaling. The extract SG1-23-1 inhibited the HCV replicon with an EC_{50} of 22 to 44 μg/mL, which is similar to the value of IC_{50}. These results suggest that the anti-HCV compound(s) included in *A. polycladia* can suppress viral replication by inhibiting NS3 helicase activity.

3. Experimental Section

3.1. Preparation of Extracts from Marine Organisms

All marine organisms used in this study were collected by hand during scuba diving off Shimoji, Okinawa, Chibishi, Kuro, Kume, and Tokashiki Islands in Okinawa Prefecture, Japan. In the case of OK-99-tagged extract, a specimen was soaked in ethanol. The ethanol-soluble fraction was concentrated, and the resulting aqueous material was suspended in ethyl acetate (EtOAc). The organic fraction was used for screening.

Each specimen from Kume was soaked in ethanol. The ethanol-soluble fraction was concentrated. The resulting material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG1 and the last digit of "1". The water layer was concentrated to dryness and suspended in methanol (MeOH). The MeOH-soluble fraction was used for screening and tagged with SG1 and the last digit of "2".

Each specimen from Tokashiki was extracted three times with acetone. After removal of acetone from the solution, the residual material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG3.

All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

3.2. High-Throughput Screening of NS3 Helicase Inhibitors

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [38] and was slightly modified with regard to the reaction mixture. A schematic diagram of the PET assay for HCV NS3 helicase activity is shown in Figure 1. The double-strand RNA was prepared as a substrate by annealing, at a 1:2 molar ratio, a 5' BODIPY FL-labeled 37-mer (5'-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUUU-3') to a 23-mer (GGUUAUGAG GGUGGAGGUAAUAG). When unwound by HCV NS3 helicase, the unlabeled ssRNA was captured by a DNA capture strand (5'-CTATTACCTCCACCCTCATAACC-3'). A fluorescent-dye-labeled

oligonucleotide was purchased from J-Bio 21 Corporation. BODIPY FL was attached to the 5'-end via an aminohexylphosphate linker with a six-carbon spacer. Unlabeled oligonucleotides were purchased from Japan Bio Services Co., Ltd. The PET NS3 helicase assay was carried out in 22 μL of 25 mM MOPS-NaOH (pH 6.5) containing 3 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 4 U RNasin, 50 nM of the double-strand RNA described above, 100 nM DNA capture strand, 5 mM ATP, and the extract (25 μg/mL) and 240 nM HCV NS3 helicase. The reaction was started by the addition of HCV NS3 helicase. The reaction mixture was incubated at 37 °C for 30 min. The fluorescence intensity was recorded every 5 s until 5 min post-reaction, and then every 30 s between 5 and 30 min post-reaction by using a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The initial reaction velocity was calculated and represented as NS3 helicase activity.

3.3. ATPase Assay

NS3 ATPase activity was determined by the method of Gallinari *et al.* [39] with slight modifications. The reaction was carried out at 37 °C for 10 min in 10 μL of the reaction mixture containing 25 mM MOPS-NaOH (pH 7.0), 1 mM DTT, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM [γ-^{32}P] ATP (Muromachi, Tokyo, Japan), 300 nM NS3, and 0.1 μg poly (U) per microliter and an indicated concentration of SG1-23-1, and then was terminated by the addition of 15 microliters of 10 mM EDTA. Two microliters of the reaction mixture were spotted onto a polyethyleneimine cellulose sheet (Merck, Darmstadt, Germany) and then developed in 0.75 M LiCl/1 M formic acid solution at room temperature for 20 min. The sheet was air-dried completely and then exposed to an image plate. Radioactive bands were visualized with an Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software (version 3.11; Fujifilm: Tokyo, Japan, 2008).

3.4. RNA Helicase Assay

NS3 RNA helicase assay was carried out by the method of Gallinari *et al.* [39] with slight modifications. The substrate for annealing two complementary RNA oligonucleotides, 5'-AGAGAGAGAGGUUGAGAGAGAGAGUUUGAGAGAGAGAG-3' (40-mer, template strand) and 5'-CAAACUCUCUCUCUCAACAAAAAA-3' (26-mer, release strand) was purchased from Shanghai GenePharma Co., Ltd. The release strand was labeled at the 5'-end with [γ-^{32}P] ATP (Muromachi, Tokyo, Japan) using the T4 polynucleotide kinase (Toyobo, Osaka, Japan) at 37 °C for 60 min and then purified by phenol chloroform extraction. The template and the labeled release strands were annealed at a molar ratio of 3:1 (template: release), denatured at 80 °C for 5 min, and slowly renatured at 23 °C for 30 min in an annealing buffer consisting of 20 mM Tris-HCl (pH 8), 0.5 M NaCl, and 1 mM EDTA. The partial duplex RNA substrate was purified on a G-50 micro column (GE Healthcare, Uppsala, Sweden) and stored at −20 °C in H_2O containing 0.25 U of RNasin Plus (Promega, Madison, WI, USA) per microliter.

SG1-23-1 extract was added at various concentrations to a helicase reaction mixture consisting of 25 mM MOPS-NaOH (pH 7.0), 2.5 mM DTT, 2.5 U of RNasin Plus (Promega), 100 μg of BSA per milliliter, and 3 mM $MgCl_2$. The mixture was supplemented with 300 nM NS3 protein and 5 fM ^{32}P-labeled partial duplex RNA substrate. It was then preincubated at 23 °C for 15 min. After adding ATP at a final concentration of 5 mM, the reaction mixture (20 μL) was incubated at 37 °C for 30 min

and stopped by adding 5 μL helicase termination buffer consisting of 0.1 M Tris-HCl (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 25% glycerol. The terminated reaction mixture was subjected to native TBE 10% polyacrylamide gel electrophoresis. The radioactive RNAs in the gel were visualized with an Image Reader FLA-9000 (Fujifilm) and quantified by Multi Gauge V 3.11 software.

3.5. RNA Binding Assay

RNA binding to NS3 helicase was analyzed by gel mobility shift assay [40]. First, let-7 single-strand RNA (5'-UGAGGUAGUAGGUUGUAUAGU-3') was incubated with [γ-^{32}P] ATP (Muromachi, Tokyo, Japan) and T4 polynucleotide kinase (Toyobo) at 37 °C for 60 min for labeling at the 5'-end of the single-strand RNA. The reaction mixture was subjected to phenol chloroform extraction for purification of labeled RNA. The reaction was carried out at room temperature for 15 min in 20 μL of the mixture consisting of 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 1 unit of RNasin Plus (Promega) per microliter, 300 nM NS3, 5 fmol let-7-labeled ssRNA, and an indicated concentration of SG1-23-1. The reaction was stopped by adding an equal volume of dye solution consisting of 0.025% bromophenol blue, 10% glycerol, and 0.5× Tris/borate/EDTA (TBE). The resulting mixture was subjected to native 6% polyacrylamide gel electrophoresis (acrylamide: bis acrylamide = 19:1). The radioactive RNA was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

3.6. Cell Lines

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/mL G418: The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b) [34]; the Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) [35]; the Huh7 Rep Feo cell line, which harbors the subgenomic replicon RNA of the N strain (genotype 1b) [33]; and the OR6 cell line, which harbors the full genomic RNA of the O strain (genotype 1b) [35].

3.7. Determination of Luciferase Activity in HCV Replicon Cells

HCV replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. The extract SG1-23-1 was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon.

3.8. Determination of Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and incubated at 37 °C for 24 h. The extract fraction of the sample code SG1-23-1 was added to the culture medium at various concentrations. These cells were treated with an indicated concentration of the extract fraction and then were harvested 72 h post-treatment. Cell viability was measured by dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

3.9. Effects on Activities of Luciferase and Internal Ribosome Entry Site (IRES)

The plasmid pEF Fluc IN and pEF Rluc EMCV IRES Feo were described previously [41]. The firefly luciferase gene was replaced with the *Renilla* luciferase gene in the plasmid pEF Fluc IN. The resulting plasmid was designated as pEF RlucIN in this study. The Huh7 cells were transfected with the pEF Fluc IN, pEF Rluc IN, or pEF Rluc EMCV IRES Feo and then were established in a medium containing 0.25 mg/mL G418 as described previously [41]. These cell lines were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with 50 μg/mL extract SG1-23-1, and then harvested at 72 h post-treatment. Activities of firefly and *Renilla* luciferases in pEF Rluc EMCV IRES Feo were measured with the dual luciferase reporter assay system (Promega). Total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

3.10. Western Blotting

The cells were lysed in lysis buffer containing Cell Culture Lysis Reagent (Promega). The cell lysate was subjected to SDS-10% polyacrylamide gel (SDS-PAGE). The proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane. The resulting membrane was incubated with the primary antibodies at 4 °C overnight and then was washed three times with PBS containing 0.02% Tween 20 (PBS-T). The resulting membrane was reacted with a horseradish peroxidase-labeled anti-IgG antibody at room temperature for 2 h and then was washed three times with PBS-T. The reacted proteins were visualized with ImmunoStar LD (Wako Pure Chemical, Osaka, Japan). The antibodies to NS3 (Abcam, Cambridge, UK), NS5A (ViroGen, Watertown, MA, USA) and beta-actin were purchased from New England Biolabs (Beverly, MA, USA) and were used as the primary antibodies in this study.

3.11. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The previously described method of RT-PCR [41] was slightly modified, as described below. Total RNA was isolated from cultured cells with the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) and then was reverse-transcribed with a Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The transcribed mRNA was amplified with PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and an appropriate primer pair. Primer sequences targeting the genes encoding 2',5'-oligoadenylate synthetase (2',5'-OAS), myxovirus resistance protein A (MxA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [41].

4. Conclusions

In conclusion, we showed that the ethyl acetate extract from *Alloeocomatella polycladia* significantly inhibits HCV replication by suppressing viral helicase activity. The purification of an inhibitory compound from the extract of *Alloeocomatella polycladia* will be required in order to improve the efficacy of chemical modification of the compound(s).

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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.

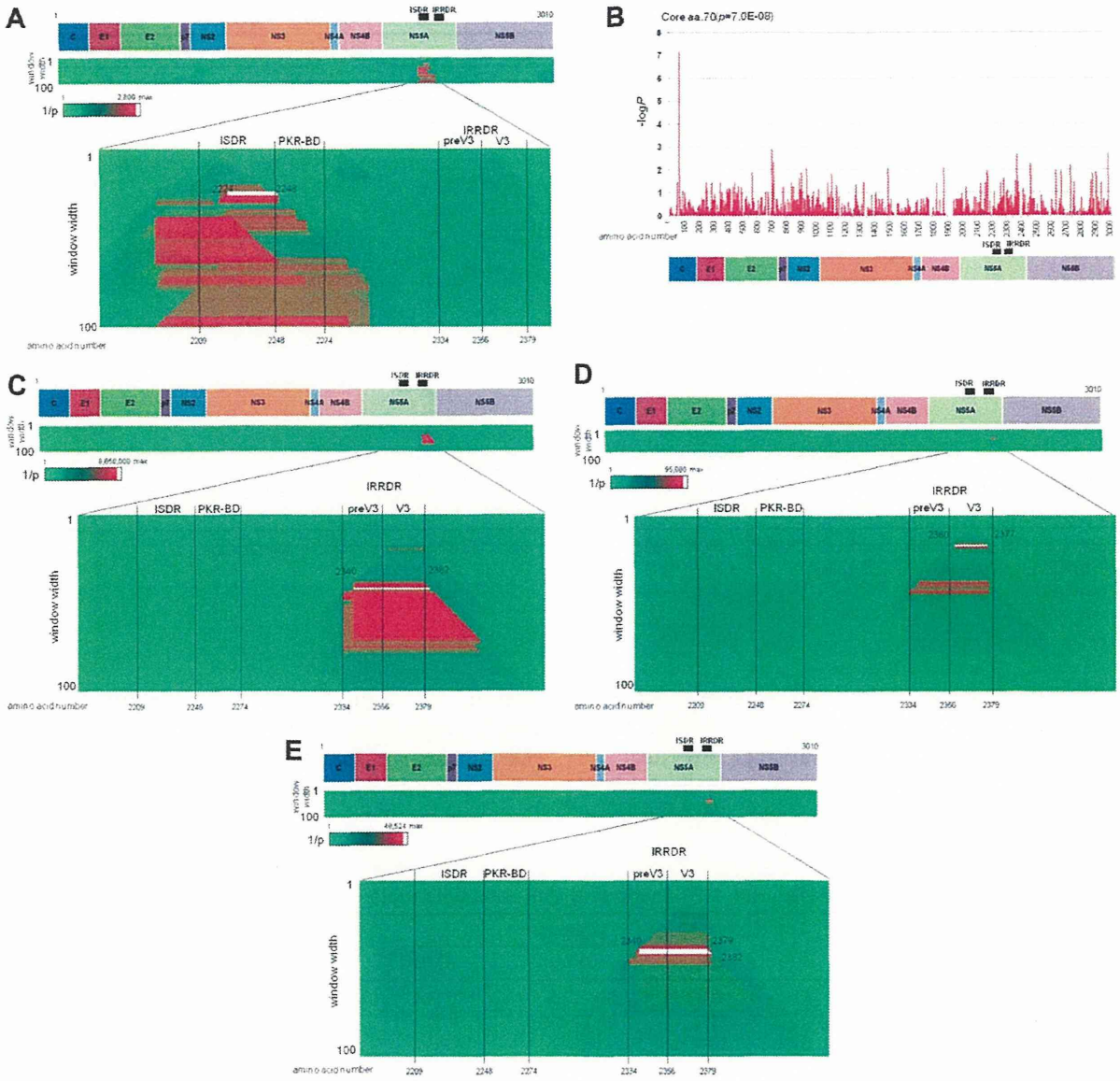

Fig. 1. The contribution of viral sequences and IL28B SNPs in the treatment response to Peg-IFN/RBV was studied. (A) Sliding-window analysis for RVR versus the remainder (n = 103). (B) Single aa analysis for nEVR versus the remainder (n = 103). (C) Sliding-window analysis for SVR versus non-SVR (n = 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/UI/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/UI/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/Ul/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

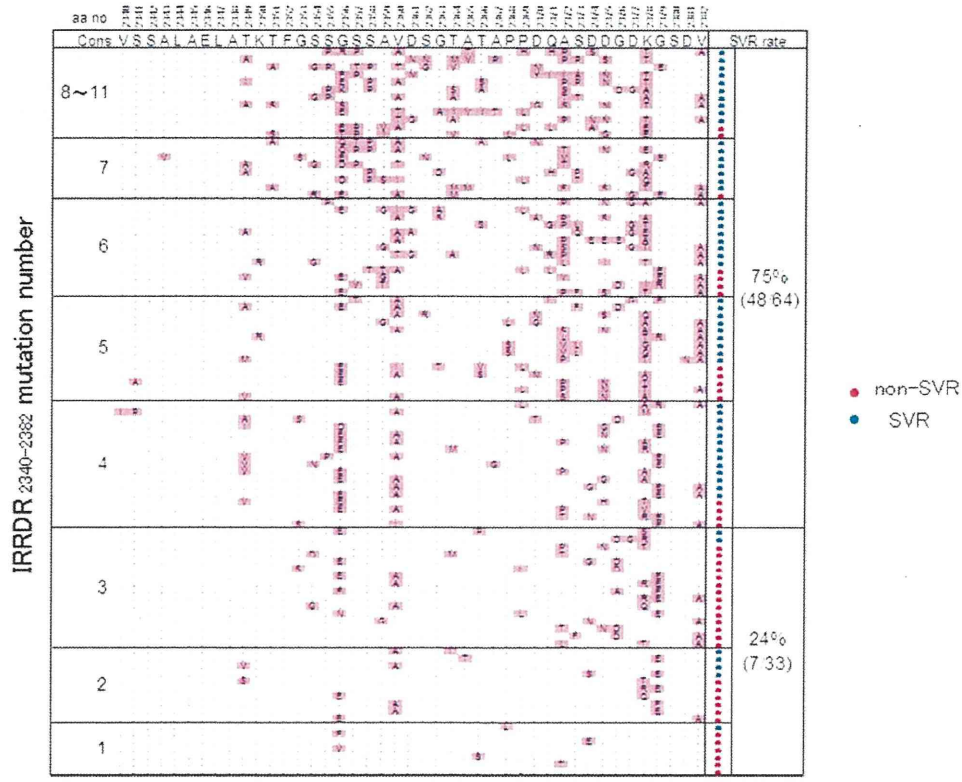

Fig. 2. Alignment of NS5A region around IRRDR aa.2340-2382, along with SVR.

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

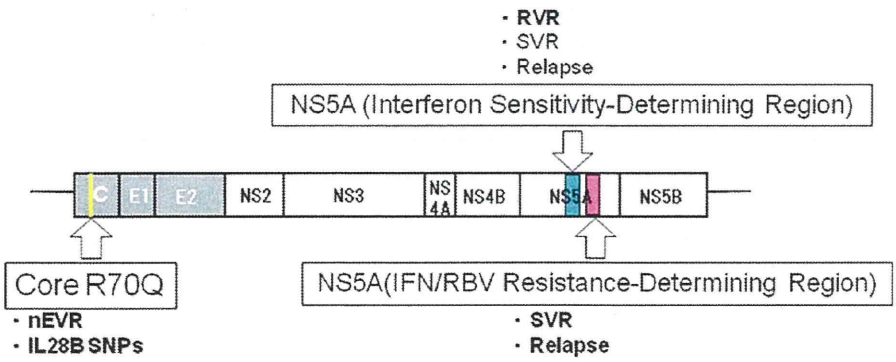

Fig. 3. Roles of three HCV-1b viral regions in the determination of time-dependent treatment response to Peg-IFN/RBV therapy.

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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