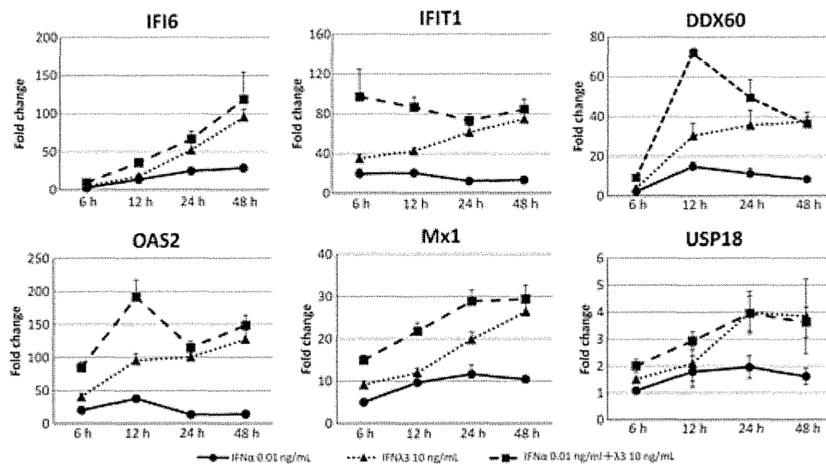

Fig. 5 Time course of ISG expression induced by 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or 0.01 ng/mL IFN-α plus 10 ng/mL IFN-λ3. Expression of the ISGs – IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 – in OR6/ORN/C-5B/KE cells treated 6, 12, 24 and 48 h were determined by qRT-PCR. Results are presented as the relative fold induction. Symbols show the mean value of triplicate wells; error bars show the SD. *Solid lines* represent 0.01 ng/mL IFN-α alone, whereas *fine dashed lines* show 10 ng/mL IFN-λ3 alone, and *coarse dashed lines* show the combination of the 2 cytokines.

the genes induced by IFN-α and IFN-λ in combination. In cDNA microarray analysis, as demonstrated in Table 1, the most strongly upregulated genes induced by IFN-α/IFN-λ3 alone or in combination were almost identical, and most of them were ISGs. As no genes showed upregulation specific to IFN-λ3, we speculate that IFN-α and IFN-λ3 share a similar antiviral intracellular mechanism at the molecular level.

Unexpectedly in microarray analyses, synergistic upregulation of ISGs was not observed. In the same manner, TaqMan real-time RT-PCR analysis showed that the combination of IFN-α and IFN-λ3 did not upregulate ISGs synergistically (Fig. 5). In addition to cDNA microarray analysis, ISRE reporter assays were performed to determine the activation of components of the JAK-STAT pathway common to both type I and III IFNs. As shown in Fig. 4, each IFN upregulated ISRE activity, and the combination of IFN-λ3 and IFN-α did not synergistically enhance ISRE activity either.

Meanwhile, the peak time of the induction of ISG expression differs for IFN-α and IFN-λ1 [9, 17]; peak gene expression occurs earlier with IFN-α than with IFN-λ1. In our study, we confirmed that the peak induction of gene expression occurred later (24 h) and lasted longer (24–48 h) with IFN-λ3 than with IFN-α (12 h). Importantly, gene expression appeared early (12 h) and was prolonged (48 h) by the combination of both IFNs. Similarly to the peak time difference between IFN-α and IFN-λ3 seem for ISG expression, a time-dependent increase in ISRE activation was observed with the combination of both IFNs. While the precise mechanism remains to be clarified, differential regulation of the time-dependent induction of ISG gene expression could be one of the mechanisms underlying the synergistic antiviral effect. One of the molecules contributing to time-dependent ISG upregulation is the ISG known as ubiquitin-specific peptidase 18 (USP18), which has been reported to bind to IFNAR2 and inhibit the interaction of Jak1 with its receptor, thereby preventing IFN-α signalling while leaving IFN-λ signalling unaffected [18, 19]. Actually, expression of USP18 is specifically upregulated with IFN-λ3 in this study as shown in Fig. 5. If the ISGs upregulated by IFN-α are downregulated by USP18, it is plausible that the expression of genes induced by IFN-α decreases early, while expression of genes induced by IFN-λ lasts longer.

A number of clinical studies have confirmed that SNPs around the IL-28B gene are associated with the response to PEG-IFN and RBV therapy, and as previously indicated, various investigations have been performed to clarify the underlying mechanism. Specifically, increased IL-28B mRNA expression in PBMC [2, 3], high serum concentrations of IFN-λ1 (IL-29) [20], low expression of ISGs in the liver prior to IFN treatment [8, 21] and high upregulation of ISG expression by IFN treatment [8, 22] were found in subjects with IL-28B SNP genotypes associated with SVR (rs12979860 CC and rs8099917 TT). Although the functional role of IFN-λ3 still needs to be investigated more thoroughly, if IFN-λ3 expression change is the essential difference in determining the clinical treatment response to PEG-IFN and RBV therapy and if its expression is decreased in patients with the specific IL-28B genotype, which is associated with non-SVR, it is possible that exogenous administration of IFN-λ3 might improve IFN-α-induced viral clearance and that such treatment would be beneficial for patients with the IFN-resistant IL-28B genotype.

In present study, the OR6-cultured cells harboured the rs8099917 TT genotype, and recombinant IFN-λ3 (IL-

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28B) protein used in the experiment was derived from cells with the rs8099917 TT genotype (data not shown). Therefore, the viral responses and/or cellular gene expression change in cells and/or proteins with different IL-28B genotypes *in vitro* should be determined in future studies.

In conclusion, we demonstrated that IFN-α and IFN-λ3 synergistically enhance anti-HCV activity *in vitro*. Although the ISGs upregulated by IFN-α and IFN-λ3 were similar, differences in time-dependent upregulation of these genes, especially prolonged ISGs expression by IFN-λ3, might contribute to their synergistic antiviral activity.

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CONFLICT OF INTEREST

Shinya Maekawa and Taisuke Inoue belong to a donation-funded department that is funded by MSD co. ltd.

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SUPPORTING INFORMATION

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

Figure S1: IFN-α and IFN-λs inhibit HCV core protein secretion. JFH1-infected Huh7.5.1 cells were incubated with various concentrations of IFN-α and IFN-λ1, -λ2, -λ3. After 48 h of treatment, HCV core protein in the medium was measured. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Figure S2: The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after OR6/ORN/C-5B/KE cells were cultured with various concentrations of (A) IFN-α, (B) IFN-λ1, (C) IFN-λ2, (D) IFN-λ3 and (E) combination of IFN-α and IFN-λ3 for 48 h. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Figure S3: The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7.5.1/JFH-1 cells were cultured with various concentrations of (A) IFN-α, (B) IFN-λ1, (C) IFN-λ2, (D) IFN-λ3 and (E) combination of IFN-α and IFN-λ3 for 48 h. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Table S1: Combination index after 48hr stimulation by CalucSyn.

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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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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 $\geq$2-log reduction, but still detectable [$\geq$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 $\times$ 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.

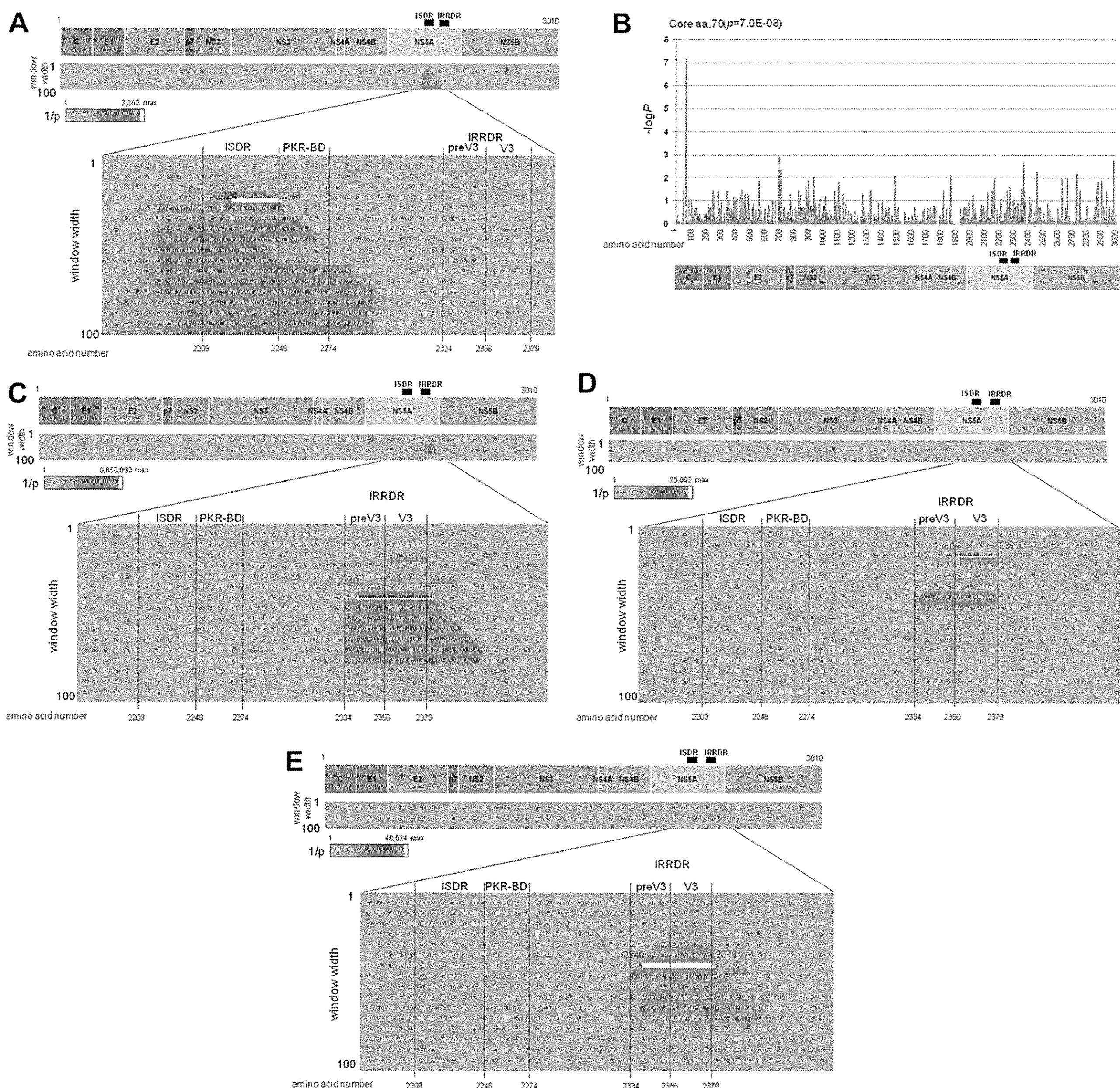

Fig. 1. The contribution of viral sequences and IL28B SNPs in the treatment response to Peg-IFN/RBV was studied. (A) Sliding-window analysis for RVR versus the remainder (n = 103). (B) Single aa analysis for nEVR versus the remainder (n = 103). (C) Sliding-window analysis for SVR versus non-SVR (n = 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/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 ($P = 2.47E\text{-}05$).

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

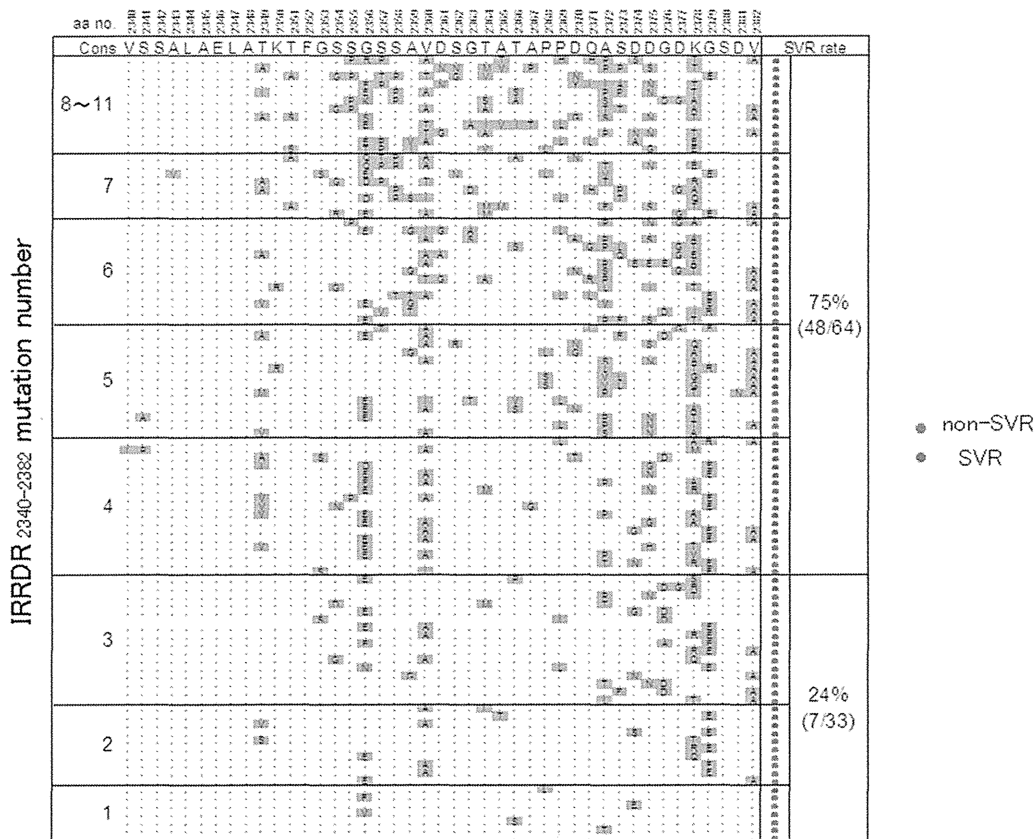

Fig. 2. Alignment of NS5A region around IRRDR aa.2340-2382, along with SVR.

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

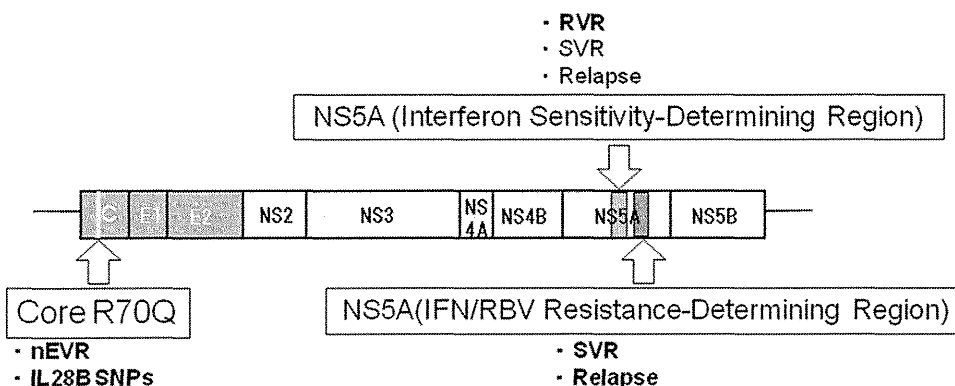

Fig. 3. Roles of three HCV-1b viral regions in the determination of time-dependent treatment response to Peg-IFN/RBV therapy.

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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ORIGINAL ARTICLE

Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C

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Abstract
Background and aims The association between hepatitis C virus (HCV) sequences with interleukin 28B (IL28B) single-nucleotide polymorphism (SNP) in the development of hepatocellular carcinoma (HCC) has not been well clarified.
Methods Complete HCV open-reading frame sequences were determined in 20 patients developing HCC and 23 non-HCC patients with HCV-1b infection in two distant time points. An additional 230 patients were studied cross-sectionally for core and NS5A sequences with HCC development. Among them, 98 patients with available samples were investigated for changes in viral core sequences over time. Finally, IL28B SNPs and HCC development were investigated in 228 patients.
Results During observation period (HCC for 10.8 years, and non-HCC for 11.1 years), changes in core a.a. 70 and three amino acid positions in NS5A were characteristics of the patients developing HCC. In 230 patients, Q (glutamine) or H (histidine) to R (arginine) ratio at core a.a. 70 was significantly higher in the HCC group (HCC group 43:22 vs. non-HCC group 66:99, $p = 0.001$). A change in core R70Q was observed over time in 11 patients associated with a decrease in platelets ($p = 0.005$) and albumin ($p = 0.005$), while a Q70R change was observed in 4 patients without associated changes in platelets (nonsignificant) and albumin (nonsignificant). IL28B SNP showed significant correlation with the core a.a. 70 residue. There was no evident link between IL28B SNPs and the occurrence of HCC.
Conclusions Hepatitis C virus core a.a. 70 residue is associated with liver disease progression and is independent factor for HCC development in genotype-1b infection. IL28B SNPs are related to core a.a. 70 residue, but not to HCC. The functional relevance of core a.a. 70 residue in hepatitis C pathogenesis should be further investigated.

Keywords HCV · HCC · Core · IL28B

M. Miura and S. Maekawa have contributed equally to this study.

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Introduction

Hepatitis C virus (HCV) infection is a major risk factor for hepatocellular carcinoma (HCC). Chronic HCV infection can result in liver cirrhosis (LC) and HCC over the course of 20–30 years [1]. However, the rate of progression is variable; some patients remain for a long time with persistently normal ALT values, while others progress rapidly to LC and HCC.

Viral factors, host factors, and their interplay appear to play an important role in determining the progression of chronic hepatitis C to LC and HCC. In terms of viral factors, most previous clinical studies have focused on searching for HCV regions correlated with the response to interferon (IFN)-based therapy. In those analyses, correlation between amino acid substitutions and treatment response have been reported for the IFN sensitivity

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determining region in nonstructural (NS)5A [2], a.a. 70 and 91 in core [3], the PKR/eIF-2alpha phosphorylation homology domain (PePHD) in envelope (E)2 [4], and the IFN–ribavirin resistance determining region (IRRDR) in NS5A [5].

Regarding the viral factors related to disease progression, among the various HCV proteins, the core protein has been thought widely to contribute because it has been shown experimentally to affect multiple cellular functions, in addition to the evidence from clinical studies [6–10]. Core protein modifies cellular apoptosis, oncogenic signaling, reactive oxygen species formation, lipid metabolism, transcriptional activation, transformation, and immune reactivity. Core protein has oncogenic potential in transgenic mice [11]. In contrast, fewer clinical studies to date have systematically investigated the correlation between the variability of HCV regions and disease progression. However, some of those limited clinical studies reported a correlation between amino acid substitutions in core or NS5A with disease progression [12–15]. Despite those reports, few studies to date support the correlation. Moreover, it is unclear whether those viral sequences change during disease progression or how the disease activity is modified by those viral sequences in the long course of chronic hepatitis.

On the other hand, regarding host factors, recent reports disclosed a significant correlation between polymorphisms in the IL28B gene and responses to pegylated-IFN plus ribavirin therapy for HCV patients [16–19]. This single-nucleotide polymorphism (SNP) also showed significant correlation with natural HCV clearance [20]. However, it remains unknown whether the IL28B SNP is related to disease progression or the development of HCC.

In this study, we first undertook the analysis to identify the viral regions related to disease progression and HCC development through the analysis of complete HCV open-reading frame (ORF) sequences. Because some regions in HCV core and NS5A showed characteristic changes over time in patients developing HCC during the observation period, we proceeded further to analyze the contribution of those regions to disease progression, in association with time and with the IL28B SNP.

Patients and methods

Patients

This study is based on the analysis of two groups of patients, 43 in Group 1 and 230 patients in Group 2.

In the first part, we tried to characterize and extract viral sequences specific to disease progression through the analysis of complete HCV ORFs (Fig. 1a). In particular, we focused our investigation on the changes in viral sequences over time in association with disease progression by comparing HCV sequences of two sufficiently distant time points. With this aim, we determined to investigate patients with a history of IFN therapy, because those patients often were followed long-term with preservation of old and recent sera. However, we excluded sustained

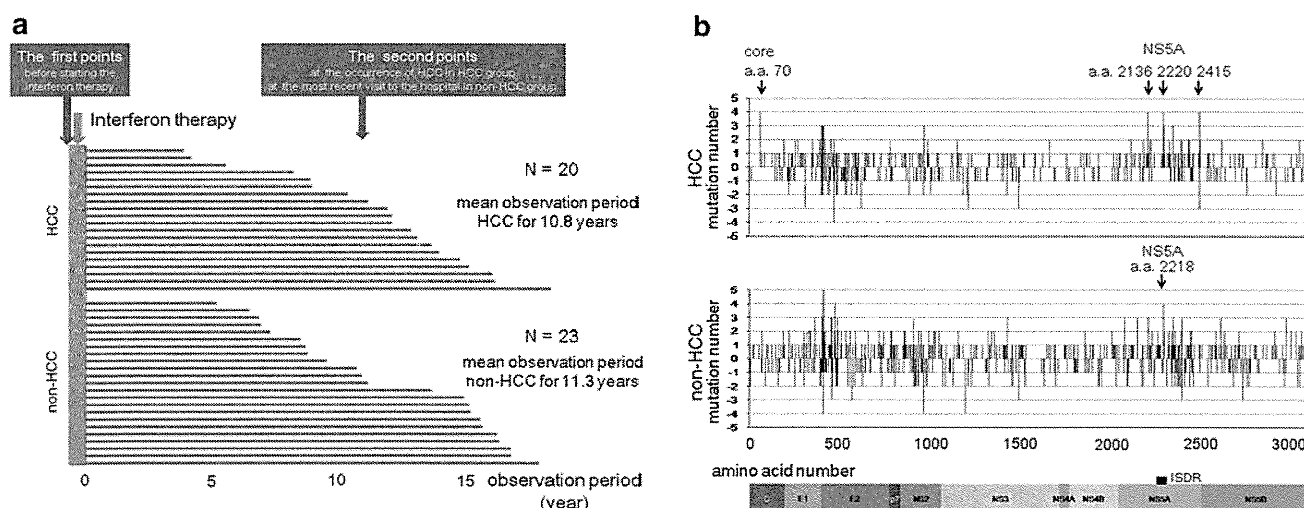

Fig. 1 **a** A total of 43 patients were analyzed for complete HCV ORF sequences. They were all non-responders to the previous IFN therapy. Twenty patients developed HCC during the observation period, while the 23 patients did not. HCV ORF sequences were determined for the paired samples and the predicted amino acid changes were compared in each patient. **b** Specific HCV amino acid changes associated with disease progression was evaluated by the analysis of the full-length viral ORF during the observation period for each patient according to the following rules: *1* +1 point for consensus to non-consensus, *2* −1 point for non-consensus to consensus, *3* 0 point for non-consensus to non-consensus. These points were added together and are shown for HCC and non-HCC patients. Patient with later HCC development (*upper panel*). Non-HCC patients (*lower panel*)

virologic response (SVR) patients because we thought that the viral clearance leads to improvement of the liver disease and, therefore, viral regions influencing the IFN response would be extracted as affecting the course of disease. Between March 1992 and April 2004, 273 consecutive patients with HCV-1b infection were given IFN monotherapy at Yamanashi University Hospital, and 133 were followed long-term. A total of 65 patients showed SVR, while 68 showed non-SVR. Among these 68 non-SVRs, 43 patients were included in the study because laboratory data and sera were available from the two distant time points (Group 1). Twenty patients developed HCC during the observation period, while the remaining 23 did not. Regarding the sera, the first time point for both groups was before starting IFN therapy, while the second points were at the occurrence of HCC for the HCC group and at the most recent visit to the hospital for the non-HCC group (Fig. 1a).

An additional 230 HCV-1b patients were recruited (Group 2) as the second study group. They were mainly outpatients at Yamanashi University Hospital and were selected randomly from those with stored sera at the time of disease diagnosis. Sixty-five had HCC, while the remaining 165 did not. They all were positive for HCV RNA at the time of study, although 74 patients had a history of IFN therapy. Parts of the core and NS5A sequences were determined at HCC onset in 65 HCC patients and at the most recent visit to the hospital in 165 non-HCC patients. Because historical sera around 10 years before were also available for 55 of these 230 patients, HCV sequence analysis was also performed for core and NS5A at those previous time points in those patients.

From these two study groups, 228 patients (68 HCCs and 160 non-HCCs) with available genomic DNAs were examined to determine the IL28B SNP.

All the patients studied all fulfilled following criteria: (1) Negative for hepatitis B surface antigen. (2) No other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease. (3) Free of coinfection with human immunodeficiency virus. (4) A signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital.

Complete and partial HCV ORF sequence determination by direct sequencing from sera

HCV RNA extraction, complementary DNA synthesis and amplification by two-step nested PCR from serum samples were done using the specific primers for full HCV ORF or partial viral regions as described previously [15]. PCR amplicons were sequenced directly by Big Dye Terminator Version 3.1 (ABI, Tokyo, Japan) with universal M13 forward and reverse primers using an ABI prism 3130 sequencer (ABI). Generated sequence files were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

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 with a model 7500 (ABI) using FAM-labeled SNP primer for the locus rs8099917 (ABI).

Statistical analysis

Statistical differences in the parameters, including all available patients' demographic, biochemic, hematologic, and virologic data, were determined between different groups of patients by Student's t test for numerical variables and Fisher's exact probability test for categorical variables. Odds ratios and their 95% confidence intervals were used to quantify the level of association. All p values of <0.05 by the two-tailed test were considered significant throughout. Multiple logistic regression analyses were used to identify the independent variables influencing core a.a. 70 residue and HCC development. Because most variables used for the analyses were generally considered to correlate with the disease progression, we entered all the variables into the multiple logistic regression analysis even if some of them did not reach significant differences in individual univariate analysis.

Results

Comparing complete HCV amino acid sequences between patients with and without HCC

The clinical characteristics of the 43 patients (Group 1) analyzed for HCV ORF changes over time are shown in Table 1. At the start of observation, clinical characteristics did not differ significantly between the HCC group and the non-HCC group. The mean observation period was comparable between the two groups and was 10.8 years for the HCC group and 11.3 years for non-HCC group ($p = 0.745$). On the other hand, platelets ($p < 0.001$), albumin ($p < 0.001$), and AFP ($p = 0.001$) became significantly lower or higher in the HCC group at the end of observation (Table 1).

We proceeded to investigate viral amino acid changes during the course of disease in each patient to determine

Table 1 Patient characteristics in Group 1

	At the start of observation			At the end of observation		
	HCC ($N = 20$)	Non-HCC ($N = 23$)	p value	HCC ($N = 20$)	Non-HCC ($N = 23$)	p value
Observation period (years)				10.8 ± 3.6	11.3 ± 3.8	0.745
Sex (male/female)	11/9	12/11	0.999	11/9	12/11	0.999
Age (years)	51.5 ± 8.0	50.0 ± 9.9	0.604	61.7 ± 10.0*	61.0 ± 10.9*	0.818
Stage of fibrosis (F1/2/3/4)	1/7/6/6	5/11/4/3	0.190	N/A	N/A	–
AST (IU/L)	102 ± 114	74 ± 40	0.695	71 ± 36*	51 ± 30	0.048
ALT (IU/L)	124 ± 86	104 ± 71	0.411	69 ± 47*	52 ± 31*	0.159
Platelets (10^{-4}/mm^3)	16.2 ± 4.8	18.3 ± 6.2	0.217	9.7 ± 3.9*	15.3 ± 5.1	<0.001
Albumin (g/dL)	4.1 ± 0.4	4.1 ± 0.2	0.639	3.6 ± 0.4	4.1 ± 0.5	<0.001
γGTP (IU/L)	90 ± 60	71 ± 46	0.275	69 ± 59	45 ± 38*	0.114
T.Chol (mg/dL)	169 ± 28	156 ± 22	0.110	146 ± 21	164 ± 5,108	0.086
Alpha-fetoprotein (ng/mL)	10.5 ± 6.8	9.3 ± 10.8	0.695	42.4 ± 41.1	4.7 ± 2.7	0.001
HCV RNA concentration (kIU/mL)	706 ± 696	614 ± 1,181	0.760	3,325 ± 415*	4,508 ± 5,108*	0.426

* Factors with significant changes over time (<0.05)

whether specific amino acid changes related to disease progression could be identified. First, the consensus amino acid was determined at each amino acid position in the HCV ORF after determination of all sequences in these 43 patients. Amino acid changes were determined according to the following rules to highlight directional changes according to disease progression: When an amino acid changed from the consensus to the non-consensus during the observation period, we scored +1 point. Conversely, a change from the non-consensus to the consensus scored −1 point. We scored 0 point for a change from one non-consensus amino acid to another. As shown in Fig. 1b, directional amino acid changes were observed throughout the HCV genome to some degree both in patients with and without HCC development during the clinical course of almost 10 years, and frequent substitutions in E2 hypervariable region were common in both groups. On the other hand, in patients with HCC development, as many as four directional changes were observed at core a.a. 70 and at three amino acid positions of NS5A (Fig. 1b, upper panel). In contrast, in patients without HCC, the significant change ($n = 4$) was observed at a.a. 2,218 of NS5A when E2 hypervariable region was excluded (Fig. 1b, lower panel).

Core and NS5A sequences in patients with and without HCC

Because the first analysis suggested that the patients with later HCC development might accumulate specific mutations in core and NS5A at the time of HCC occurrence, additional sequences were analyzed from 230 HCV-1b patients to confirm the result. The clinical backgrounds of the additional 230 patients are shown in Table 2 (Group 2).

Table 2 Patient characteristics in Group 2

	HCC ($N = 65$)	Non-HCC ($N = 165$)	p value
Observation period (years)			
Sex (male/female)	42/23	76/89	0.018
Age (years)	68.2 ± 9.2	62.4 ± 11.7	<0.001
AST (IU/L)	66 ± 35	41 ± 21	<0.001
ALT (IU/L)	67 ± 47	44 ± 47	<0.001
Platelets (10^{-4}/mm^3)	11.3 ± 5.8	15.3 ± 6.2	<0.001
Albumin (g/dL)	3.6 ± 0.5	4.4 ± 2.9	0.025
γGTP (IU/L)	59 ± 53	38 ± 40	0.001
T.Chol (mg/dL)	153 ± 30	165 ± 31	0.004
Alpha-fetoprotein (ng/mL)	302 ± 1,670	10 ± 25	0.025
HCV RNA concentration (kIU/mL)	5,400 ± 13,574	7,990 ± 8,512	0.104

All patients were positive for HCV RNA. Between the HCC (65 patients) and non-HCC (165 patients) groups, HCC patients were older ($p < 0.001$) and more frequently tended to be males ($p = 0.018$). Moreover, AST, ALT, γGTP, and AFP were significantly higher, and platelets, albumin, and cholesterol were significantly lower in the HCC group. Different predicted amino acids in the core and NS5A regions, between the two groups, are demonstrated in Fig. 2a. The ratio of the core a.a. 70Q (glutamine) or H (histidine) to R (arginine) was significantly higher with the existence of HCC as demonstrated in Fig. 2a (left panel). On the other hand, evident correlations were not confirmed between mutations in NS5A and disease progression (Fig. 2a, right panel). The ratio of Q or H

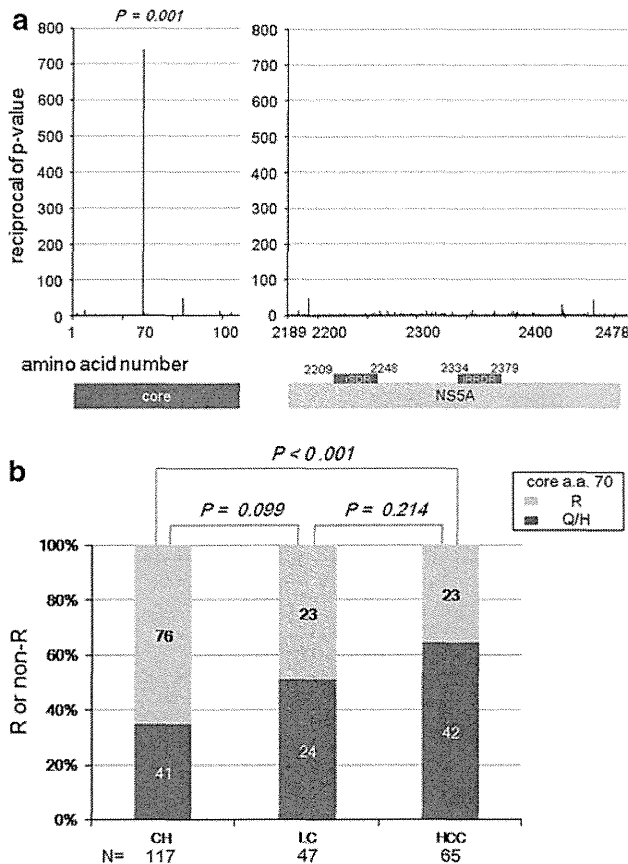

Fig. 2 Core and NS5A sequences in additional patients were studied. **a** Using sera from 230 additional patients at the time of diagnosis, amino acid usage was compared between HCC and non-HCC patients for the part of core and the NS5A region, and this difference is shown as the *bar height* expressed as reciprocal *p* values. **b** In 230 patients, the association between polymorphisms of core a.a. 70 and the state of liver disease (chronic hepatitis, LC, or HCC) is shown

to R progressively increased in patients in the three major groups of disease activity: chronic hepatitis, cirrhosis, and HCC (Fig. 2b). The association between the disease progression and core a.a. 70 polymorphism also was observed irrespective of IFN-based therapy (data not shown).

Changes in core a.a. 70 over time in patients with and without HCC

We then examined changes in core a.a. 70 over time in association with disease progression (Fig. 3). For this analysis, 55 patients from Group 2, for whom sera from two distant time points were available, were added to the 43 patients in Group 1 and a total of 98 patients were enrolled. When they were classified into two groups according to later HCC onset, the mean observation period was comparable between the groups, 10.4 years for the HCC group and 12 years for the non-HCC group. The occurrence of core a.a. 70Q was 61% (22/36) at the time of

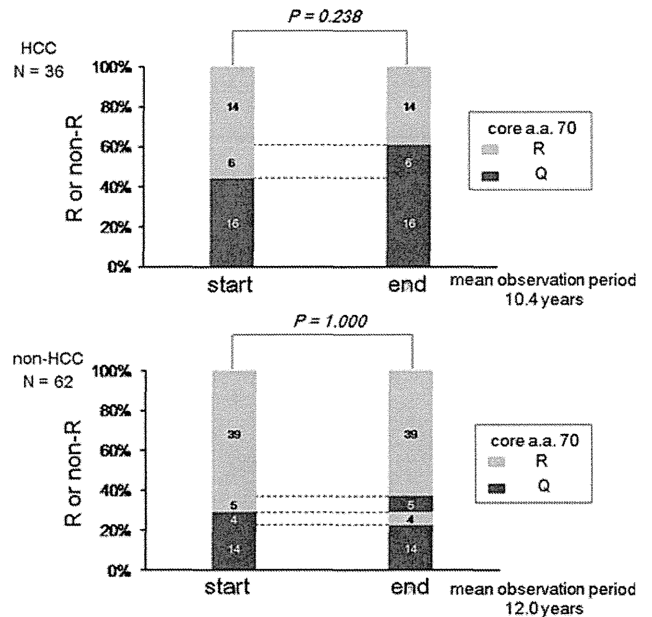

Fig. 3 Changes in core a.a. 70 over time were studied. In 98 patients available for the analysis of 2, sufficiently distant time points, amino acid changes of core a.a. 70 were investigated over time in each patient group. Patients with later HCC development (*upper panel*). Non-HCC patients (*lower panel*)

HCC onset in the HCC group (Fig. 3, upper panel), but 31% (19/62) for the non-HCC group (Fig. 3, lower panel). In contrast, it was 44% (16/36) at the start of observation in the HCC group (Fig. 3, upper panel) and 29% (18/62) for the non-HCC group (Fig. 3, lower panel). Regarding the core a.a. 70 changes over time, R was dominant throughout the observation period in the non-HCC group (71% at the start and 69% at the end), while the dominant amino acid changed from R (56%) to Q (61%) in the HCC group, so that the core a.a. 70 residues of 17% of the HCC patients changed from R to Q during the course of HCC development. In other words, 6 of 22 patients with 70Q at the onset of HCC had 70R originally (6/22, 27%), while 0 of 14 (0%) with 70R at the most recent observation time had 70Q at the beginning. There were no patients with core a.a. 70H throughout the observation period in this population. The result demonstrates that the relationship between 70Q and HCC development is significant at the time of HCC development. At the start of observation, there was also a tendency that the patients with 70Q compared with 70R develop HCC. However, this difference did not reach statistical significance as shown in Supplementary Fig. 1.

Core a.a. 70 changes over time and their association with disease progression

These 98 patients were classified into four groups according to the pattern of core a.a. 70 change (Table 3) and their

Table 3 Progression of liver disease in 98 patients categorized by core a.a. 70 changes over time

	R → R ($N = 53$)			Q → R ($N = 4$)		
	Start	End	*p* value	Start	End	*p* value
HCC rate (HCC/non-HCC)	–	26.4% (14/39)	–	–	0% (0/4)	–
Sex (male/female)	–	25/28	–	–	4/0	–
Observation period (years)	–	11.1 ± 3.4	–	–	12.9 ± 3.5	–
Age (years)	51.3 ± 11.7	62.4 ± 12.1	<0.001	48.0 ± 11.6	61.0 ± 9.1	0.128
AST (IU/L)	68 ± 73	48 ± 26	0.066	56 ± 32	83 ± 61	0.456
ALT (IU/L)	80 ± 71	48 ± 35	0.003	114 ± 71	96 ± 42	0.678
Platelets (10^{-4}/mm^3)	17.0 ± 5.8	15.0 ± 6.7	0.104	21.3 ± 3.9	17.2 ± 5.2	0.251
Albumin (g/dL)	4.1 ± 0.4	3.9 ± 0.6	0.225	4.4 ± 0.4	4.3 ± 0.4	0.647
γGTP (IU/L)	56 ± 51	38 ± 40	0.052	95 ± 51	61 ± 46	0.371
T.Chol (mg/dL)	172 ± 36	158 ± 33	0.032	152 ± 14	175 ± 32	0.222
Alpha-fetoprotein (ng/mL)	8.3 ± 9.5	12.5 ± 22.1	0.202	6.0 ± 6.0	5.2 ± 2.2	0.816
HCV RNA concentration (kIU/mL)	4,634 ± 8,509	7,070 ± 14,159	0.291	5,798 ± 7,970	13,676 ± 1,881	0.162
	R → Q ($N = 11$)			Q → Q ($N = 30$)		
	Start	End	*p* value	Start	End	*p* value
HCC rate (HCC/non-HCC)		54.5% (6/5)			53.3% (16/14)	
Sex (male/female)		6/5			13/17	
Observation period (years)		13.7 ± 1.65			10.8 ± 3.5	
Age (years)	56.4 ± 7.5	69.3 ± 9.3	0.002	54.6 ± 8.5	64.9 ± 9.9	<0.001
AST (IU/L)	62 ± 47	46 ± 12	0.285	79 ± 51	60 ± 31	0.087
ALT (IU/L)	100 ± 69	37 ± 15	0.008	95 ± 58	59 ± 36	0.006
Platelets (10^{-4}/mm^3)	17.7 ± 3.9	11.8 ± 4.8	0.005	16.3 ± 6.5	11.9 ± 5.6	0.007
Albumin (g/dL)	4.2 ± 0.2	3.8 ± 0.4	0.005	4.1 ± 0.3	3.8 ± 0.5	0.009
γGTP (IU/L)	73 ± 53	33 ± 16	0.025	101 ± 55	71 ± 65	0.065
T.Chol (mg/dL)	157 ± 21	144 ± 27	0.245	163 ± 28	150 ± 32	0.100
Alpha-fetoprotein (ng/mL)	7.1 ± 4.3	97.8 ± 63.6	0.267	20.8 ± 50.0	35.1 ± 54.7	0.295
HCV RNA concentration (kIU/mL)	2,415 ± 3,163	2,349 ± 1,851	0.957	2,869 ± 3,984	3,229 ± 4,026	0.731

clinical characteristics were investigated. Significant decreases of platelets ($p = 0.007$) and albumin ($p = 0.009$) were observed in the Q unchanged group during the observation period, but not in the R unchanged group ($p = 0.104$ and 0.225, respectively). Because platelets and albumin are markers of liver disease progression, it was considered that the Q unchanged group progressed rapidly with frequent HCC occurrence (53%, 16/30) while the R unchanged group showed stable disease with less frequent HCC occurrence (26%, 14/53). In contrast, the R to Q group showed progressive disease ($p = 0.005$ and 0.005, respectively) similar to the Q unchanged group, while the Q to R group showed stable disease similar to the R unchanged group ($p = 0.251$ and 0.647, respectively), demonstrating that amino acid changes of core a.a. 70 were significantly associated with disease progression.

The IL28B SNP and its association with core a.a. 70 and disease progression

Next, the association between the state of liver disease and IL28B SNP was analyzed for a total of 228 patients through the analysis of the rs8099917 locus. Among them, 162 patients (71%) had the major homozygous TT alleles, while 66 patients (29%) had the minor homozygous or heterozygous alleles (GG/TG). Although some patients had a history of IFN therapy, all patients were positive for HCV RNA at the time of study. The clinical characteristics related to disease progression were compared, as shown in Table 4. Each group consisted of patients with similar distributions of age and sex. Though most clinical factors showed no evident differences in these groups, γGTP was high ($p = 0.020$) and HCV RNA concentration was apt to be low ($p = 0.085$) in TG/GG group. Moreover, the ratio