lamivudine resistance.

FIGURE LEGENDS

Fig. 1 Clinical course of HBV-infected patients treated with lamivudine. "X" indicates the emergence of lamivudine resistance. Asterisks indicate patients selected for HBV nucleotide sequence analysis.

Fig. 2 Codon differences in each viral ORF between lamivudine sensitive and resistant groups. The differences are indicated by a vertical line representing the inverse of the *P* value. (a) pre-S1/S2, and S ORF, (b) polymerase ORF, (c) precore and core ORFs, (d) X ORF.

Although a few genotype A and B viruses were included in the analysis, for convenience, the sequences are numbered according to the system for genotype C HBV. Viral amino acids are numbered according to the adopted standardized numbering system for the HBV polymerase [Stuyver et al., 2001].

Fig. 3 Kaplan-Meier analysis of relationship of substitutions with the emergence of lamivudine resistance.

The sequences are numbered according to the system for genotype C HBV.

Fig. 4 Amino acid sequence alignment of the pre-S1, pre-S2, and polymerase ORFs associated with the lamivudine resistance. Duration of the LAM administration indicates the period for HBV to become LAM resistant in the resistant group, while it indicates the overall observation period in the non-resistant group. Above the sequences observed in each patient, representative viral sequences of genotype A, B, and C around those areas also are shown to indicate genotype-specific viral amino acids.

(a) Part of pre-S1 ORF.

(b) Part of pre-S2 ORF.
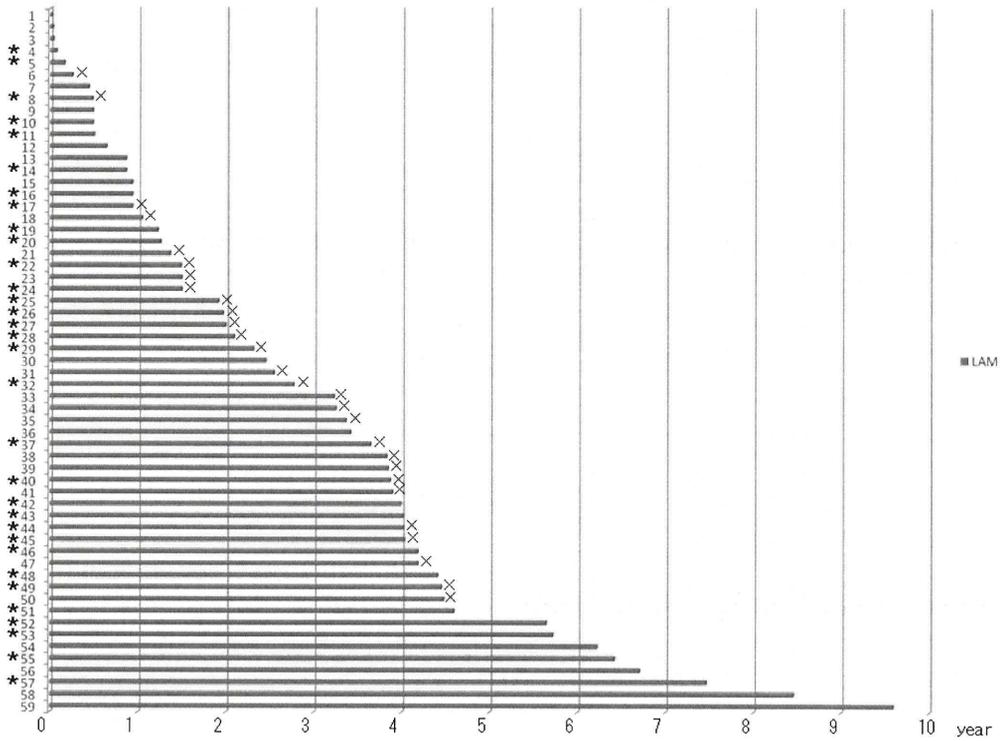
(c) Part of polymerase ORF.

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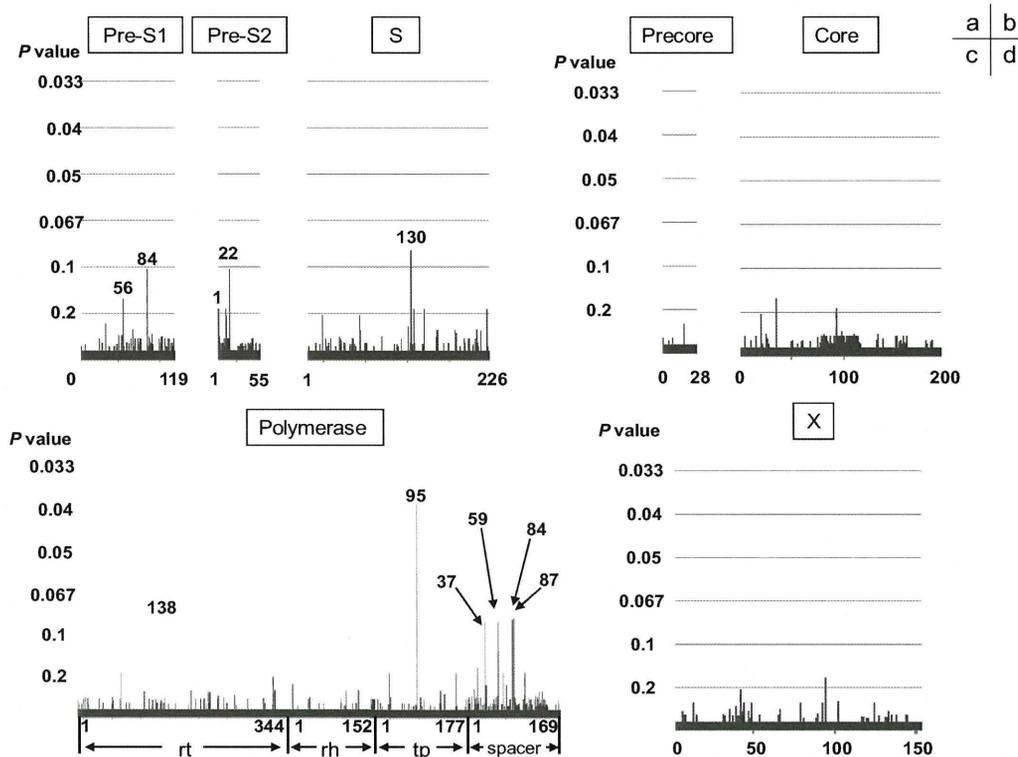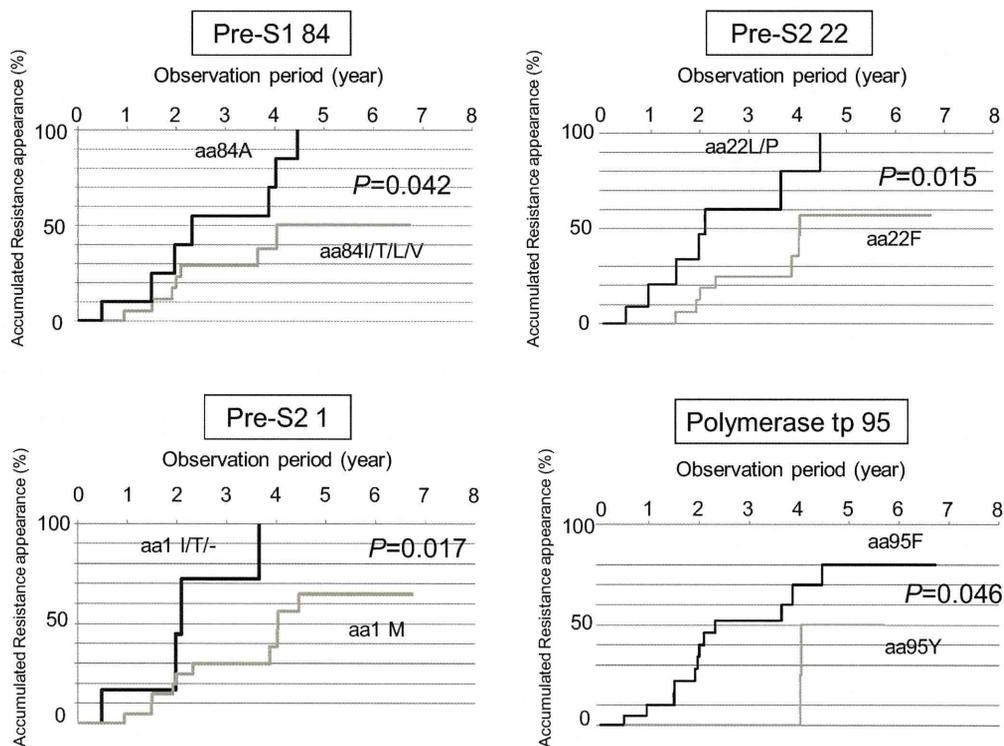
FIGURE

Fig.1

Sueki et al.

Fig.2

Fig. 3

Sueki et al.

Fig. 4

Reference sequences (aa positions: Pre-S1 = 84, Pre-S2 = 1 / 22, Polymerase tp = 95)

genotype	accession	Q	G	A	L	T	M	Q	W	N	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	G	L	Y	F	P	Q	Q	F	V	G
A	AY233290	-	-	I	-	A	-	-	-	-	-	-	A	-	-	-	-	-	-	-	Q	-	-	-	-	R	-	-	-	-	-	-	-	-	-
A	AY128092	-	-	I	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	Q	-	-	-	-	R	-	-	L	-	-	-	-	-	-
A	AB194950	-	-	T	-	-	I	K	-	-	-	P	A	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	AB033554	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	-	A	-	-	-	-	K	-	-	-	-
B	AB031267	-	-	I	-	-	-	-	-	-	-	A	-	-	-	-	T	-	Q	-	-	-	-	-	-	A	-	-	L	-	K	-	-	-	-
B	AB219429	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	-	A	-	-	-	-	K	-	-	-	-
C	AB112063	-	-	I	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	Y	-	-
C	AF241410	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-
C	X52939	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	Y	V	-

Patient sequences

group	Pt No.	Duration of LAM administration (Months)	Q	G	A	L	T	M	Q	W	N	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	G	L	Y	F	P	Q	Q	F	V	G
Lamivudine non-resistant	1	0.9	-	-	I	-	-	-	-	-	-	-	-	-	-	Y	-	K	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	Y	-	-
	2	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	5.8	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	5	10.4	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-
	6	11.2	-	-	-	-	-	I	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	S	-	-	L	-	-	-	Y	-	-	
	7	14.7	-	-	I	-	-	-	/	/	H	-	-	A	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	8	15.1	-	-	T	-	-	I	H	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	Y	-	-	
	9	33.3	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	47.9	-	-	I	-	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	-	A	-	-	L	-	K	-	-	-	-	
	11	48.1	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	12	50.2	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	13	52.9	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	14	55.1	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	15	67.7	-	-	L	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-
	16	68.6	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	17	77.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18	89.6	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
Lamivudine resistant	1	5.8	-	-	-	-	-	I	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	S	-	-	L	-	S	-	-	L	-
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	10	43.8	-	-	T	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	Y	L	-
	11	46.4	-	-	L	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12	48.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	13	48.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	53.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	S	-	L	-	S	-	Y	L	-

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TABLES

TABLE I. Baseline Clinical Charactaristics

Clinical factor	LAM non-resistant n=18	LAM resistant n=14	P-value
Demographic Characteristics			
Age, years [1]	53.9 (±13.2)	55.6 (±7.7)	0.662
Sex, no. male/female	13/5	9/5	0.712
CH/LC	5/13	3/11	0.261
HCC (+/-)	11/7	7/7	0.721
Biochemical Characteristics			
Alanine aminotransferase level, IU/l [2]	91 (13-1780)	70.5 (17-2739)	0.805
Platelets count, ×10^{-4}/ml [1]	11.8 (±5.8)	12.1 (±5.3)	0.900
Total bilirubin, mg/dl [2]	0.95 (0.3-19.7)	1.1 (0.4-5.0)	0.634
Albumin, g/dl [2]	3.2 (±0.6)	3.5 (±0.9)	0.270
ChE, IU/l [1]	196.4 (±105.0)	207.1 (±92.4)	0.566
T-chol, mg/dl [1]	156.1 (±39.6)	163.6 (±37.4)	0.590
Prothrombin time, % [1]	64.5 (±16.1)	69.9 (±15.9)	0.358
α-fetoprotein, ng/ml [2]	16.1 (1.9-35194)	11.5 (1.6-611.5)	0.506
Virological Characteristics			
HBV Genotype (A/B/C)	1/1/16	0/1/13	0.662
HBV DNA level Log10copies/ml [1]	5.80 (±1.45)	6.61 (±0.97)	0.078
HBeAg, positive/negative	6/12	8/6	0.283
Precore mutation ratio (%)	38.9	28.6	0.712
Core promotor mutation	4/14	3/11	0.880
Duration of LAM administration until HBV PCR negative (Month) [2]	2.1 (0.4-7.7)	3.7 (1.4-69.0)	0.024

[1] average (±SD) student's t test

[2] median (range) Mann-Whitney U test

TABLE II. Amino acid substitution number in each region of the HBV ganome

HBV protein	LAM non-resisitant	LAM resisitant	P-value
Pre-S1, median (range)	2.0 (0-6)	2.0 (0-11)	0.460
Pre-S2, median (range)	0 (0-4)	2.0 (0-8)	0.060
S, median (range)	3.0 (1-9)	4.0 (2-8)	0.372
Pre-S1/Pre-S2/S, median (range)	7.0 (3-15)	7.0 (4-23)	0.206
Polymerase, median (range)	15.5 (9-30)	17.0 (8-35)	0.448
Precore, median (range)	0.5 (0-1)	0 (0-1)	0.144
Core, median (range)	3.5 (0-9)	5.0 (0-35)	0.859
X, median (range)	4.0 (1-7)	3.0 (1-9)	0.706

Mann-Whitney U test

TABLE III. Baseline Clinical Charactaristics classified by the mutation at codon 84 in pre-S1

Clinical factor	Pre-S1 84I/T/L/V n=20	Pre-S1 84A n=12	*P*-value
HBV DNA level Log10 copies/ml[*1]	5.75 (±1.38)	6.83 (±0.86)	0.022
Duration of LAM administration until HBV PCR negative (Months)[*2]	2.1 (0.4-7.6)	4.0 (1.9-69.0)	0.005

[*1] average (±SD) student's t test

[*2] median (range) Mann-Whitney U test

TABLE IV. Baseline Clinical Charactaristics classified by the mutation at codon 22 in pre-S2

Clinical factor	Pre-S2 22F n=21	Pre-S2 22L/P n=11	*P*-value
Age, years [*1]	50.7 (±9.6)	62.3 (±9.7)	0.003

[*1] average (±SD) student's t test

TABLE V . Baseline Clinical Charactaristics classified by the mutation at tp aa95 in polymerase

Clinical factor	Polymerase tp 95Y n=21	Polymerase tp 95F n=11	*P*-value
Alanine aminotransferase level, IU/l [*1]	52 (13-810)	133 (23-2739)	0.0495
Total bilirubin, mg/dl [*1]	0.9 (0.3-5.0)	1.2 (0.5-19.7)	0.049
α-fetoprotein, ng/ml [*1]	8 (1.6-35194)	81 (4-214.3)	0.034

[*2] median (range) Mann-Whitney U test

TABLE VI. Factors asociated with LAM resistance identified by multivariate analysis

Variable	Hazard Ratio (95% CI)	*P*-value
Duration of LAM administration until HBV PCR negative	1.1 (1.0 - 1.1)	0.700
Albumin	1.2 (0.6 - 2.4)	0.682
Pre-S1 84	8.5 (1.5 - 49.3)	0.017
Pre-S2 1	12.4 (1.1 - 139.7)	0.041
Pre-S2 22	1.2 (0.2 - 5.9)	0.833
Polymerase tp 95	0.3 (0.4 - 32.2)	0.275

CI = confidence interval
Cox proportional-hazards regression

COMPREHENSIVE ANALYSIS FOR VIRAL ELEMENTS AND IL28B POLYMORPHISMS IN RESPONSE TO PEGINTERFERON PLUS RIBAVIRIN THERAPY IN HCV-1B INFECTION

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FOOTNOTES

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ABSTRACT

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 sequence study in consecutive 103 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 SNPs analyses (rs8099917). In each comparison of the patients between with and without rapid viral response (RVR), non-early viral response (nEVR), sustained virological response (SVR) or relapse, following regions were extracted as most significantly associated with the different responses respectively: 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 sensitivity-determining region (ISDR, NS5A aa.2209-2248) and the IFN/RBV resistance-determining region (IRRDR, NS5A aa.2339-2379). In a multivariate analysis, the IL28B SNP (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 importantly, NS5A aa.2340-2382 have a tremendous influence on the treatment response in association with the viral kinetics, resulting in significantly different outcomes in chronic HCV-1b infection.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide, causing chronic liver disease that may progress to hepatocellular carcinoma (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 (sustained virological response: SVR) (2). Recently, direct acting antiviral agents (DAAs) are under development, and Telaprevir and Boceprevir have just been included in the HCV treatment regimens in the Unites States. However, it has gradually became loomed that HCVs showing resistance to the 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 comprehensive characterization of viral and host determinants for the PEG-IFN/RBV therapy and to determine the most appropriate candidates for the new therapies.

In interferon-based therapy, the treatment response is influenced by multiple host and viral factors. Among the host factors, younger age, milder fibrosis stage, being non-obese (4), 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 1/4 show favorable responses (9). On the other hand, the contribution of other viral factors, polymorphisms in several restricted viral genetic regions, has long been debated in terms of their association with treatment responses. HCV genetic elements, including the interferon sensitivity-determining region (ISDR) in NS5A (10, 11), PKR-binding domain (PKR-BD) in NS5A(12, 13), V3 region in NS5A(14), IFN/RBV resistance-determining region (IRRDR) in NS5A(15), PKR-eIF2 phosphorylation homology domain (PePHD) of E2 (16), C-terminal region of NS5A (G404S, and E442G) (17), F415Y in NS5B (18), polymerase motif in NS5B(19), and aa.70 and 91 in core (20) have been investigated for their correlation with the clinical outcome of IFN-based therapy or ribavirin in genotype 1 infection. Complete open reading frame (ORF) analyses in PEG-IFN/RBV therapy also revealed the link between the treatment response at day 28, or the treatment outcome with viral diversities in several viral genomic regions in genotype 1 infection (21, 22). Importantly, most recent studies reported 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 the treatment response has not been presented clearly yet, especially along with IL28B SNPs. Moreover, inconsistent results that have been reported 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 the 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 the 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 non-early viral response (nEVR), respectively (24). In the present study, we undertook more comprehensive and detailed analysis to disclose the impact of HCV ORF on determining the early viral response, the final outcome and the 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 analyzed retrospectively consecutive patients with chronic HCV-1b infection treated with combination therapy of PEG-IFN/RBV at the Yamanashi University Hospital between December 2004 and July 2008. Eligible patients were 18 to 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 level, available pretreatment serum sample conserved for HCV sequence analysis. Patients were excluded if they had decompensated liver cirrhosis or hepatocellular carcinoma. Consequently, 103 patients were eligible for this study. In addition to those 103 patients, 30 consecutive patients who received the standard length of PEG-IFN/RBV at the Yamanashi University Hospital for August 2008 to April 2011 and were meeting the above criteria were also included in the study to perform univariate and multivariate analysis for SVR and relapse. The study was approved by the ethics committees of 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, Labour, and Welfare, Japan. Patients were treated with PEG-IFN alpha-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 (non-EVR), 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 patients' characteristics were analyzed: age, sex, stage of fibrosis on liver

biopsy, body mass index, ALT, hemoglobin, γ-GTP, total cholesterol, albumin, platelet counts, alpha

fetoprotein, serum HCV RNA, PEG-IFN dose, RBV dose. Liver-biopsy specimens were evaluated

blindly by an independent interpreter. HCV RNA was determined by PCR (Amplicor HCV RNA kit,

version 2.0, Roche Diagnostics).

Viral Response

Patients were subdivided into 4 groups according to the initial response at week 12. Each

group was defined as follows: Rapid viral response (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 non-EVR (nEVR;

HCV RNA <2 log drop at week 12). Sustained virological response (SVR) was defined as

undetectable HCV RNA 24 weeks after the completion of therapy. Viral relapse after achievement

of end of treatment response (ETR) were also evaluated. In some analysis, cEVR was further

divided into 2 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, cDNA synthesis, and nested-PCR was performed using patients' serum

collected before starting the therapy, as described previously (25). The full-length HCV genome was

amplified by nested PCR with 20 partially overlapping primer sets. Both strands of the PCR products

were cycle-sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,

Tokyo, Japan) according to the manufacturer's instructions using an M13 forward and reverse

primers. The products were sequenced by an automated DNA sequencer 3130 (Applied Biosystems).

The nucleotide and predicted amino acid 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 the treatment response. Briefly, the total number of amino acid substitutions compared to the consensus sequence within a given number of consecutive amino acids (window) was counted at each amino acid position in each HCV sequence. The distribution of amino acid 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 U test for each amino acid window. In each comparison, the length of peptide window was changed from 1 to 100 amino acids to search for those regions. Consequently, about 300,000 windows (100 width x 3010 amino acids) were analyzed for each HCV amino acid sequence. To visualize the result, windows showing significantly low p-values were colored in red and non-significant p-values were colored in green to generate a "heat map" appearance using Microsoft Excel, while 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 with a model 7500 (Applied Biosystems) using FAM-labeled SNP primer for the locus rs8099917 (purchased from Applied Biosystems).

Statistical Analysis

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

Variables with p <0.05 in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors with the odds ratios as well as 95% confidence

intervals. All p values of <0.05 by the two-tailed test were considered significant.

RESULTS

Patients Characteristics

Clinical background factors of the 103 patients are shown in Table 1. The 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 3 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.

In Supplementary Table 1, clinical background factors of the 30 patients who were additionally included for the univariate and multivariate analysis for SVR and relapse receiving the standard period of PEG-IFN/RBV therapy are also shown.

IL28B SNPs and Their Relationship to Viral Diversity

To evaluate the contribution of IL28B polymorphism in the 103 study group, we investigated the rs8099917 SNPs in 89 patients available for the analysis. As shown in Table 2, the polymorphism was closely related to the viral response at weeks 12. In order to clarify the relationship between the viral diversity and the 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). As shown in Supplementary Fig.1, IL28B SNP was significantly correlated with the amino acid 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.

HCV Sequences Related to RVR and nEVR

To characterize the HCV sequences related to the RVR and nEVR, we determined the full dominant HCV ORF sequences by direct sequencing and searched for polymorphic amino acid 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 amino acid position discriminating the two groups (p=7.0E-8,

Fig. 1b). In this comparison of the nEVR versus the reminder, a sliding window analysis also

extracted regions around aa.70 to be the most significantly different (data not shown).

HCV Sequences Related to the 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 amino

acid, various differences were observed 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 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 the SVR patients and the

non-SVR patients among 57 patients with standard therapy achieving ETR (40 non-relapsers 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 most strongly related to

relapse (p=1.1E-05, Fig. 1d).

Univariate 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). Since all the RVR patients