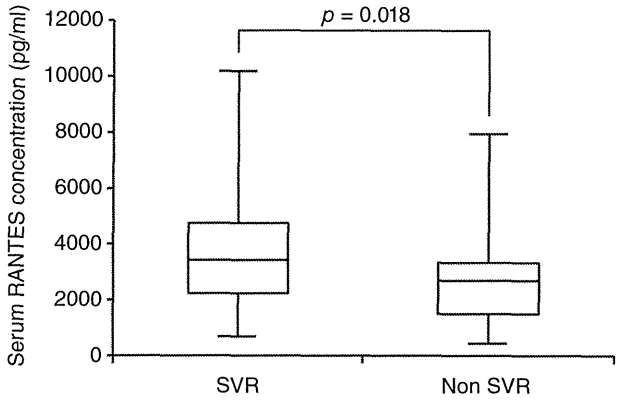


Figure 1 Difference in serum *RANTES* levels between the sustained virological response (SVR) group and the non-SVR group. Ninety-six patients who underwent the standard therapy for 48 weeks were analyzed for serum *RANTES* level using the bead array method. They were divided into the SVR ($n = 57$)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

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

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

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

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

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

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

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

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

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

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

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

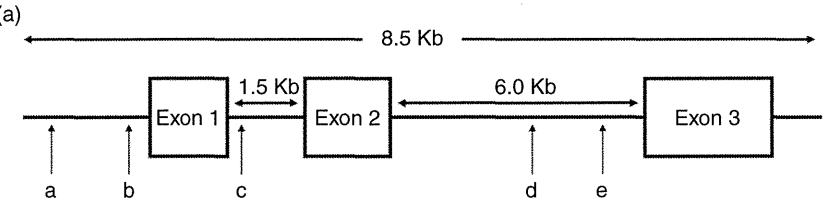
after commencement of therapy) was also significantly correlated with high pretreatment serum *RANTES* level ($P = 0.015$, data not shown). Moreover, we could also show that high serum *RANTES* levels correlated with the clinical background factors low alanine aminotransferase values and high platelet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

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

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

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

(a)



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

(b)

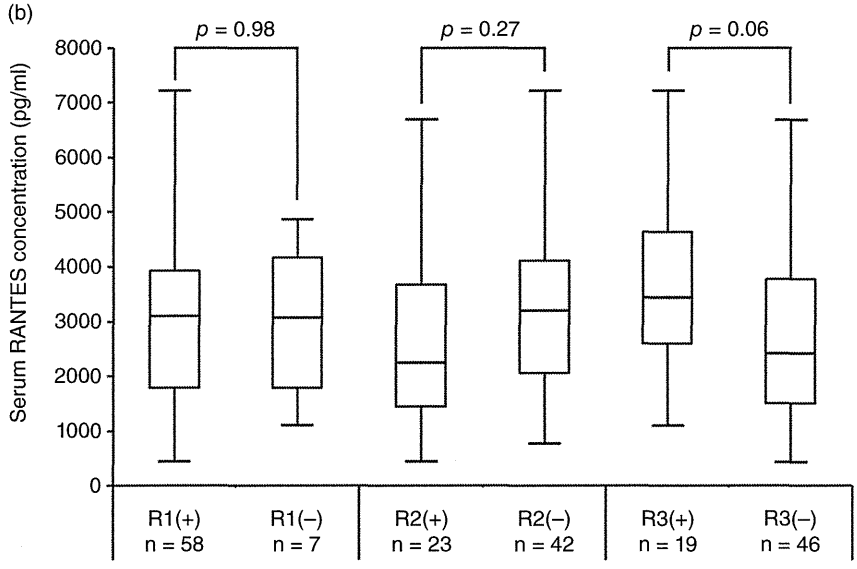


Figure 2 *RANTES* haplotypes and serum *RANTES* level. (a) *RANTES* haplotypes in the patients studied. The human *RANTES* gene spans 8.5 kb on chromosome 17q11-q12 and has the characteristic three exon and two intron organization of the CC chemokine family.[22] Exons are shown as open boxes while introns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

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

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

ACKNOWLEDGMENTS

THIS WORK WAS supported by Grants-in-Aid from the Scientific Research Fund of the Ministry of Education, Science, Sports and Culture (21590836, 21590837, 23390195); and a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan (H22-kanen-006).

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Journal of Medical Virology 84:1360–1368 (2012)

Correlation Between Pretreatment Viral Sequences and the Emergence of Lamivudine Resistance in Hepatitis B Virus Infection

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The emergence of amino acid or nucleotide substitutions leads to lamivudine resistance in hepatitis B virus (HBV) infected patients. The aim of this study was to investigate whether viral sequences help predict the emergence of lamivudine resistance. The study subjects comprised 59 consecutive patients infected with HBV treated with daily therapy of 100 mg lamivudine. Among those, 32 patients with adequate pretreatment serum preservation were investigated for the correlation between viral amino acid substitutions and the appearance of lamivudine resistance with consideration of clinical background by determining dominant HBV full open reading frames. Viral resistance to lamivudine emerged in 28 of 59 patients (47%) in a median period of 2.45 years. Sequence comparisons of HBV genomes between patients who later developed lamivudine resistance and patients who did not revealed the existence of significant differences between the two groups in the pre-S1 84 ($P = 0.042$), pre-S2 1 ($P = 0.017$) and 22 ($P = 0.015$), and polymerase tp 95 ($P = 0.046$), judged by a log-rank test. Viral sequence analyses revealed the presence of amino acid substitutions in HBV pre-S1 and pre-S2 that may be associated with the emergence of lamivudine resistance during chronic HBV infection. ***J. Med. Virol. 84: 1360–1368, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV; lamivudine resistance pre-S substitution; ORF sequence analysis

INTRODUCTION

Hepatitis B virus (HBV) infects persistently more than 350 million people worldwide [Liang, 2009], and increases their risk of developing liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) over the typically long disease course. High serum virus titers have been shown recently to promote significantly disease progression, as well as the development of HCC [Chen et al., 2006b; Iloeje et al., 2006]. Therefore, effective suppression of the serum viral load by an antiviral agent might inhibit disease progression [Lim et al., 2009].

Lamivudine was introduced clinically as one of the first-generation nucleoside analogs to inhibit HBV replication [Liaw et al., 2004]. Lamivudine is safe [Lok et al., 2003], effectively decreases serum viral load, improves alanine aminotransferase (ALT) levels and liver fibrosis [Leung, 2000; Villeneuve et al., 2000], and enhances hepatitis B e antigen (HBeAg) seroconversion rates [Liaw et al., 2000; Leung et al., 2001; Chen et al., 2006a], which lead to the suppression of HCC development [Liaw et al., 2004]. In contrast, prolonged use of lamivudine may lead to the emergence of drug-resistant HBV mutants in a substantial percentage of patients. When resistance emerges, patients should be treated with a different nucleoside analog, which does not show cross-resistance, alone or in combination with lamivudine [Rizzetto et al., 2005; Carey and Harrison, 2009; Chen et al., 2009]. Newly introduced second-generation nucleoside analogs, such as entecavir and tenofovir, have been shown to be superior in suppressing viral load and preventing the emergence of drug-resistant viruses. However, because of its high economical efficacy compared to other, newer-generation

Ryota Sueki and Shinya Maekawa contributed equally to the work.

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Accepted 17 April 2012

DOI 10.1002/jmv.23314

Published online in Wiley Online Library (wileyonlinelibrary.com).

nucleoside analogs, the appropriate selection of patients suitable for lamivudine therapy by accurate prediction of the emergence of resistance would benefit economically challenged patients worldwide. On the other hand, prediction of the eventual emergence of resistance to lamivudine has been difficult.

Many previous studies have shown a correlation between lamivudine resistance and the HBV mutations that appear with viral acquisition of lamivudine resistance. These mutations lead to amino acid mutations in the HBV polymerase, including rt M204I/V in the C domain and rt V173L and rt L180M in the B domain [Ling et al., 1996; Tipples et al., 1996; Ghany and Doo, 2009]. However, it is not known whether any specific sequences of viral genomes not exposed to lamivudine might predict the development of resistance following the commencement of lamivudine treatment. Typically, the emergence of lamivudine resistance has been predicted by pretreatment or in-treatment clinical variables, such as HBeAg positivity, higher baseline HBV DNA levels, female sex, lower ALT levels and a poor early viral response to lamivudine [Andersson and Chung, 2009; Zhou et al., 2009].

The present study was conducted to clarify and characterize pretreatment HBV sequences associated with the subsequent emergence of lamivudine resistance by determining the complete sequences of HBV ORFs by direct nucleotide sequencing, using patients' sera as the source of HBV DNA.

PATIENTS AND METHODS

Patients

Fifty-nine patients with chronic hepatitis or liver cirrhosis, infected with HBV and who underwent lamivudine therapy at Yamanashi University Hospital from May 2001 to June 2010 were enrolled initially in the study. All patients received lamivudine orally, initially at a dosage of 100 mg per day. Although all 59 patients responded initially to lamivudine therapy and HBV DNA became undetectable, lamivudine resistance was diagnosed in 28 patients (47%) because HBV DNA reappeared during the observation period, while in the other 31 patients it did not (Fig. 1). Because pretreatment serum from 32 of the patients had been preserved adequately for determination of the complete HBV nucleotide sequence, the final analysis was based on these 32 patients. All patients included were positive for hepatitis B surface antigen (HBsAg) and were tested for HBV DNA by the Quantiplex HBV DNA assay (Bayer Diagnostics, Emeryville, CA), transcription-mediated amplification assay (Chugai Diagnostics Science Co., Ltd, Tokyo, Japan), or COBAS® Amplicor HBV Monitor Test v2.0 (Roche Diagnostics, Indianapolis, IN). Patients with co-existing autoimmune hepatitis, alcoholic liver disease, drug-induced liver injury, chronic hepatitis C, or human immunodeficiency virus infection were excluded from the study. For patients with emerging drug resistance, adefovir dipivoxil was started at a dosage of

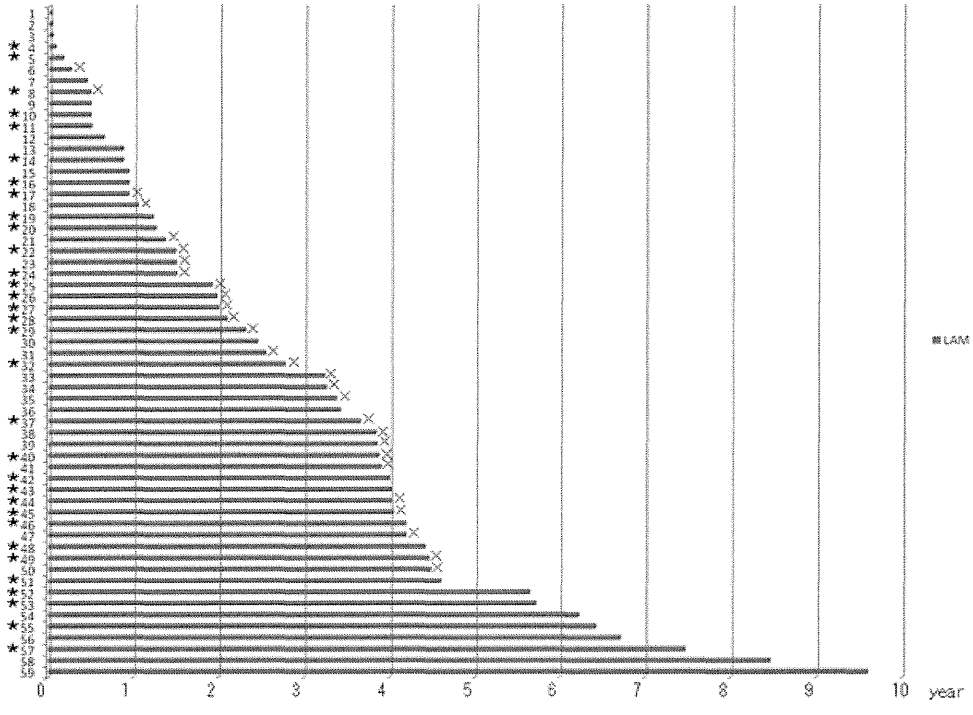

Fig. 1. Clinical course of HBV-infected patients treated with lamivudine. "X" indicates the emergence of lamivudine resistance. Asterisks indicate patients selected for HBV nucleotide sequence analysis.

10 mg per day, in addition to lamivudine, according to the guideline established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan. A signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital.

DNA Extraction, PCR, and Direct Sequencing

Full-length HBV DNA was amplified by two-step PCR from patients' sera and sequenced directly as described elsewhere [Sugauchi et al., 2001]. Sequence reads were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram. For ambiguous reads, only the dominant base was assigned after evaluation of all overlapping fragments. Full-length HBV genome sequences were assembled using this information and translated in silico and the ORFs of drug-resistant and sensitive genomes were compared.

Statistical Analysis

Statistical differences in the parameters, including all available demographic, biochemical, hematological, and virological statuses, were determined for the different patient groups by Student's *t*-test for numerical variables and Fisher's exact probability test for categorical variables. The odds ratio and 95% confidence intervals were calculated. *P* values of <0.05 by the two-tailed test were considered to indicate statistical significance. In order to evaluate the contribution of pretreatment viral amino acid sequences to the development of lamivudine resistance, Kaplan–Meier analysis and Cox proportional hazards model was performed.

RESULTS

Patients' Clinical Parameters

The pretreatment clinical and virological characteristics of the 32 patients, prior to starting lamivudine therapy, are shown in Table I, sorted according to the subsequent emergence of lamivudine resistance. Although HBV DNA became undetectable initially after the commencement of lamivudine therapy, drug resistance was diagnosed in 14 patients because of reappearance of HBV DNA during the observation period. No statistical difference was observed in age, sex, ALT, total bilirubin, choline esterase, total cholesterol, prothrombin time, platelets, α-fetoprotein, HBeAg/anti-HBe positivity, viral genotypes, liver disease (chronic hepatitis or liver cirrhosis), or pretreatment HBV DNA level. Genotype C was most prevalent in both groups (16/18 in the non-resistant group and 13/14 in the resistant group). In contrast, the time for HBV DNA to become undetectable was longer in this group, compared to that in the susceptible group ($P = 0.024$). Figure 1 shows the length of therapy for all 59 patients; "×" denotes the time of lamivudine resistance onset. Lamivudine resistance was diagnosed in 28 (47%) of 59 patients during a median observation period of 2.45 years.

Comparison of the HBV ORFs of the Lamivudine Resistant and Non-Resistant Groups

Full-length HBV genomic sequences from the 32 patients were determined by direct nucleotide

TABLE I. Baseline Clinical Characteristics

Clinical factor	LAM non-resistant (n = 18)	LAM resistant (n = 14)	*P*-value
Demographic characteristics			
Age, years[a]	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[b]	91 (13–1,780)	70.5 (17–2,739)	0.805
Platelets count, $\times 10^{-4}$/ml[a]	11.8 (±5.8)	12.1 (±5.3)	0.900
Total bilirubin, mg/dl[b]	0.95 (0.3–19.7)	1.1 (0.4–5.0)	0.634
Albumin, g/dl[b]	3.2 (±0.6)	3.5 (±0.9)	0.270
ChE, IU/L[a]	196.4 (±105.0)	207.1 (±92.4)	0.566
T-chol, mg/dl[a]	156.1 (±39.6)	163.6 (±37.4)	0.590
Prothrombin time, %[a]	64.5 (±16.1)	69.9 (±15.9)	0.358
α-fetoprotein, ng/ml[b]	16.1 (1.9–35,194)	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 Log_{10} copies/ml[a]	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)[b]	2.1 (0.4–7.7)	3.7 (1.4–69.0)	0.024

[a]Average (±SD) Student's *t*-test.
[b]Median (range) Mann–Whitney *U*-test.

TABLE II. Amino Acid Substitution Number in Each Region of the HBV Genome

HBV protein	LAM non-resistant	LAM resistant	*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.

sequencing. Conceptual in silico translation of the dominant pretreatment HBV DNA sequences allowed correlation of the amino acid substitution numbers in each viral ORF with the drug resistance of the virus. Table II shows that the number of amino acid changes in each viral ORF did not differ significantly between the two groups. However, although not significant, there was a tendency that amino acid substitutions in the pre-S2 region were more frequent in patients with eventual development of lamivudine resistance (the median numbers of non-synonymous mutations were 0 and 2 in the sensitive and resistant groups, respectively; $P = 0.06$).

Next, the amino acid residues differing between the two groups at each position in each viral protein were compared. The vertical line representing the *P*-value for each HBV ORF (Fig. 2a–d) indicates the difference between the two groups. Comparison of the two groups revealed amino acid differences at the residues indicated as follows: pre-S1 56, 84, pre-S2 1 and 22, S 130 (Fig. 2a), and polymerase rt 138, tp 95, spacer 37, 59, 84, and 87 (Fig. 2c). The polymerase was numbered according to the standardized numbering system [Stuyver et al., 2001]. The most significant difference was observed at polymerase tp 95 in the (Fig. 2c). In contrast, only a slight difference was observed in the precore and core and X (Fig. 2b,d). In particular, the changes at pre-S1 84 and polymerase spacer 87 were seen to be coexistent because the pre-S1 and polymerase ORFs overlap. In contrast, the

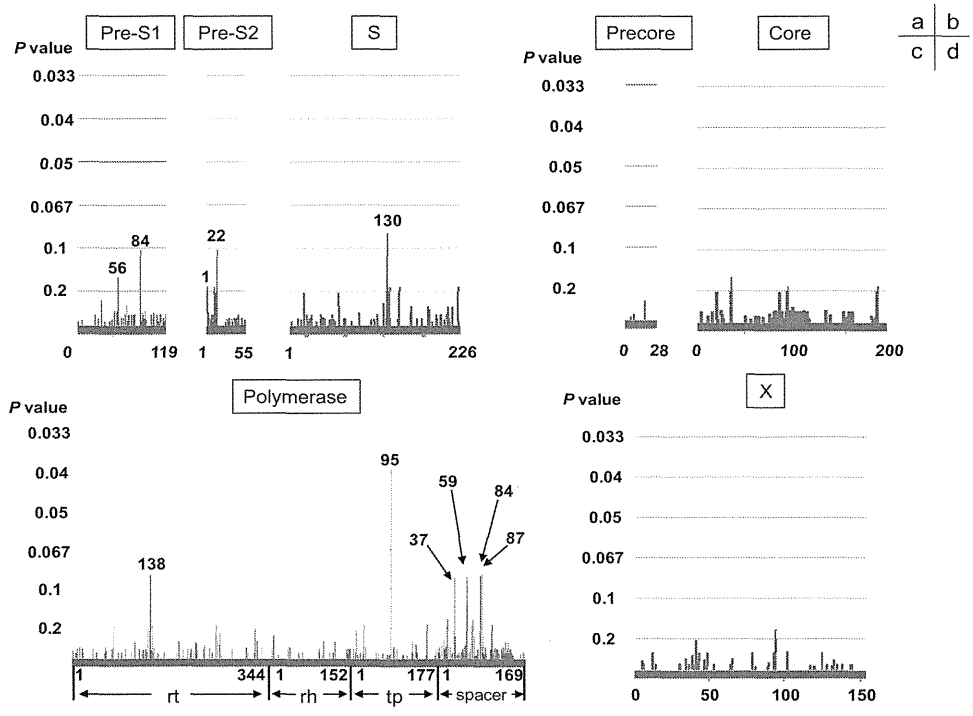

Fig. 2. Codon differences in each viral ORF between lamivudine sensitive and resistant groups. The differences are indicated by a vertical line representing the inverse of the *P*-value. (**a**) Pre-S1/S2, and S ORF, (**b**) polymerase ORF, (**c**) precore and core ORFs, (**d**) X ORF. Although a few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

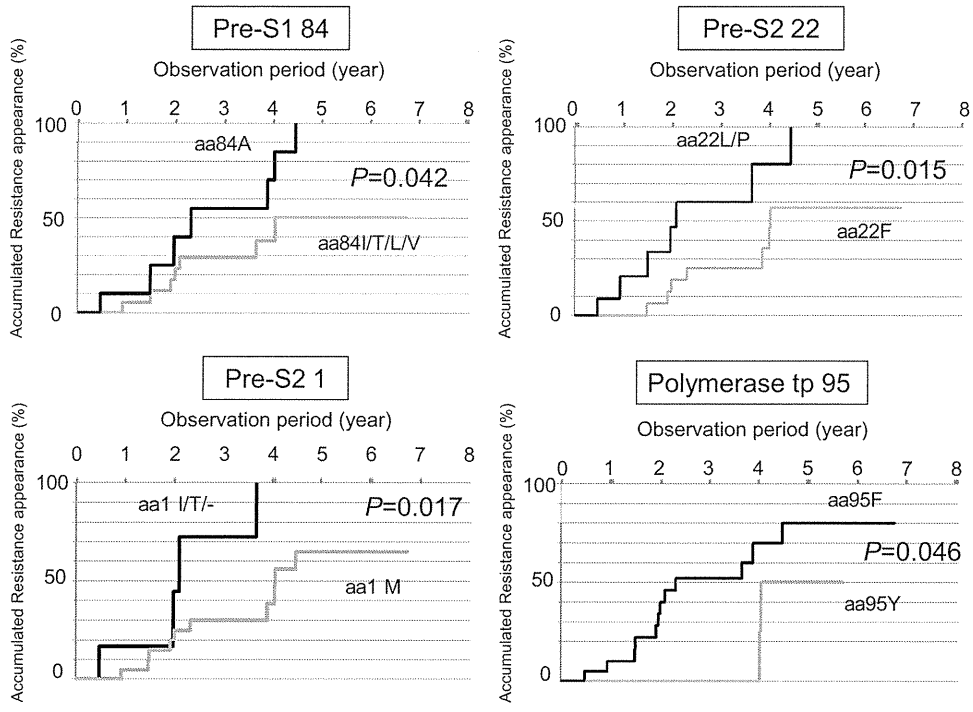

Fig. 3. Kaplan–Meier analysis of relationship of substitutions with the emergence of lamivudine resistance. The sequences are numbered according to the system for genotype C HBV.

coding changes at polymerase rt M204I/V, rt L180M, rt 173L, rt A181V, and rt N236T, and at S I195M, S W196L, and S W196 (stop), previously reported to result from mutations associated with viral acquisition of resistance to lamivudine or adefovir, were not observed prior to lamivudine therapy in any patients in this study.

Thereafter, Kaplan–Meier curves were constructed to understand better the potential influence of the amino acid changes, as revealed above, on the emergence of lamivudine resistance (Fig. 3, log-rank test). When the time of emergence of resistance was considered, a significant difference was observed with respect to the substitutions of pre-S1 84 ($P = 0.042$), pre-S2 1 ($P = 0.017$) and 22 ($P = 0.015$), and polymerase tp 95 ($P = 0.046$). Figure 4 shows a multiple alignment of amino acid sequences within the pre-S1, pre-S2, and polymerase ORFs.

Patient Characteristics Related to HBV ORF Substitutions

As shown in Table III, patients with isoleucine, threonine, leucine, or valine at pre-S1 84 had significantly lower HBV DNA levels, which became undetectable earlier than in patients with alanine at pre-S1 84. There were no evident differences between the characteristics of patients with and without substitutions at pre-S2 1 (data not shown). Patients with substitutions at pre-S2 22 were older ($P = 0.003$, Table IV). On the other hand, patients with substitutions in the polymerase tp 95 had increased total bilirubin ($P = 0.049$), ALT values ($P = 0.495$) and α-fetoprotein values ($P = 0.034$, Table V).

Multivariate Analysis to Reveal Independent Factors Predicting Lamivudine Resistance

In an attempt to define independent factors that might predict the emergence of lamivudine resistance, a multivariate analysis using the Cox proportional hazards model was performed. As shown in Table VI, the duration of lamivudine treatment until HBV DNA became undetectable, serum albumin level, pre-S1 84 substitutions or pre-S2 1 and 22 substitutions, and polymerase tp 95 substitution were entered into the analysis. As a result, the pre-S1 84 and pre-S2 1 substitution could be identified as independent variables.

DISCUSSION

In this study, the correlation between pretreatment HBV genomic sequences and the emergence of resistance in patients administered lamivudine to treat chronic HBV infection were investigated. Investigation was focused on determining whether a correlation exists between the viral genome diversity and emergence of lamivudine resistance. This was accomplished by determining the complete nucleotide sequences of HBV genomes amplified from the patients' pretreatment sera. Sequence comparisons

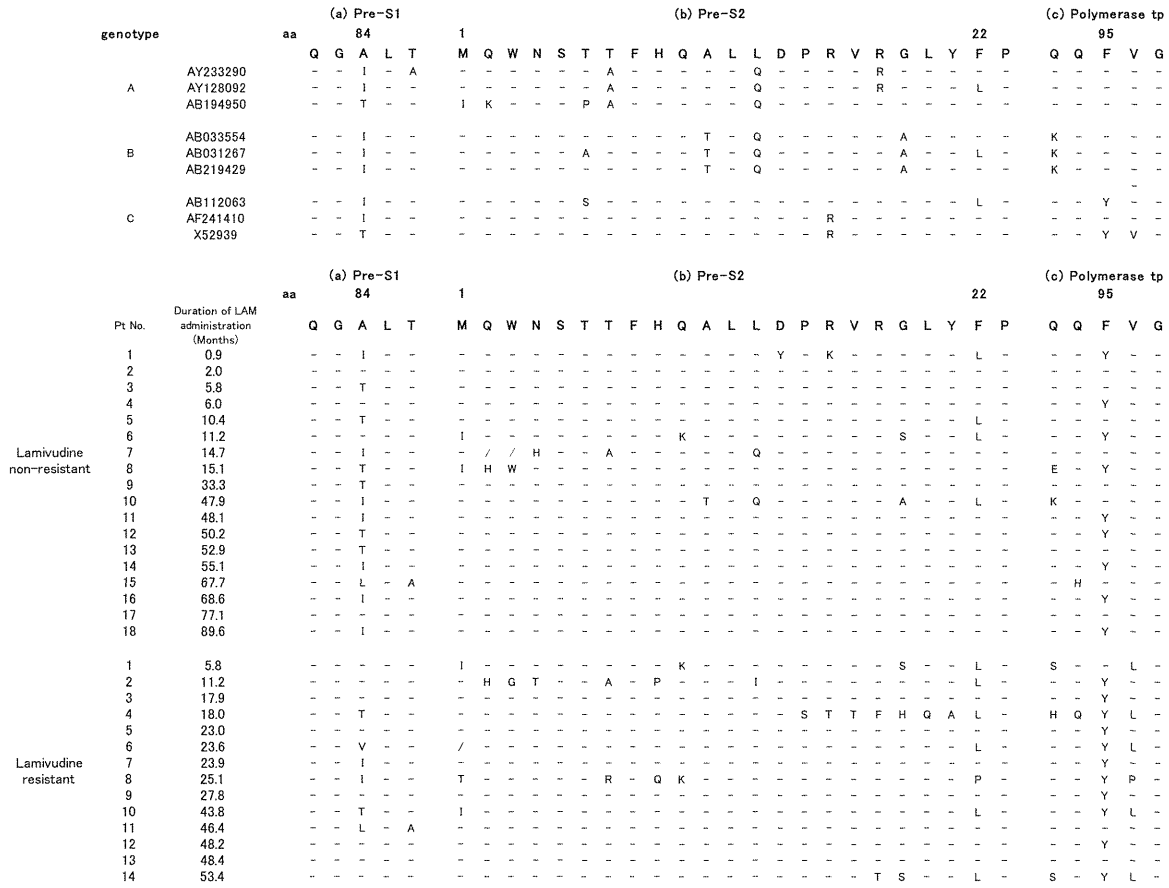

Fig. 4. Amino acid sequence alignment of the pre-S1, pre-S2, and polymerase ORFs associated with the lamivudine resistance. Duration of the LAM administration indicates the period for HBV to become LAM resistant in the resistant group, while it indicates the overall observation period in the non-resistant 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.

revealed that substitutions in the pre-S1 and pre-S2 ORFs serve as predictors of emergence of lamivudine resistance.

In previous studies reporting the correlation between drug resistance and mutations in the HBV genome, the focus was confined to the HBV polymerase [Ghany and Doo, 2009]. Because the polymerase protein is the direct target of nucleoside analogues, amino acid residue changes in the HBV polymerase are considered to result from selective and mutational pressure exerted by those agents. Therefore, prominent amino acid sequence changes are generally considered to appear during therapy [Kobayashi et al., 2009]. However, the emergence of resistance obviously cannot be predicted by these mutations, and the emergence of resistance usually is predicted by studying clinical factors. Among these conventional pretreatment and in-treatment predictors of lamivudine resistance, it was observed that longer periods of HBV persistence (determined by DNA detection) after

TABLE III. Baseline Clinical Characteristics 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 [a]	5.75 (±1.38)	6.83 (±0.86)	0.022
Duration of LAM administration until HBV PCR negative (months)[b]	2.1 (0.4–7.6)	4.0 (1.9–69.0)	0.005

[a]Average (±SD) student's t test.
[b]Median (range) Mann–Whitney *U*-test.

TABLE IV. Baseline Clinical Characteristics 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[a]	50.7 (±9.6)	62.3 (±9.7)	0.003

[a]Average (±SD) Student's *t*-test.

TABLE V. Baseline Clinical Characteristics 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[a]	52 (13–810)	133 (23–2,739)	0.0495
Total bilirubin, mg/dl[a]	0.9 (0.3–5.0)	1.2 (0.5–19.7)	0.049
α-fetoprotein, ng/ml[a]	8 (1.6–35,194)	81 (4–214.3)	0.034

[a]Median (range) Mann–Whitney *U*-test.

commencing lamivudine therapy correlated with the appearance of resistance, an observation that was consistent with most previous studies [Andersson and Chung, 2009; Zhou et al., 2009]. This demonstrates that studied patients did not represent outliers from random populations studied previously.

Here, amino acid differences between patients were compared, according to their responses to lamivudine treatment, at each position in each viral ORF, and showed that patients who developed resistance accumulated more substitutions within specific regions of the pre-S1, pre-S2, and polymerase ORFs. Thereafter, a statistical analysis was conducted to investigate whether these substitutions correlated with the emergence of drug resistance. It was found that preexisting substitutions in pre-S1 84 and pre-S2 1 correlated significantly and independently with lamivudine resistance. Because the HBV polymerase genes evaluated all encoded rt 204V/I mutations at the time of appearance of lamivudine-resistance, it is considered that the preexisting substitutions in those pre-S regions enabled the later mutation of rt 204V/I in the polymerase gene. On the other hand, although regions of the polymerase gene overlapping with pre-S1 84 and pre-S2 1, 22 genes were evaluated for their association with lamivudine resistance, the corresponding amino acid changes in the polymerase gene did not correlate with lamivudine resistance according to Kaplan–Meier analysis, demonstrating the importance of the pre-S regions in the development of resistance (data not shown). Interestingly, patients with a substitution in pre-S1 84 exhibited high viral loads and displayed longer times until HBV DNA became undetectable compared to patients without this substitution. In contrast, a substitution in pre-S2 22 correlated with increased age, and the substitution in polymerase tp 95 with advanced disease.

TABLE VI. Factors Associated 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.

Although the study was focused on the viral amino acid substitutions, viral nucleotide differences also were compared between patients, according to their responses to lamivudine treatment (data not shown). In this analysis, pretreatment substitutions at nucleotide position 53 in the polymerase/pre-S1 region and at nucleotide position 2151 in the core region correlated significantly with the later appearance of lamivudine resistance. In fact, nucleotide position 53 corresponds to the pre-S1 84, and its substitution causes an amino acid change at pre-S1 84. On the other hand, the substitution at nucleotide position 2152 in the core region is synonymous and the role of this substitution should be investigated in a further study.

The pre-S1/pre-S2/S region encodes the small surface (S), middle (M), and large (L) proteins using alternative codons for the initiation of translation [Gao et al., 2007]. These proteins are considered to have crucial functional roles in the life cycle of HBV [De Meyer et al., 1997; Cooper et al., 2003; Kay and Zoulim, 2007; Watanabe et al., 2007; Lian et al., 2008; Ni et al., 2010]. Apart from the HBV life cycle, recent studies have shown that pre-S sequences significantly impact on the pathogenesis of liver disease [Sugauchi et al., 2003; Zhang et al., 2007; Fang et al., 2008]. The pre-S1 and pre-S2 regions serve as immune targets for T and B cells accumulating in the liver [Bauer et al., 2002], while mutant HBV pre-S epitopes stimulated a lower T cell response than wild-type HBV. HBV with pre-S substitutions leads to cellular retention of viral proteins and a dramatic reduction of virion production [Ni et al., 2010]. The appearance of pre-S substitutions inhibits apoptosis of infected hepatocytes [Ni et al., 2010]. Patients with progressive liver disease or HCC experience a higher frequency of pre-S substitutions or deletions than patients with stable disease [Chaudhuri et al., 2004]. In association with nucleoside analog therapy, Ohkawa et al. [2008] showed the possibility that pre-S2 substitutions might support the replication capacity of lamivudine-resistant HBV.

On the other hand, there have been no previous studies reporting the correlation between pretreatment pre-S substitutions and the development of lamivudine resistance to date. While the mechanisms need further clarification, it is possible to hypothesize a model explaining the correlation, considering these previous findings. Because those previous reports indicate that HBVs with pre-S substitutions function as

immune escape mutants, it is possible that HBVs with pre-S substitutions are advantageous for viral survival and replication in hepatocytes, despite that virion production is reduced. In addition, those infected cells are themselves protected from apoptosis. In those circumstances of persistent viral replication, the chances of acquiring the essential substitutions in the polymerase gene conferring lamivudine resistance might increase.

Before these findings can be applied confidently in clinical settings, some caveats must be considered. First, the number of patients analyzed in the study was quite small, and therefore the potential role of the substitutions detected in drug susceptibility must be evaluated by studies of larger populations. Second, because HBV sequences were determined directly, as opposed to first cloning multiple genomes, the dynamics of minor HBV populations and their contribution to the appearance of resistance are not known. Subcloning analysis or deep sequencing might help further to establish the clinical importance and role of these substitutions in drug resistance. The utility of these viral substitutions for designing HBV therapies with the second-generation nucleoside analogs requires additional research. As for the stability of these predictive viral regions during the treatment period, five patients were available for the analysis of the complete HBV genome sequence after the acquisition of lamivudine resistance. Interestingly, the predictive positions of Pre-S1 84 and Pre-S2 1 changed after the acquisition of lamivudine resistance in some patients. However, the role of those changes needs to be further clarified by larger sample sizes.

In conclusion, it was demonstrated that the presence of pre-S1 and pre-S2 substitutions in the HBV genome prior to treatment might play an important role in the subsequent evolution of lamivudine resistance.

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JOURNAL OF VIRAL HEPATITIS

Journal of Viral Hepatitis, 2012 doi:10.1111/j.1365-2893.2012.01649.x

IL-28B (IFN-λ3) and IFN-α synergistically inhibit HCV replication

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Received March 2012; accepted for publication June 2012

SUMMARY. Genetic variation in the IL-28B (interleukin-28B; interferon lambda 3) region has been associated with sustained virological response (SVR) rates in patients with chronic hepatitis C treated with peginterferon-α and ribavirin. However, the mechanisms by which polymorphisms in the IL-28B gene region affect host antiviral responses are not well understood. Using the HCV 1b and 2a replicon system, we compared the effects of IFN-λs and IFN-α on HCV RNA replication. The anti-HCV effect of IFN-λ3 and IFN-α in combination was also assessed. Changes in gene expression induced by IFN-λ3 and IFN-α were compared using cDNA microarray analysis. IFN-λs at concentrations of 1 ng/mL or more exhibited concentration- and time-dependent HCV inhibition. In combination, IFN-λ3 and IFN-α had a synergistic anti-HCV effect; however, no synergistic enhancement was observed for interferon-stimulated response element (ISRE) activity or upregulation of interferon-stimulated genes (ISGs). With respect to the time course of ISG upregulation, the peak of IFN-λ3-induced gene expression occurred later and lasted longer than that induced by IFN-α. In addition, although the genes upregulated by IFN-α and IFN-λ3 were similar to microarray analysis, interferon-stimulated gene expression appeared early and was prolonged by combined administration of these two IFNs. In conclusion, IFN-α and IFN-λ3 in combination showed synergistic anti-HCV activity *in vitro*. Differences in time-dependent upregulation of these genes might contribute to the synergistic antiviral activity.

Keywords: HCV, IFN-λ, IL-28B, ISG, synergistic inhibition, microarray.

INTRODUCTION

In 2009, reports from three genome-wide association studies revealed that several single-nucleotide polymorphisms (SNPs) (rs12979860, rs12980275 and rs8099917) around the IL-28B (interleukin-28B; interferon lambda 3) gene are strongly associated with sustained viral response (SVR) to PEG-IFN and RBV treatment for chronic hepatitis C [1–3]. Specifically, patients with the TG or GG genotype at rs8099917 infected with genotype 1b are more resistant to PEG-IFN and RBV treatment than patients with the TT genotype. IL-28B haplotypes were also reported to be strongly associated with spontaneous HCV clearance [1, 4, 5].

IL-28B is a member of the type III IFN family [6], consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). IFN-λs bind to their cognate receptor, composed of IL28R1 and IL10R2, and then activate the receptor-associated Janus-activated kinases (Jak) 1 and tyrosine kinase (Tyk) 2, leading to the activation of downstream signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2. Similar to type I IFN signalling, the Jak-STAT signalling pathway activates the IFN-stimulated response element (ISRE) within the promoter region of interferon-stimulated genes (ISGs) [7].

Concerning the functional role of IL-28B in HCV infection, two of *in vivo* studies assessed the correlation of IL-28A/B mRNA levels in whole blood and peripheral blood mononuclear cells (PBMC) with IL-28B haplotypes at position rs8099917. IL-28A mRNA and IL-28B mRNA levels in subjects with the TT genotype were higher than in subjects with other genotypes (TG or GG), suggesting an association between higher amounts of endogenous IFN-λs and HCV clearance [2, 3]. On the other hand, subjects

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; ISG, interferon-stimulated genes; MTS, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium; PBMC, peripheral blood mononuclear cells; SNP, single-nucleotide polymorphisms; STAT, signal transducer and activator of transcription; SVR, sustained viral response.

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with the TT genotype at SNP rs8099917 were reported to have lower expression levels of ISGs in the liver during the pretreatment period as compared with subjects with the TG or GG genotypes [8]. Several *in vitro* studies support a direct role of IFN-λs in the control of HCV replication through the innate immune pathway. In a cell culture system, Marcello *et al.* [9] showed that IFN-λ1 inhibited HCV replication with similar kinetics to that of IFN-α, although IFN-λ1 induced stronger upregulation of ISGs and this effect lasted longer. Moreover, combinations of IFN-λ1 and IFN-α had a greater inhibitory effect on HCV replication compared with the individual agents [10].

As suggested by the studies performed to date, a change in IFN-λ3 expression might be a key mechanism by which IL-28B SNPs determine the response to PEG-IFN and RBV. Considering that IFN-λ1 plays a direct role in the control of HCV replication and that IFN-λ1 enhances the antiviral activity of IFN-α, it seems reasonable to speculate that IFN-λ3 plays a similar antiviral role. Therefore, in this study, we investigated the direct antiviral role of IFN-λ3 alone and in combination with IFN-α using an HCV replicon system. In addition, we used microarray analysis to investigate the influence of IFN-λ3, alone or in combination with IFN-α, on the regulation of ISG-mediated antiviral pathways.

MATERIALS AND METHODS

Cell culture and HCV replicon

The human hepatoma cell lines OR6 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) at 37°C in 5% CO_2. JFH-1-infected Huh7.5.1 cells were grown in DMEM supplemented with 10% FBS. The OR6 cell line, harbouring full-length genotype 1b HCV RNA and co-expressing *Renilla* luciferase (ORN/C-5B/KE) [11], was established in the presence of 500 μg/mL G418 (Promega, Madison, WI, USA).

Reagents

IL-28A (IFN-λ2), IL-28B (IFN-λ3) and IL-29 (IFN-λ1) were obtained from R&D Systems (Minneapolis, MN, USA). IL-28A and IL-29 are recombinant proteins generated from an NSO-derived murine myeloma cell line, and IL-28B is a recombinant protein generated from the CHO cell line. Interferon alpha-2b (INTRON®A 300 IU) was obtained from Schering-Plough Corporation (Kenilworth, NJ, USA).

Reporter plasmids and luciferase assay

HCV replication in OR6 replicon cells was determined by monitoring *Renilla* luciferase activity (Promega). To monitor IFN signalling directed by the interferon-stimulated response element (ISRE), the plasmids pISRE-luc expressing firefly luciferase were cotransfected using FuGENE®6 Transfection Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Luciferase activity was quantified using the dual-luciferase assay system (Promega) and a GloMax 96 Microplate Luminometer (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD percentage of the control values.

Quantification of HCV core protein and RNA

We quantified HCV core protein in culture supernatant using Lumipulse Ortho HCV Ag (Ortho Clinical Diagnostics, Tokyo, Japan) as specified by the manufacturer. The principle of the measurement method is based on the chemiluminescent enzyme immunoassay (CLEIA) [12].

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Intracellular genomic JFH-1 HCV RNA as well as cellular mRNA of IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 was quantified by TaqMan RT-PCR. The cells were lysed and subjected to reverse transcription without purification of RNA using a Cells-to-Ct kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative PCR was performed in triplicate using a 7500 Real-Time PCR System (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The sequences of the sense and antisense primers and the TaqMan probe for 5′UTR region of HCV were 5′-TGCGG AACCGGTGAGTACA-3′, 5′-CTTAAGGTTTAGGATTCGTGCT CAT-3′ and 5′-(FAM)CACCCTATCAGGCAGTACCACAAGG CC(TAMRA)-3′, respectively. TaqMan probes for IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 were purchased from Applied Biosystems. Primers for 18s rRNA (Applied Biosystems) were used as internal control.

Microarray analysis

OR6 replicon cells were harvested by centrifugation after exposure to 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both for 6, 12, 24 and 48 h. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Quality control of extracted RNA was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

The RNA was then amplified and labelled using the Ambion® WT Expression Kit and GeneChip® WT Terminal Labelling and Control Kit (Affymetrix, Santa Clara, CA, USA). cDNA was synthesized, labelled and hybridized to the GeneChip® array according to the manufacturer's protocol, starting with 200-ng total RNA. The GeneChips were finally washed

and stained using the GeneChip® Fluidics Station 450 (Affymetrix) and then scanned with the GeneChip® Scanner 3000 7G (Affymetrix).

Affymetrix CEL files were imported into GeneSpring GX v.11.5 (Agilent Technologies, Santa Clara, CA, USA) analysis software. Data were normalized using robust multichip average analysis (RMA).

Dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays

To evaluate the cell viability, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

IFN-λ1, IFN-λ2 and IFN-λ3 demonstrate antiviral activity against HCV

To determine the antiviral effect of IL-29 (IFN-λ1), IL-28A (IFN-λ2) and IL-28B (IFN-λ3) against HCV, OR6/ORN/C-5B/KE cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various concentrations for another 24, 48 and 72 h. In this system, the *Renilla* luciferase activity reflects the amount of HCV RNA synthesized. As shown in Fig. 1, at concentrations of 1 ng/mL or more, all IFN-λs led to a concentration- and time-dependent decrease in luciferase activity of the OR6/C-5B replicon. IFN-λ3 at 10 ng/mL inhibited HCV replication (32% reduction, $P < 0.05$) to a similar extent as 0.01 ng/mL IFN-α (49% reduction, $P < 0.05$) by 48 h.

We also assessed the effects of IFN-λ1, IFN-λ2 and IFN-λ3 on Huh7.5.1/JFH-1 cells. JFH-1 cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various doses for another 48 h. To determine their antiviral effect, HCV core protein in the medium and intracellular HCV RNA were measured by CLEIA and quantitative real-time RT-PCR, respectively. HCV RNA quantitative PCR assays were multiplexed for 18s ribosomal RNA to control for the amount of input RNA. As shown in Fig. 2, all IFN-λs inhibited HCV replication in JFH-1 cells in a concentration-dependent manner. Similarly, all of the IFN-λs caused suppression of HCV core protein secretion into the cell culture medium (Figure S1).

In C-5B system, there was no evident cytotoxicity below 100 ng/mL in any interferons except for IFN-λ1 (Figure S2). On the other hand, cytotoxicity was observed in lesser concentrations by those IFNs in JFH-1 system. However, as demonstrated in Fig. 2 and Figure S3, antiviral effect exceeded the cytotoxicity in the JFH-1 system.

Synergistic inhibition of HCV replication by IFN-λ3 and IFN-α in combination

We examined whether the combination of IFN-λ3 and IFN-α induces greater antiviral activity as compared with the individual cytokines alone. OR6/ORN/C-5B/KE cells were treated with the combinations of IFN-λ3 and IFN-α at various concentrations for 48 h. As shown in Fig. 3a, the relative concentration–inhibition curves of IFN-α were plotted for each fixed concentration of IFN-λ3, and the curves shifted to the left with increasing concentrations of IFN-λ3. The results indicate a synergistic effect of IFN-λ3 and IFN-α against HCV replication. We confirmed the synergistic effect of IFN-λ3 and IFN-α by isobologram (Fig. 3b). The inhibitory effects of the combination were quantified according to the method of Chou *et al.* using the CalcuSyn software program (Biosoft, Cambridge, UK). At the ED_{50} of each drug, the combination index was 0.40-0.61, indicating significant synergism. We also assessed the effect of the combination on Huh7.5/JFH-1 cells by HCV RNA quantitative PCR assays and HCV core protein secretion. At the ED_{50} of each drug, the combination index was 0.55 and 0.48, respectively (Table S1). The cytotoxicity was not observed at the range of concentration tested (Fig. S2E, S3E).

IFN-λ3 induces ISRE promoter activity

We used the ISRE luciferase reporter assay to assess activity downstream of the JAK-STAT signalling pathway. The ISRE-firefly luciferase plasmid was transfected into OR6/ORN/C-5B/KE cells for 24 h, and these cells were cultured with various concentrations of IFN-λ3 and IFN-α for another 12, 24 or 48 h. Firefly and *Renilla* luciferase activity was then measured.

IFN-λ3 induced ISRE luciferase activity in a time-dependent manner; activity was elevated threefold after treatment with 100 ng/mL IFN-λ3 for 48 h (Fig. 4). In contrast, IFN-α induced ISRE luciferase more rapidly, producing maximal activation of the response to IFN-α at 12 h. The combination of IFN-λ3 and IFN-α induced ISRE luciferase activity similarly to IFN-λ3 alone.

IFN-α and IFN-λ3 induce expression of similar genes in HCV 1b replicon cells

OR6/ORN/C-5B/KE cells were stimulated for 6, 12, 24 and 48 h with 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both, while controls were left unstimulated for the same time interval. Induction of gene expression by IFNs was analysed in microarray experiments.

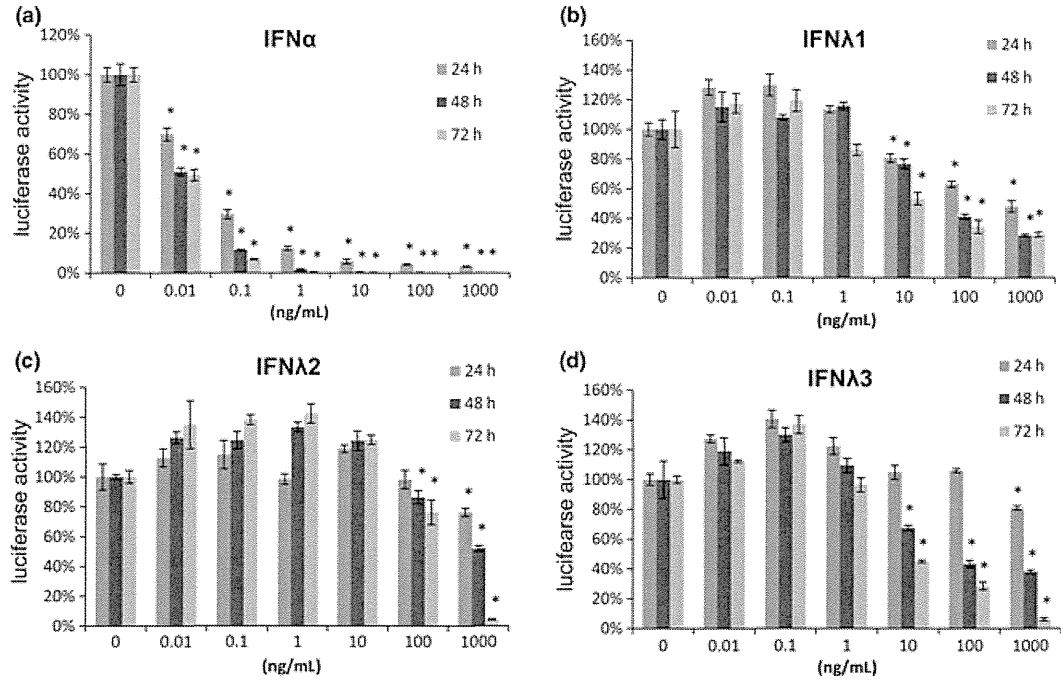

Fig. 1 IFN-α and IFN-λs inhibit HCV replicon in OR6 cells. Specific inhibition of the replication of a full-length HCV genotype 1b replicon by (a) IFN-α and (b) IFN-λ1, (c) IFN-λ2, (d) IFN-λ3 were quantified on the basis of luciferase activity. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$ *vs* control (IFN 0 ng/mL) of each time point.

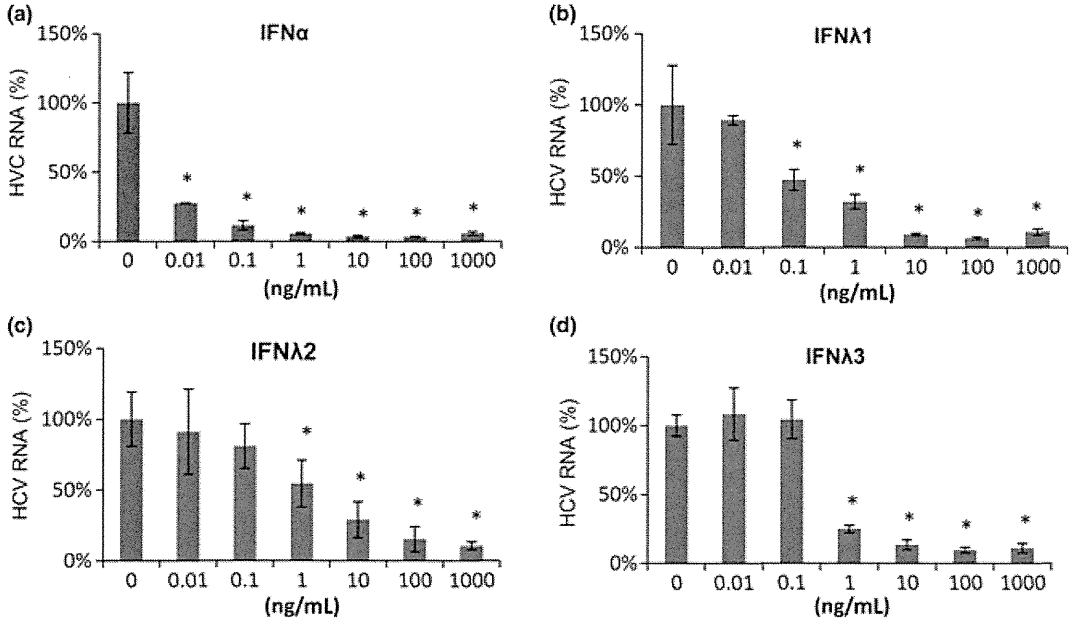

Fig. 2 IFN-α and IFN-λs inhibit HCV replicon in Huh7.5.1 cells. JFH1-infected Huh7.5.1 cells were incubated with various concentrations of (a) IFN-α and (b) IFN-λ1, (c) IFN-λ2, (d) IFN-λ3. After 48 h of treatment, total RNA was isolated and reverse transcribed, after which quantitative PCR was performed. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$ *vs* control (IFN 0 ng/mL).

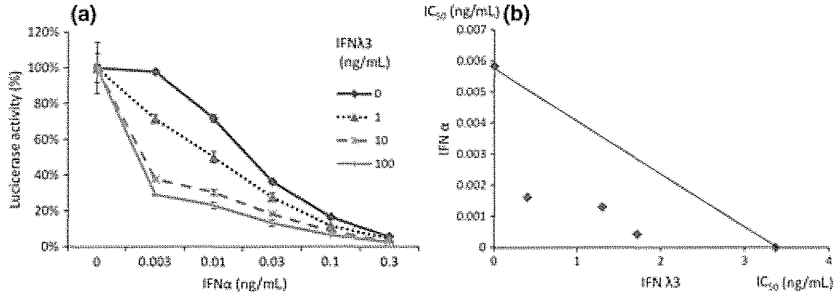

Fig. 3 Synergistic inhibitory effect of IFN-λ3 with IFN-α on hepatitis C virus replication. OR6/ORN/C-5B/KE cells were treated with combinations of IFN-λ3 with IFN-α at various concentrations. (a) The relative concentration–inhibition curves of IFN-α plotted for each fixed concentration of IFN-λ3 (0, 1, 10 and 100 ng/mL). (b) Classic isobologram for IC_{50} of IFN-λ3 with IFN-α in combination.

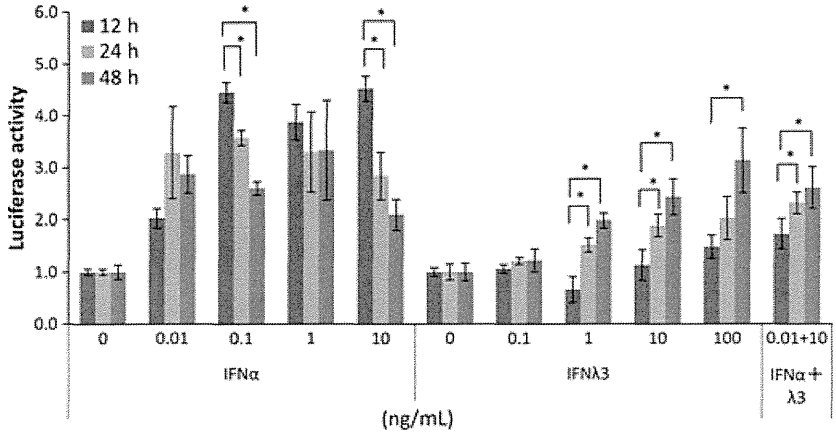

Fig. 4 IFN-stimulated response element (ISRE) promoter activity induced by IFN-α, IFN-λ3 or combination of IFN-α and IFN-λ3. OR6/ORN/C-5B/KE cells transfected with ISRE-firefly luciferase were cultured with various concentrations of IFN-α alone, IFN-λ3 alone or 0.01 ng/mL IFN-α plus 10 ng/mL IFN-λ3. ISRE-firefly luciferase activity at 24 h after transfection. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$.

At all time points, the IFN-λ3-treated samples showed a tendency for the induction of a larger number of genes than samples treated with IFN-α. However, as shown in Table 1 listing the top 25 genes that were upregulated by both IFN-α and IFN-λ3 at 12 h, most of the upregulated genes are previously identified ISGs and the genes with high ranks were similar irrespective of the type of IFN or time point.

The time course of ISGs regulation differs between IFN-α and IFN-λ3

By microarray analysis, ISGs were more rapidly induced after the addition of IFN-α *vs* IFN-λ3 (data not shown). To confirm the rapid induction of ISGs by IFN-α, six ISGs, that is, IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18, were quantified for time-dependent expressional change by real-time RT-PCR. Expression of most of the genes upregulated by IFN-α peaked at 12 h and fell thereafter. In contrast, expression of IFN-λ3-induced genes peaked at 24 h and lasted up to 48 h. Combination of IFN-α and IFN-λ3 induced ISG with peak effects occurring at 12–24 h and lasting up to 48 h (Fig. 5).

DISCUSSION

In this study, we demonstrated that IFN-λ family members have distinctive time-dependent antiviral activities in an HCV replicon system and that IFN-λ3 and IFN-α have a synergistic effect in combination. Moreover, we attempted to identify the antiviral mechanism of IFN-λ3 by conducting a cDNA microarray analysis.

In previous studies, anti-HCV activity of IFN-λ1, IFN-λ2 and IFN-λ3 was reported in JFH-1 and OR6/C-5B systems [13]. Time-dependent anti-HCV activity has also been observed with IFN-λ1 [9]. In this study, we confirmed the previous results and added the further finding that time-dependent antiviral activity is not limited to IFN-λ1, but rather is common among all IFN-λs.

Table 1 Top 25 genes that were upregulated by both IFN-α and IFN-λ3 at 12 h

Gene bank ID	Gene symbol	Gene description	IFN-α 0.01 ng/mL fold increase	IFN-λ3 10 ng/mL fold increase	IFN-α+IFN-λ3 fold increase
BC007091	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	4.01	4.49	4.87
BC049215	OAS2	2′-5′-oligoadenylate synthetase 2, 69/71kDa	3.06	3.88	4.48
M33882	MX1	Myxovirus (influenza virus) resistance 1	3.24	3.29	3.69
AF095844	IFIH1	Interferon induced with helicase C domain 1	2.73	3.02	3.54
BC038115	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	2.70	2.92	3.51
BC011601	IFI6	Interferon, alpha-inducible protein 6	3.07	3.24	3.42
BC042047	HERC6	Hect domain and RLD 6	2.56	2.75	3.34
AF442151	RSAD2	Radical S-adenosyl methionine domain containing 2	1.32	2.59	3.28
U34605	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	2.47	2.91	3.25
AY730627	OAS1	2′,5′-oligoadenylate synthetase 1, 40/46kDa	2.32	2.57	3.05
AB006746	PLSCR1	Phospholipid scramblase 1	2.37	2.51	3.03
AF307338	PARP9	Poly (ADP-ribose) polymerase family, member 9	2.39	2.46	2.94
M87503	IRF9	Interferon regulatory factor 9	2.61	2.59	2.85
AK297137	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.90	2.36	2.79
AK290655	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.47	2.45	2.77
BX648758	PARP14	Poly (ADP-ribose) polymerase family, member 14	2.07	2.25	2.66
BC132786	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.83	2.17	2.59
AF445355	SAMD9	Sterile alpha motif domain containing 9	2.07	2.08	2.56
	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	1.63	1.92	2.39
BC014896	USP18[1] USP41	Ubiquitin-specific peptidase 18/ubiquitin-specific peptidase 41	1.52	1.78	2.11
AB044545	OAS3	2′-5′-oligoadenylate synthetase 3, 100kDa	1.44	1.57	2.10
BC010954	CXCL10	Chemokine (C-X-C motif) ligand 10	0.76	1.66	1.99
BC014896	USP18	Ubiquitin-specific peptidase 18	1.33	1.55	1.99
AL832618	IFI44L	Interferon-induced protein 44-like	0.58	1.31	1.95

We also assessed whether IFN-λ3 and IFN-α in combination could produce additive or synergistic effects on antiviral activity. In previous studies, additive antiviral activity against HCV was reported with the combination of IFN-λ1 and IFN-α [9, 10]. However, there have been no previous reports on the combined effects of IFN-λ3 and IFN-α. In this study, the focus was on IFN-λ3, because IFN-λ3 is suspected to be the key molecule, mediating the effect of SNPs in the IL-28B gene region on the anti-HCV response to IFN-α. As shown in Fig. 3 and Table S1, synergistic induction of anti-HCV activity occurred in both the OR6/C-5B and Huh7.5/JFH-1 HCV replicon systems. Synergy was demonstrated by the combination index values (Table S1).

Although it has been reported that the upregulated genes induced by IFN-λ are similar to those induced by IFN-α [9, 14–16], there have been no previous reports on