Table 1. Patient Characteristics and *IL28B* Genotype

	IL28B Major*	*IL28B* Minor†	*P*-value‡
Patients, n	54	34	
Age (SD), year	58.8 (10.0)	59.1 (10.3)	0.918§
Sex, n (%)			0.051‖
Male	13 (24.1)	15 (44.1)	
Female	41 (75.9)	19 (55.9)	
BMI (SD), kg/m^2	22.7 (3.5)	23.5 (3.6)	0.193§
ALT (SD), IU/L	61.3 (50.7)	62.4 (44.7)	0.962§
γ-GTP (SD), IU/L	36.7 (25.9)	57.3 (52.4)	0.010§
LDL-cholesterol (SD), mg/dL	103.3 (29.8)	91.8 (26.9)	0.067§
Hemoglobin (SD), g/dL	14.1 (1.4)	14.4 (1.3)	0.186§
Platelet count (SD), $\times 10^3 3/\mu L$	161 (6.4)	163 (4.4)	0.489§
Fibrosis stage, n (%)			0.532‖
F1, 2	38 (70.4)	26 (76.5)	
F3, 4	16 (29.6)	8 (23.5)	
Viral load (SD), $\times 10^{63}$ IU/mL	1.7 (1.4)	1.9 (2.0)	0.788§
%HCV core 70 & 91 a.a. double mutation¶	8.9	43.5	0.001‖
%ISDR wild**	43.5	51.7	0.486‖
Viral response, n (%)			<0.001‖
SVR	17 (31.5)	13 (38.2)	
TVR	26 (48.1)	3 (8.8)	
NVR	11 (20.4)	18 (52.9)	

Unless otherwise indicated, data are given as mean (SD).

*rs8099917 TT and rs12979860 CC.

†rs8099917 TG and rs12979860 CT.

BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; SVR, sustained virological response; TVR, transient virological response; NVR, nonvirological response.

‡Comparison between *IL28B* major and minor genotypes.

§Mann-Whitney *U* test.

‖Chi-square test.

¶HCV core mutation was determined in 68 patients.

**ISDR was determined in 75 patients.

the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of the positive and negative cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*), the adaptor molecule (*IPS-1*), the related ubiquitin E3-ligase (*RNF125*), the modulators of these molecules (*ISG15* and *USP18*), and *IFNλ* (*IL28A/B*) was quantified by real-time quantitative polymerase chain reaction (PCR) using target gene-specific primers. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen reagent (Nippon Gene, Toyama, Japan) from the liver biopsy specimen, which was 0.2-0.4 cm in length and 13G in diameter. Complementary DNA (cDNA) was transcribed from 2 μg of total RNA template in a 140-μL reaction mixture using the SYBR RT-PCR Kit (Takara Bio, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio) with the SYBR RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Assays were performed in duplicate and the expression levels of target genes were normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and hydroxymethylbilane synthase (*HMBS*), an enzyme that is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two housekeeping genes was used in the present study. Sequences of the primer sets were as follows: *RIG-I*, 5′-AAAGCATGCA TGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTC ACTGATAA-3′; *MDA5*, 5′-ACATAACAGCAACATG GGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGG AG-3′; *LGP2*, 5′-ACAGCCTTGCAAACAGTACAAC CTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; *IPS-1*, 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGC ACGCCAGGCTTACTCA-3′; *RNF125*, 5′-AGGGCA CATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAAC GGCAAAGTGG-3′; *ISG15*, 5′-AGCGAACTCATCT TTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACA TGGA-3′; *USP18*, 5′-TGGTTCTGCTTCAATGACT CCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACT C-3′; *IFNλ*: 5′-CAGCTGCAGGTGAGGGA-3′, 5′-G GTGGCCTCCAGAACCTT-3′; *GAPDH*, 5′-GCACC GTCAAGGCTGAGAAC-3′, 5′-ATGGTGGTGAAGA CGCCAGT-3′; *HMBS*, 5′-AAGCGGAGCCATGTCT GGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Genotyping for* IL28B *(rs8099917 and rs12979860) Polymorphism. Genetic polymorphism in a tagged SNP located near the *IL28B* gene (rs8099917 and rs12979860) was determined by direct sequencing of PCR-amplified DNA. In brief, after extraction from whole blood samples, genomic DNA was amplified by PCR. Sequences of the primer sets were: rs8099917, 5′-ATCCTCCTCTCATCCCTCA TC-3′, 5′-GGTATCAACCCCACCTCAAAT-3′; rs129 79860, 5′-GGACGAGAGGGCGTTAGAG-3′, 5′-AG GGACCGCTACGTAAGTCAC-3′.

Both strands of the PCR products were sequenced by the dye terminator method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Chiba, Japan); nucleotide sequences were determined by a capillary DNA sequencer ABI3730xl (Applied Biosystems). Homozygosity (rs8099917 GG and rs12979860 TT) or heterozygosity (rs8099917 TG and rs12979860 CT) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (rs8099917 TT and rs12979860 CC) was defined as having the *IL28B* major allele.

Western Blotting. Western blotting was performed using samples from 14 patients (six from *IL28B* major patients and eight from *IL28B* minor patients) as described.[19] In brief, liver biopsy specimens of

approximately 10 mg were homogenized in 100 μL of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30 μg of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti-β-actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon[20] were used for a positive control for cleaved IPS-1.

Definitions of Response to Therapy. A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFNα-2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

Statistical Analysis. Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney U test for two groups. All tests of significance were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics and* IL28B *Genotype. Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report of the rarity of a minor homozygote in Japanese patients.[3] *IL28B* minor patients were significantly associated with a higher γ-glutamyl transpeptidase (γ-GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

Gene Expression Involving Innate Immunity and* IFNλ *in the Liver. Hepatic expression levels of cytoplasmic viral sensors (*RIG-I, MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFNλ (IL28A/B)* expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFNλ* based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the ISG15/USP18 system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFNδ* was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the RIG-I/IPS-1 and ISG15/USP18 systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

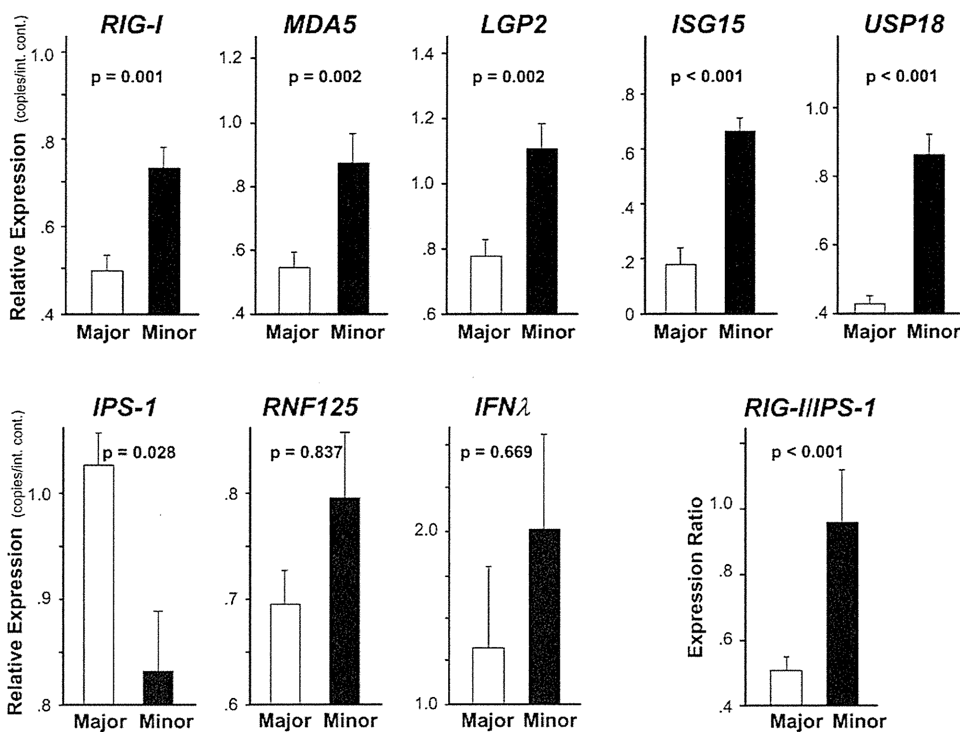

Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

Receiver Operator Characteristic (ROC) Analysis. To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

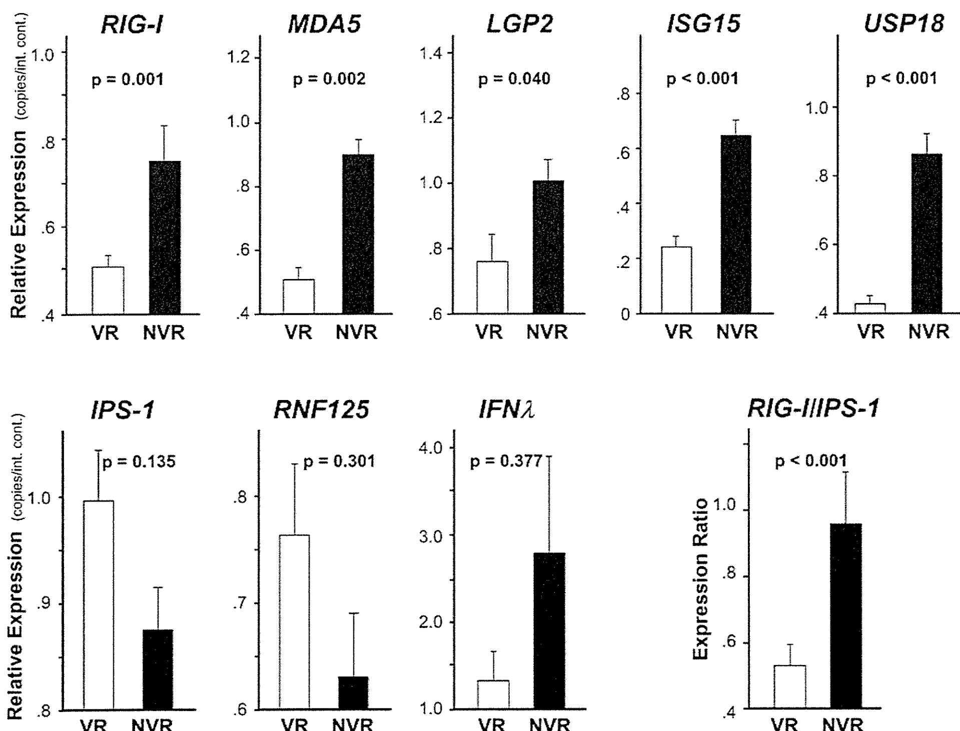

Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

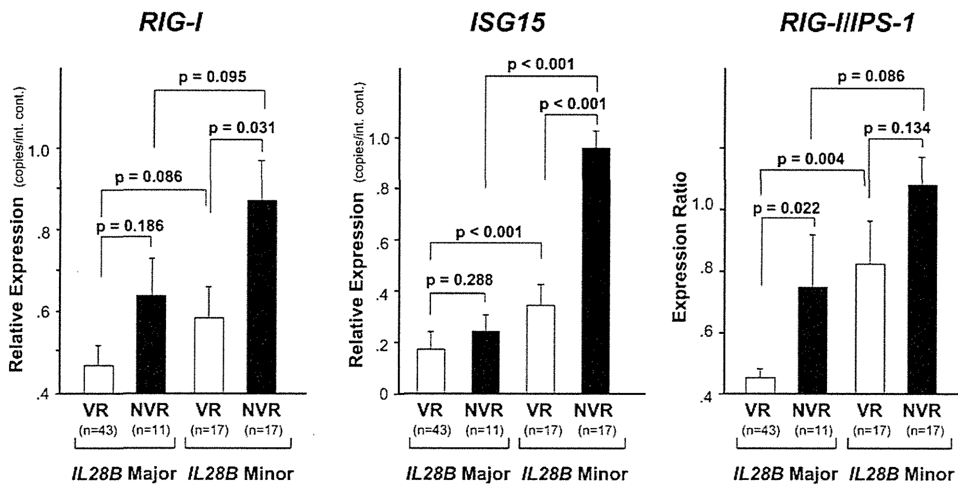

Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIGI/IPS-1* ratio, no statistically significant difference was found in NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

Factors Associated with NVR. In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic expressions of *RIG-I, MDA5, LGP2, ISG15,* and *USP18,* and *RIG-I/IPS-1* ratio were significantly

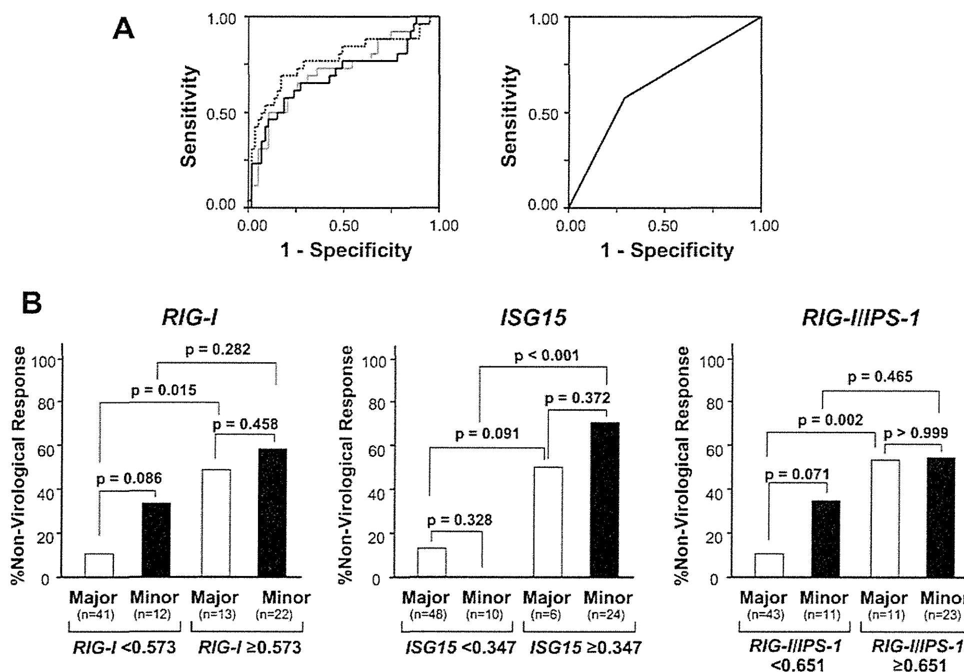

Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/ISG15* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
RIG-I (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
ISG15 (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
RIG-I/IPS-1 (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
IL28B genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.
*Genotype at rs8099917.
†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

IPS-1 and RIG-I Protein Expression in the Liver. Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

Table 3. Factors Associated with Nonvirological Response

	Univariate Analysis		Multivariate Analysis*	
Factors	Risk Ratio (95% CI)	*P*-value	Risk Ratio (95% CI)	*P*-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m^2)	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10^4/μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
IL28B genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/*int. control*)				
RIG-I	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
MDA5	1.53 (1.12-2.00)	0.001		
LGP2	1.34 (1.04-1.74)	0.026		
IPS-1	0.90 (0.78-1.04)	0.143		
RNF125	0.93 (0.83-1.04)	0.204		
ISG15	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
USP18	1.67 (1.27-2.20)	<0.001		
IFNλ	1.02 (0.99-1.05)	0.170		
RIG-I/IPS-1 ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.
*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5, LGP2, USP18,* and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.
†rs8099917 TT and rs12979860 CC.
‡rs8099917 TG and rs12979860 CT.

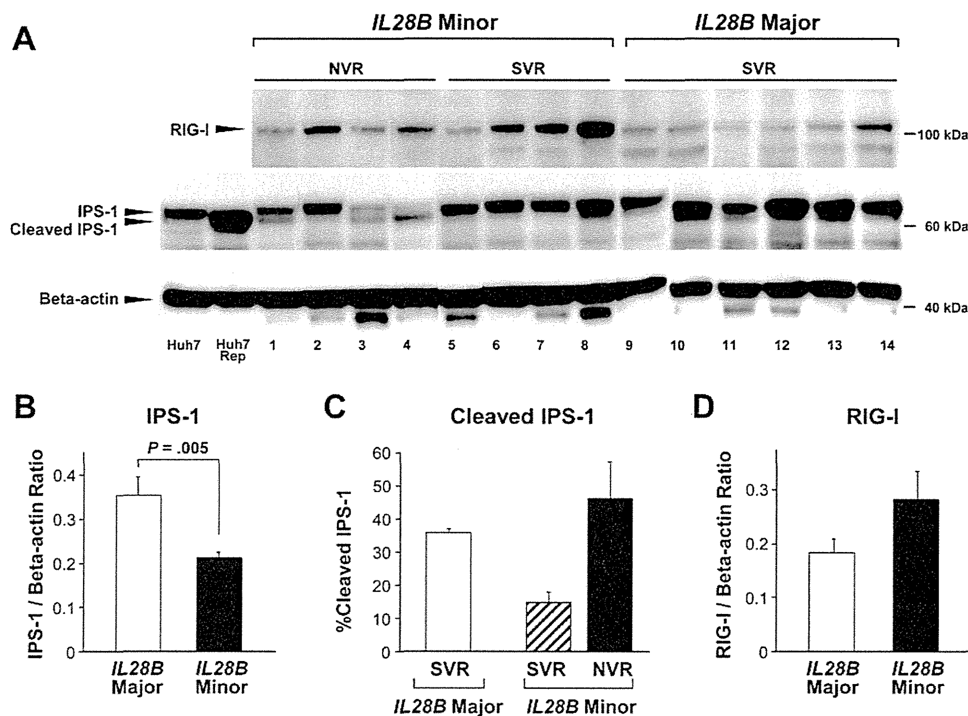

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β-actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFNα/RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFNα/RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.[7,8] Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,[19] suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFNα/RBV response as well as with *IL28B* genotype.[21-23]

In contrast to cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,[9,18]

transcriptional and translational *IPS-1* expression are probably suppressed by HCV with resistant phenotype, which may be more adaptive in *IL28B* minor patients than in *IL28B* major patients. When we analyzed the proportion of full-length or cleaved IPS-1 to the total IPS-1 protein in a subgroup of *IL28B* minor patients, cleaved IPS-1 product was less dominant in SVR than in NVR, whereas uncleaved full-length IPS-1 protein was more dominant in SVR than in NVR. Therefore, the ability of HCV to evade host innate immunity by cleaving IPS-1 protein and/or host capability of protection from IPS-1 cleavage is probably responsible for the variable treatment responses in *IL28B* minor patients.

Our results indicated a close association between *IL28B* minor patients with higher γ-GTP level and higher frequency of HCV core double mutants, which are known factors for NVR. In contrast, no significant association was observed between *IL28B* genotype and age, gender, or liver fibrosis, which are also known to be unfavorable factors for virological response to PEG-IFNα/RBV. Therefore, certain factors other than the *IL28B* genotype may independently influence virological response. To elucidate whether gene expression involving innate immunity independently associates with a virological response from the *IL28B* genotype, we performed further analysis in a subgroup and conducted a multivariate regression and ROC analyses. Our multivariate and ROC analyses demonstrate that higher expressions of *RIG-I* and *ISG15* as well as a higher ratio of *RIG-I/IPS-1* are independently associated with NVR, and quantification of these values is more useful in predicting final virological response to PEG-IFNα/RBV than determination of *IL28B* genotype in each individual patients. However, the SVR rates in our patients were similar among *IL28B* genotypes, which suggests more SVR patients with the *IL28B* minor allele were included in the present study than those in the general CH-C population. Hence, our data did not necessarily exclude the possibility of the *IL28B* genotype in predicting NVR, although our multivariate analysis could not identify the *IL28B* minor allele as an independent factor for NVR. Interestingly, an association between *IL28B* genotype and expressions of *RIG-I* and *ISG15* as well as *RIG-I/IPS-1* expression ratio is still observed even in patients with the same subgroup of virological response (Fig. 3).

In the present study, although hepatic *IFNλ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of *IL28B* from that of *IL28A*, and moreover, we could not specify which cell expresses *IFNλ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFNλ* in relation to treatment response need further clarification by specifying type of IFNλ and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I/IPS-1* ratio was significantly higher in NVR with HCV genotype 2.[19] However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.[24] The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

Differences in allele frequency for *IL28B* SNPs among the population groups has been reported. The frequency of *IL28B* major allele among patients with Asian ancestry is higher than that among patients with European and African ancestry.[25] Because *IL28B* polymorphism strongly influences treatment responses within each population group,[5] our data obtained from Japanese patients can be applied to other population groups. However, the rate of SVR having African ancestry was lower than that having European ancestry within the same *IL28B* genotype.[5] Hence, further study is required to clarify whether this difference among the population groups with the same *IL28B* genotype could be explained by differences in expression of genes involved in innate immunity.

In a recent report, an SVR rate of telaprevir with PEG-IFNα/RBV was only 27.6% in *IL28B* minor patients.[26] Because new anti-HCV therapy should still contain PEG-IFNα/RBV as a platform for the therapy, our findings regarding innate immunity in addressing the mechanism of virological response and predicting NVR remain important in this new era of directly acting anti-HCV agents, such as telaprevir and boceprevir.

In conclusion, this clinical study in humans demonstrates the potential relevance of the molecules involved in innate immunity to the genetic variation

of *IL28B* and clinical response to PEG-IFNα/RBV. Both the *IL28B* minor allele and higher expressions of *RIG-I* and *ISG15* as well as higher *RIG-I/IPS-1* ratio are independently associated with NVR. Innate immune responses in *IL28B* minor patients may have adapted to a different equilibrium compared with that in *IL28B* major patients. Our data will advance both understanding of the pathogenesis of HCV resistance and the development of new antiviral therapy targeted toward the innate immune system.

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ORIGINAL ARTICLE

Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice

Niu Shi,[1,2] Nobuhiko Hiraga,[1,2] Michio Imamura,[1,2] C Nelson Hayes,[1,2] Yizhou Zhang,[1,2] Keiichi Kosaka,[1,2] Akihito Okazaki,[1,2] Eisuke Murakami,[1,2] Masataka Tsuge,[1,2] Hiromi Abe,[1,2] Hiroshi Aikata,[1,2] Shoichi Takahashi,[1,2] Hidenori Ochi,[2,3] Chise Tateno-Mukaidani,[2,4] Katsutoshi Yoshizato,[2,4] Hirotaka Matsui,[5] Akinori Kanai,[6] Toshiya Inaba,[5] Fiona McPhee,[7] Min Gao,[7] Kazuaki Chayama[1,2,3]

[1]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
[2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan
[3]Laboratory for Digestive Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan
[4]PhoenixBio Co., Ltd., Higashihiroshima, Japan
[5]Department of Molecular Oncology and Leukemia Program Project, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
[6]Radiation Research Center for Frontier Science, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
[7]Bristol-Myers Squibb, Research and Development, Wallingford, Connecticut, USA

Correspondence to
Professor Kazuaki Chayama, Department of Gastroenterology and Metabolism, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; chayama@mba.ocn.ne.jp

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ABSTRACT

Objective We recently demonstrated that combination treatment with NS3 protease and NS5B polymerase inhibitors succeeded in eradicating the virus in genotype 1b hepatitis C virus (HCV)-infected mice. In this study, we investigated the effect of combining an NS5A replication complex inhibitor (RCI) with either NS3 protease or NS5B inhibitors on elimination of HCV genotypes 1b, 2a and 2b.

Design The effects of Bristol-Myers Squibb (BMS)-605339 (NS3 protease inhibitor; PI), BMS-788329 (NS5A RCI) and BMS-821095 (NS5B non-nucleoside analogue inhibitor) on HCV genotypes 1b and 2a were examined using subgenomic HCV replicon cells. HCV genotype 1b, 2a or 2b-infected human hepatocyte chimeric mice were also treated with BMS-605339, BMS-788329 or BMS-821095 alone or in combination with two of the drugs for 4 weeks. Genotypic analysis of viral sequences was achieved by direct and ultra-deep sequencing.

Results Anti-HCV effects of BMS-605339 and BMS-821095 were more potent against genotype 1b than against genotype 2a. In in-vivo experiments, viral breakthrough due to the development of a high prevalence of drug-resistant variants was observed in mice treated with BMS-605339, BMS-788329 and BMS-821095 in monotherapy. In contrast to monotherapy, 4 weeks of combination therapy with the NS5A RCI and either NS3 PI or NS5B inhibitor succeeded in completely eradicating the virus in genotype 1b HCV-infected mice. Conversely, these combination therapies failed to eradicate the virus in mice infected with HCV genotypes 2a or 2b.

Conclusions These oral combination therapies may serve as a Peg-alfa-free treatment for patients chronically infected with HCV genotype 1b.

Significance of this study

What is already known on this subject?

- Anti-HCV drug monotherapy for chronic hepatitis C patients often results in viral breakthrough due to the emergence of drug-resistant clones.
- Combination treatment of NS3 PI and NS5A inhibitor can eradicate genotype 1b HCV in chronic hepatitis C patients without interferon.

What are the new findings?

- Combination treatment of NS5A inhibitor with either NS3 PI or NS5B inhibitor can eradicate HCV, but the effect differs among HCV genotypes.

How might it impact on clinical practice in the foreseeable future?

- Short-term combination of NS5A inhibitor with either NS3 PI or NS5B inhibitor might provide an effective interferon-free treatment for genotype 1b chronic hepatitis C patients; however, the combination treatment might be less effective against genotype 2.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases, such as cirrhosis and hepatocellular carcinoma.[1 2] A number of new selective inhibitors of HCV proteins, termed direct-acting antiviral agents (DAA), are currently under development. HCV inhibitors targeting NS3 protease and NS5A and NS5B polymerase activity have proceeded to clinical trials for HCV-infected patients. DAA are used in combination with Peg-alfa and ribavirin because monotherapy with these drugs results in the early emergence of drug-resistant variants.[3 4] As Peg-alfa/ribavirin treatment is frequently associated with serious adverse events, an oral Peg-alfa/ribavirin-free DAA combination therapy would offer an ideal treatment option for chronic hepatitis C patients. The first proof-of-concept study to combine NS3 protease and NS5B inhibitors (INFORM-1) reported that 13 days of this combination treatment achieved robust antiviral suppression in genotype 1 HCV-infected patients, and no evidence of resistance to either compound was observed.[5] Following the INFORM-1 study, we and other groups have also reported that a DAA-only

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combination comprising NS3 protease inhibitor (PI), Bristol-Myers Squibb (BMS)-650032 (asunaprevir) and NS5A replication complex inhibitor (RCI), BMS-790052 (daclatasvir) can achieve high sustained virological response (SVR) rates in patients with HCV genotype 1b infection.[6] A number of DAA-only combination studies are now entering phase 2 clinical trials.[7] The effect of telaprevir was recently analysed in genotype 2 HCV-infected patients. Fifteen days of telaprevir monotherapy decreased the serum HCV RNA titre by 3.7 log_{10} IU/ml, and 3 months of telaprevir plus 24 weeks of Peg-alfa/ribavirin triple therapy resulted in SVR in 100% of genotype 2 HCV-infected patients.[8] However, the effect of Peg-alfa/ribavirin-free DAA combination therapy on genotype 2 HCV has not been reported.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes that can be infected with HCV.[9] This animal model is useful for evaluating anti-HCV drugs such as Peg-alfa and NS3 PI.[10 11] Using this animal model, we recently described the successful elimination of HCV genotype 1b by treatment with a combination of NS3 protease and NS5B inhibitors.[12] In this study, we investigated whether short-term combination treatments with NS5A RCI and either NS3 protease or NS5B site I inhibitors could eliminate HCV *in vivo* in human hepatocyte chimeric mice, and we compared the efficacy of the drugs against HCV genotype 1 versus genotype 2.

METHODS

Compounds and cells

BMS-605339 (NS3 PI, analogue of asunaprevir), BMS-788329 (NS5A RCI, analogue of daclatasvir) and BMS-821095 (NS5B non-nucleoside analogue inhibitor; NNI) were synthesised by BMS. Huh-7 cells that stably maintain HCV replicons were propagated as subconfluent monolayers in Dulbecco's modified essential medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 0.5 mg/ml geneticin (G418; Invitrogen Corp., Carlsbad, California, USA) at 37°C under 5% carbon dioxide.[13]

Determination of IC_{50} in culture systems

The genotype 1b (Con 1) replicon cell line was constructed as described previously.[14] A genotype 2a (JFH-1) cell line was generated by introducing the JFH-1 sequence from NS3 to NS5B into the genotype 1b (Con 1) backbone.[15] Inhibition of HCV RNA replication by either BMS-605339, BMS-788329 or BMS-821095 for 72 h was monitored using a luciferase reporter assay. Antiviral activities of the compounds, for example, the 50% inhibitory concentration (IC_{50}), were determined as described previously.[16]

Human serum samples

Human serum containing a high titre of HCV genotypes 1b, 2a and 2b was obtained from patients with chronic hepatitis who had given written informed consent to participate in the study. Serum samples were divided into small aliquots and stored in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee.

Animal treatment

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously.[17] All mice were transplanted with frozen human hepatocytes obtained from the same donor. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples and killing of animals were performed under ether anaesthesia. Eight weeks after hepatocyte transplantation, mice were injected intravenously with 100 μl of HCV-positive human serum samples. Mice serum samples were obtained every 1 or 2 weeks after HCV infection, and HCV RNA levels were measured.

Treatment of HCV-infected mice with anti-HCV inhibitors

Eight weeks after HCV infection when the mice developed stable viraemia (6–8 log_{10} copies/ml), mice were administered orally with one of the following: 75 mg/kg of BMS-605339 (twice a day); 10 or 30 mg/kg of BMS-788329 (once a day); or 30 or 100 mg/kg of BMS-821095 (once a day) for 4 weeks. To analyse the effect of the combination treatment, BMS-788329 was mixed with either BMS-605339 or BMS-821095 and given together as a cocktail. To analyse susceptibility to Peg-alfa, 10 μg/kg of human Peg-alfa (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) were administered by intramuscular injection twice a week for weeks.

RNA extraction and amplification

RNA extraction, nested PCR and quantitation of HCV by real-time PCR were performed as described previously.[11 12] Briefly, RNA was extracted from serum samples and extracted livers using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV complementary DNA was performed using a light cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limit of real-time PCR is 3 log_{10} copies/ml.

Sequence analysis

The nucleotide and amino acid sequences of the NS3, NS5A and NS5B regions of HCV were determined by direct sequencing as described previously.[12] The primers used to amplify the NS3 region were 5′–GTGCTCCAAGCTGGCATAAC–3′ and 5′–AGGACCGAGGAATCGAACAT–3′ as the first (outer) primer pair and 5′–CTAGAGTGCCGTACTTCGTG–3′ and 5′–ACT GATCCTGGAGGCGTAGC–3′ as the second (inner) primer pair. The primers used to amplify the NS5A region were 5′–GAA TGCAGCTCGCCGAGCAA–3′ and 5′–CCATGTTGTGGTGGC GCAGC–3′ as the first (outer) primer pair and 5′–GCAGCTGT TGGCAGCATAGGTC–3′ and 5′–GATGGTAGTGCATGTCG CC–3′ as the second (inner) primer pair. The primers used to amplify the NS5B region were 5′–TAAGCGAGGAGGCT GGTGAG–3′ and 5′–CCTATTGGCCTGGAGTGTTT–3′ as the first (outer) primer pair and 5′–GACTCAACGGTCAC TGAGAG–3′ and 5′–CCTATTGGCCTGGAGTGTTT–3′ as the second (inner) primer pair. The amplified DNA fragments were separated onto a 2% agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., California, USA). The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype 1b HCV-J (GenBank accession no.: D90208)[18].

Ultra-deep sequencing

We have adapted multiplex sequencing by synthesis to sequence multiple genomes simultaneously using the Illumina genome analyser. Briefly, cDNA was fragmented using sonication, and the resultant fragment distribution was assessed using the Agilent BioAnalyzer 2100 platform. A library was prepared

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Table 1 In-vitro activity of BMS-605339, BMS-788329 and BMS-821095 in HCV replicon assays

	IC_{50} (nM)		
Genotype (strain)	BMS-605339	BMS-788329	BMS-821095
1b (Con 1)	3.5±0.8	0.012±0.005	3.8±0.6
2a (JFH-1)	81±27	0.014±0.007	365±266

Data are represented as means±SD from at least three independent experiments.
HCV, hepatitis C virus.##

using the Multiplexing sample preparation kit (Illumina Inc., California, USA). Imaging analysis and base calling were performed using Illumina Pipeline software with default settings. The N-terminal 1344 nucleotides of NS3 protease, 1146 nucleotides of NS5A RCI and 1133 nucleotides of NS5B polymerase were analysed. This technique revealed an average coverage depth of over 1000 sequence reads per base pair in the unique regions of the genome. Read mapping to a reference sequence was performed using BWA.[19] Direct sequencing consensus data were used to improve alignment to the reference sequence. Codon counts were merged and analysed using R V.2.14.

Statistical analysis

Mice serum HCV RNA titres were compared using the Mann–Whitney U test. A p value less than 0.05 was considered statistically significant.

RESULTS

Antiviral activities of BMS-605339, BMS-788329 and BMS-821095 in cell culture systems

The inhibitory effects of BMS-605339, BMS-788329 and BMS-821095 on HCV replication were analysed *in vitro* using HCV replicon cells (genotype 1b, Con 1 and genotype 2a, JFH1). A summary of the IC_{50} values for each drug is shown in table 1. Antiviral activities of BMS-605339 and BMS-788329 were similar to asunaprevir[15] and daclatasvir,[20] respectively. BMS-605339 and BMS-821095 IC_{50} values were 23-fold and 116-fold more potent against genotype 1b than against genotype 2a, respectively.

Peg-alfa treatment on mice infected with HCV genotypes 1 and 2

We first analysed the effect of Peg-alfa on mice infected with HCV genotypes 1 and 2. Mice were injected with 10^5 copies of HCV obtained from patients infected with HCV genotypes 1b, 2a, or 2b. Administration of 10 μg/kg of human Peg-alfa twice a week for 2 weeks resulted in only a 0.53 log_{10} decrease in the serum HCV RNA titre in HCV genotype 1b-infected mice (figure 1). In contrast, the same therapy resulted in 1.9 log_{10} and 1.5 log_{10} decreases in serum HCV RNA titres in mice with HCV genotypes 2a ($p<0.05$) and 2b (not significant), respectively. No decline in HCV RNA titre was observed in control mice infected with HCV genotype 1b during this 2-week period (figure 1). These results are consistent with the clinical observation that genotype 2 demonstrates a higher susceptibility to Peg-alfa treatment compared to HCV genotype 1.

Effects of BMS-605339, BMS-788329, or BMS-821095 on HCV genotype 1b in mice

We analysed the effect of DAA monotherapy on mice infected with HCV genotype 1b. Nine mice were injected with 10^5 copies

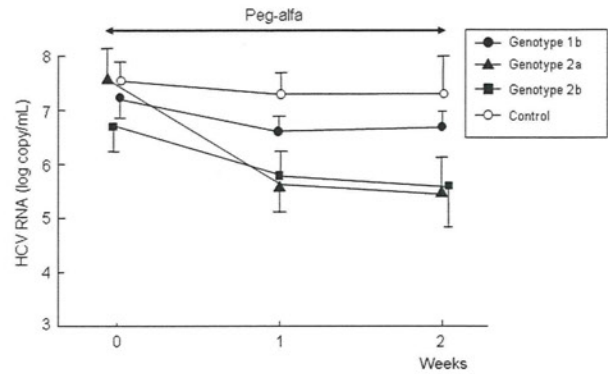

Figure 1 Antiviral effects of Peg-alfa treatment in mice. Mice were infected with hepatitis C virus (HCV) genotypes 1b (n=3), 2a (n=4) or 2b (n=4), then treated with 10 μg/kg of Peg-alfa twice per week for 2 weeks. HCV-infected mice without treatment (n=3) were also analysed (control). Mice serum HCV RNA titres were measured at the indicated times. Data are presented as mean±SD.

of HCV obtained from a patient infected with genotype 1b. Eight weeks after injection when stable viraemia had developed, mice were treated with BMS-605339 (N3PI) (figure 2A), BMS-788329 (NS5A RCI) (figure 2B) or BMS-821095 (NS5B site I inhibitor) (figure 2C) for 4 weeks. Although all BMS-605339 and BMS-788329-treated mice showed an initial reduction of serum HCV RNA titres, all later showed rebound during treatment. Nucleotide analysis by direct sequencing revealed the emergence of a mutation coding for D168E in the NS3 region (NS3 PI-resistant variant)[21] in a BMS-605339-treated mouse (figure 2A), and a mutation coding for Y93H in the NS5A region (NS5A RCI-resistant variant)[14] in a BMS-788329-treated mouse at week 4 of treatment (figure 2B). Almost all mice treated with BMS-821095 showed an initial reduction in serum HCV RNA titres, and also showed rebound with the emergence of mutations coding for P495A and P495S in the NS5B region (NS5B site I inhibitor-resistant variant)[22] at week 4 of treatment (figure 2C). HCV RNA titre reduction was not obvious in some mice treated with 30 mg/kg of BMS-821095 (figure 2C), suggesting that exposure of this inhibitor at 30 mg/kg dosing was not sufficient to suppress viral replication. Ultra-deep sequence analysis showed the development of a high prevalence of drug-resistant variants in mice sera in the NS3 PI, NS5A RCI-treated mice, and enrichment of pre-existing resistance variants in the NS5B NNI-treated mouse 4 weeks after the beginning of the treatment (figure 2D).

Combination treatment of BMS-788329 with either BMS-605339 or BMS-821095 in HCV genotype 1b mice

As monotherapies with either the NS3 PI, or the NS5A RCI or the NS5B NNI were unable to eradicate HCV RNA due to the emergent resistance variants, we analysed the effects of combining the NS5A RCI with either the NS3 PI or NS5B NNI. Mice infected with HCV genotype 1b (two mice per combination group) were treated with 10 mg/kg of BMS-788329 and either 75 mg/kg twice daily of BMS-605339 or 100 mg/kg of BMS-821095 for 4 weeks. In all mice, HCV RNA became negative by nested PCR 1 week after the beginning of combination therapy and remained undetectable after cessation of treatment (figure 3A,B). Elimination of the virus was assumed as HCV RNA was undetectable by nested PCR in mice livers treated with BMS-788329 and either BMS-605339 or BMS-821095 8 weeks

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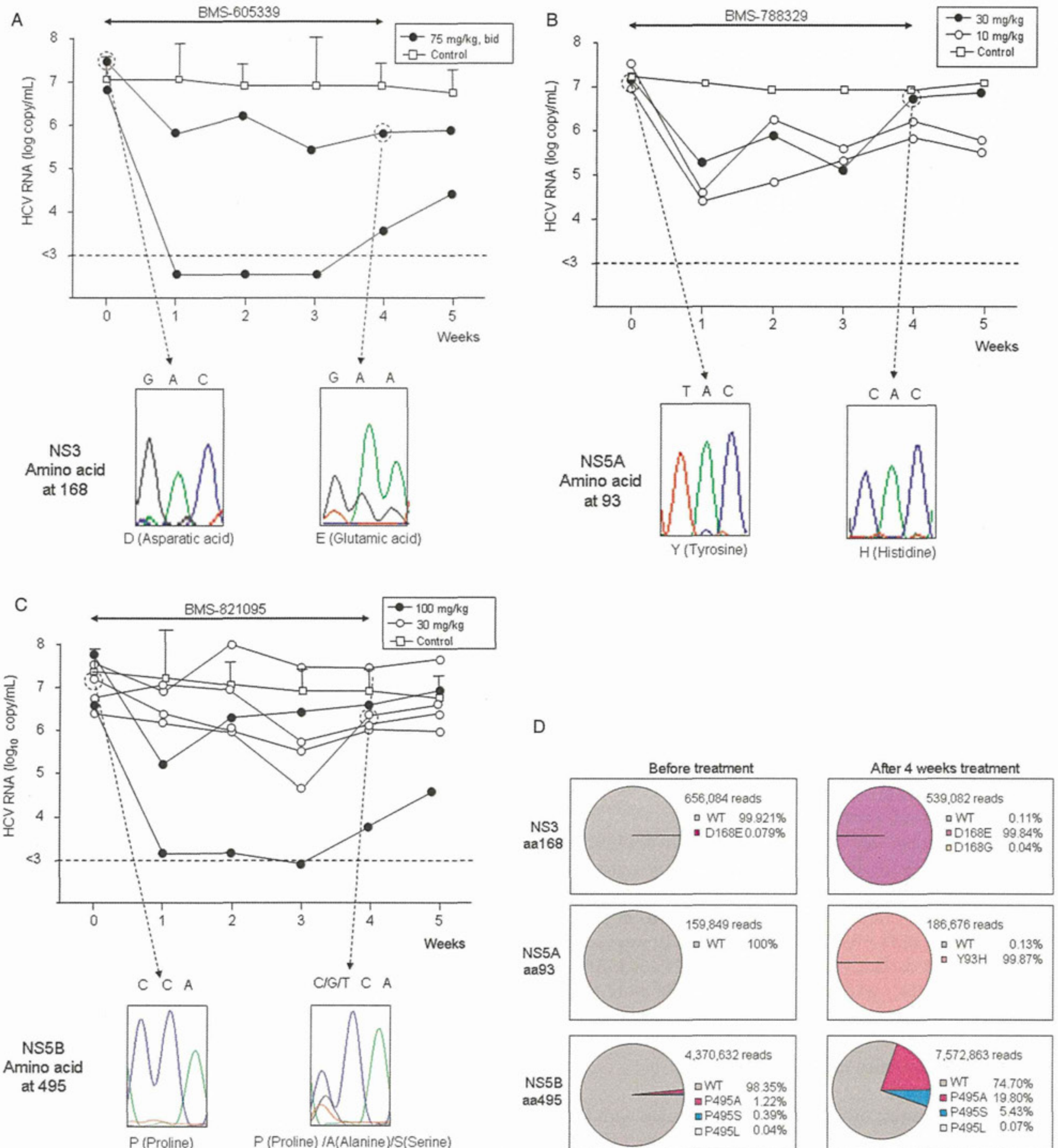

Figure 2 Antiviral effects of BMS-605339, BMS-788329 or BMS-821095 monotherapy in mice infected with hepatitis C virus (HCV) genotype 1b. Mice were injected intravenously with 10^5 copies of HCV genotype 1b. Eight weeks after HCV infection, mice were treated with the indicated concentrations of BMS-605339 (A), BMS-788329 (B) or BMS-821095 (C) for 4 weeks. Serum samples were obtained at the indicated times, and HCV RNA titre and nucleotide and amino acid (aa) sequences were analysed. HCV-infected mice without treatment were also analysed (control). The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml). (D) The aa frequencies in the BMS-605339 (top), BMS-788329 (middle bottom) or BMS-821095 (bottom) treated mice by ultra-deep sequencing before treatment and at 4 weeks are shown.

(week 12) and 7 weeks (week 11) after cessation of therapy, respectively (figure 3C).

Combination treatment of BMS-788329 with either BMS-605339 or BMS-821095 in HCV genotype 2 mice

We analysed the effect of DAA combination therapies on mice infected with HCV genotypes 2a and 2b. In contrast to mice with genotype 1b, mice with genotypes 2a or 2b failed to respond to 4 weeks of treatment with BMS-788329 and BMS-605339 (figure 4A,B). Although the combination of BMS-788329 with BMS-821095 revealed no detectable viral load decline at the time points examined in genotype 2a mice, viral load reductions were detected in genotype 2b mice. Sequence analysis revealed no emergence of resistance variants in the NS3, NS5A or NS5B regions before and 4 weeks after the end of each of these combination treatments, suggesting insufficient drug selection pressure (data not shown).

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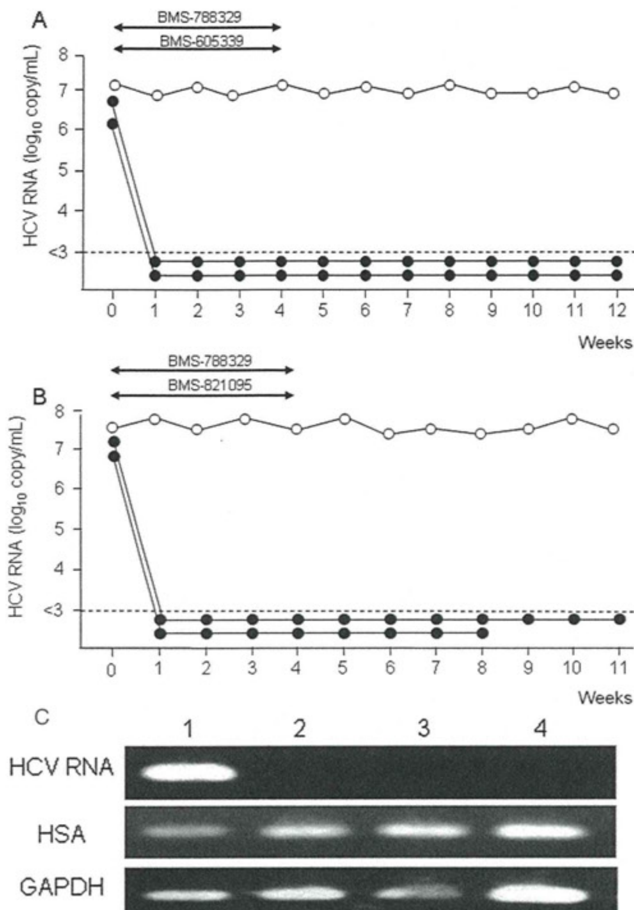

Figure 3 Antiviral effects of NS5A replication complex inhibitor combinations with either an NS3 protease inhibitor or an NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 1b. The four mice were treated with 10 mg/kg of BMS-788329 and either 75 mg/kg twice daily of BMS-605339 (A) or 100 mg/kg of BMS-821095 (B) for 4 weeks (closed circles). Mice without treatment were also analysed (open circles). Serum samples were obtained at the indicated times, and HCV RNA titres were measured. The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml). (C) Nested PCR of HCV RNA, human serum albumin and GAPDH in mouse livers. Livers from mice treated with BMS-788329 and either BMS-605339 (lane 2) or BMS-821095 (lane 3) were obtained. Mouse livers with (lane 1) or without (lane 4) HCV infection were also analysed.

DISCUSSION

DAA-only therapy may offer a promising option to eradicate HCV without incurring the severe side effects of Peg-alfa. However, the emergence of drug-resistant variants is expected for all DAA[21] and has already been observed in combination therapies with two DAA.[5 23 24] If the exposure of the drugs can be safely increased, as we recently reported for a two-drug combination administered to human hepatocyte chimeric mice,[12] eradication of virus is still possible. In this study, we tested the ability of different two-DAA combination therapies to eradicate HCV. Although DAA monotherapies resulted in a viral breakthrough due to the development of a high prevalence of drug-resistant variants (figure 2A–D), DAA combination therapies with the NS5A RCI and either the NS3 PI or NS5B NNI were shown to eradicate virus successfully from HCV genotype 1b-infected mice with only 4 weeks of treatment (figure 3). These two-DAA combination treatments resulted in more rapid, robust declines within the first week of treatment when compared with the suboptimal antiviral responses from each of their respective monotherapies. Furthermore, regimens containing NS5A RCI appeared equally effective in treating mice chronically infected with hepatitis C genotype 1b.

In contrast to the rapid decrease in HCV RNA in mice infected with HCV genotype 1b, HCV genotype 2a and 2b-infected mice either did not respond or responded poorly to treatment with the NS5A RCI combined with either the NS3 PI or NS5B NNI (figure 4A,B). In this study, NS3 PI and NS5B NNI IC_{50} values against genotype 1b were markedly more potent than against genotype 2a in cell culture systems (table 1). These findings are consistent with previous experimental results that reported reduced activity of these drug classes against genotype 2.[25–28] In clinical trials, telaprevir monotherapy was found to result in a rapid decrease in serum HCV RNA levels in patients infected with HCV genotype 2; however, another protease inhibitor, BILN-2061, was less effective in patients with HCV genotype 2 compared to genotype 1.[29] Sequence analysis revealed a pre-existing A156G variant in the NS3 region, a L31M variant in the NS5A region and a I482L variant in the NS5B region in both HCV genotypes 2a and 2b infecting strains used in this study (data not shown). These NS3-A156G and NS5A-L31M variants confer resistance to inhibitors with similar chemical structures to BMS-605339 and BMS-788329, respectively, in genotype 2a replicon cell culture assays.[30–32] Although BMS-788329 was very potent against the genotype-2a JFH-1 replicon (IC_{50} 0.014 nM; table 1), its activity was significantly less against other genotype 2a and 2b viruses, such as genotype 2a HC-J6CF. The loss in potency observed in these viruses is not surprising because these viruses have a methionine at NS5A amino acid residue 31. The IC_{50} of a genotype 2a hybrid replicon containing HC-J6CF NS5A with L31M substitution is approximately 10 nM (data not shown). The minimal antiviral response in mice infected with genotypes 2a and 2b receiving treatments containing BMS-788329 with either BMS-605339 or BMS-821095 can therefore be explained by pre-existing NS3, NS5A and NS5B resistance variants. Nevertheless, it is possible that mice infected with wild-type genotype 2 viruses and subsequently treated with higher doses of each of these DAA in dual or even triple combination therapy may have resulted in more robust reductions in viral load. The human hepatocyte chimeric mouse model offers a viable approach for identifying effective DAA-only combinations that not only act against HCV genotype 1 but against all HCV genotypes.

In summary, we demonstrated that an NS5A RCI can be effectively combined with different inhibitor classes to cure human hepatocyte chimeric mice infected with HCV genotype 1b after 4 weeks of treatment. However, these treatment combinations were not effective against HCV genotype 2. Oral combinations incorporating an NS5A RCI might offer Peg-alfa-free treatment options for genotype 1b chronic hepatitis C patients.

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Competing interests MG and FM are employees of Bristol-Myers Squibb. All other authors declare no competing interests.

Ethics approval The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care.

Patient consent Obtained.

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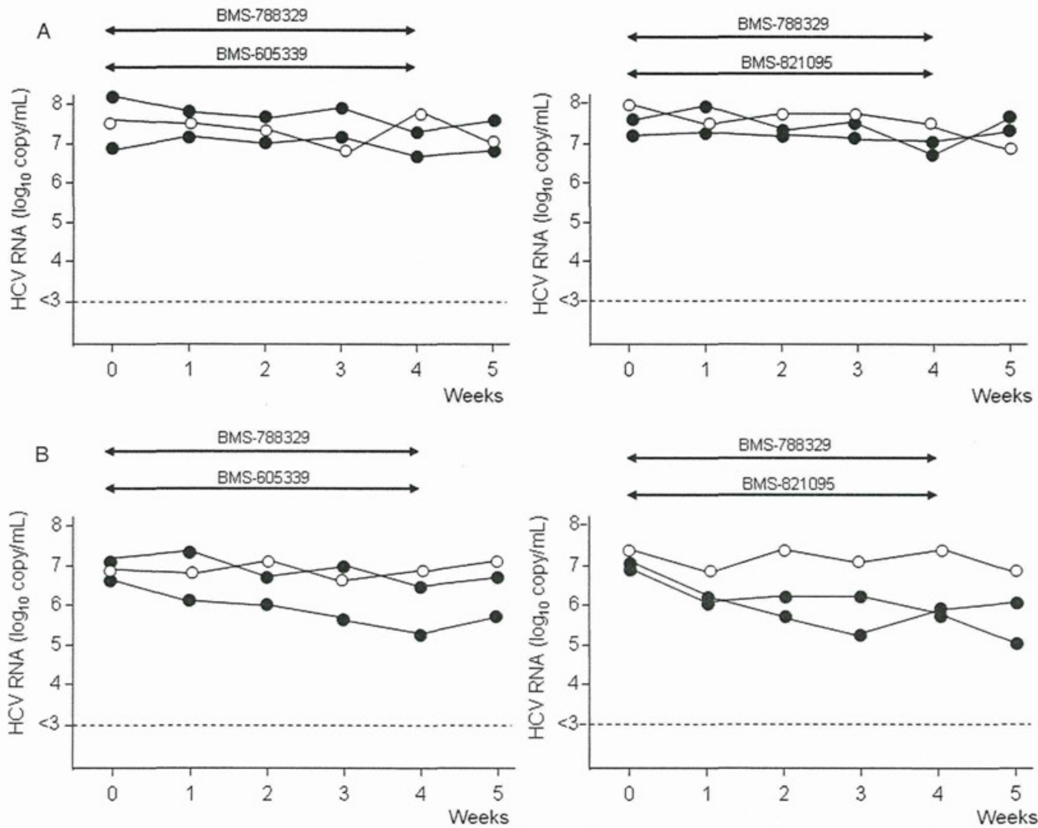

Figure 4 Antiviral effects of NS5A replication complex inhibitor combinations with either NS3 protease inhibitor or NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 2. Each of the four HCV genotype 2a (A) or 2b (B) infected mice were treated with 10 mg/kg of BMS-788329 combined with either 75 mg/kg twice daily of BMS-605339 (left panel) or 100 mg/kg of BMS-821095 (right panel) for 4 weeks (closed circles). Mice without treatment were also analysed (open circles). Serum samples were obtained at the indicated times, and HCV RNA titres were measured. The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml).

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Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice

Niu Shi, Nobuhiko Hiraga, Michio Imamura, et al.

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ORIGINAL PAPER

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Clinical Relevance and Sequence Analysis of the *Helicobacter pylori dupA* Region from Two Areas in Japan with Different Gastric Cancer Risks

Hidetaka Matsuda[1], Yoshiyuki Ito[1], Hiroyuki Suto[1], Akiyo Yamakawa[1], Satoko Satomi[1], Masahiro Ohtani[1,2], Yukinao Yamazaki[1,2], Yukinori Kusaka[3], Yoshiki Shimabukuro[4], Kaoru Kikuchi[4], Yoshihide Keida[4], Takeshi Azuma[5], Yasunari Nakamoto[1]

[1]Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan; [2]Department of Endoscopic Medicine, University of Fukui Hospital, University of Fukui, Fukui 910-1193, Japan; [3]Department of Environmental Health, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan; [4]Division of Internal Medicine, Okinawa Chubu Hospital, Okinawa 904-2293, Japan; [5]Frontier Medical Science in Gastroenterology, International Center for Medical Research and Treatment, Kobe University School of Medicine, Kobe 650-0017, Japan

Abstract

Background & Aims: We investigated the virulence and sequences of duodenal ulcer-promoting gene A (*dupA*) of *Helicobacter pylori* (*H. pylori*) isolated from 2 areas in Japan with different gastric cancer risks.

Methods: The *dupA* status of 248 Japanese *H. pylori* strains (111 from Fukui and 137 from Okinawa) isolated from patients with duodenal ulcers, gastric ulcers, chronic gastritis, or gastric cancer was evaluated by dot-blot hybridization followed by polymerase chain reaction. Sequence analyses of *dupA* and its upstream region were performed in 15 strains.

Results: In both areas, no significant association was observed between *dupA* and the evaluated clinical outcomes. In a multivariable logistic regression, *dupA* positivity was independently associated with strains isolated from Okinawa, showing the lowest incidence of gastric cancer in Japan, and with East Asian-type *cagA* positivity (odds ratio [OR]=2.128, 95% confidence interval [CI] =1.146–3.949 and OR=12.924, 95% CI=1.689–98.901, respectively). Sequence alignment showed *dupA* was divided into 2 genotypes: a Shi470-type (2499 bp) and a J99-type (1839 bp). Among 60 *dupA*$^+$ Japanese strains, 58 (96.7%) were of the Shi470-type and 2 of the J99-type. The nucleotide sequences of 13 Shi470-type Japanese isolates from both areas were highly homologous (98.9%) to each other and a remote Amazonian strain, Shi470. Of these, 12 (92.3%) carried intact full-length *dupA*.

Conclusion: In Japan, *dupA* appeared to be associated with East Asian-type *cagA* and with host residence in Okinawa. The intact genotype of the Shi470-type *dupA* was the major form in Japan.

Immunogastroenterology 2012; 1:127-135

Key words

Helicobacter pylori; *dupA*; *cagA*; virulence factors; type IV secretion system; Okinawa

Introduction

Chronic *Helicobacter pylori* (*H. pylori*) infection is a definitive risk factor for various gastroduodenal diseases such as peptic ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma.[1-3] Although the mechanism of this infection in humans has been extensively studied, no definitive explanation has been obtained. Although infection with strains producing cytotoxin-associated gene A antigen (CagA) and vacuolating cytotoxin (VacA) are reported to increase the risk of severe gastroduodenal diseases in the West, this association has not been demonstrated in East Asia.[4-10] In addition, none of the reported virulence factors including *cagA* and *vacA* expression has been confirmed to be a definitive marker for any single specific clinical outcome.[11-13]

H. pylori exhibits a higher degree of genomic and allelic diversity than most other bacterial species, and significant geographic differences exist among strains.[14-16] Geographic diversity in the *H. pylori* virulence genes has been associated with different regional prevalences in gastroduodenal diseases; the prevalence of gastric cancer (GC) is higher in Japan than in other countries.[17,18] However, Okinawa has the lowest prevalence of GC in Japan and approximately half of that in other prefectures.[19] On the other hand, the prevalence of *H. pylori* in Okinawa does not differ significantly from those in other parts of Japan.[20] To investigate the differences in the prevalence of GC between Okinawa and other prefectures in Japan, we previously analyzed the sequences of *H. pylori* virulence genes isolated from patients in 2 distinct Japanese areas (Okinawa and Fukui) and found that the genotypes of *cagA* were the most important factor causing the discrepancy.[19,21-27]

Recently, Lu et al. described a novel *H. pylori* virulence factor,

Yasunari Nakamoto, Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan; Email: nakamoto-med2@med.u-fukui.ac.jp
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designated duodenal ulcer-promoting gene A (*dupA*).[28] *dupA* comprises 2 genes—*jhp0917* and *jhp0918*—present in the *H. pylori* plasticity zone. A single base-pair insertion (C or T) in the 3′ region of *jhp0917* confers a continuous 1839 bp open reading frame (ORF). As *dupA* shows homology to *virB4*, a *virB/D* homolog gene encoding the type IV secretion system (T4SS) of *Agrobacterium tumefaciens*, this gene is considered a component of a novel TFSS of *H. pylori*.[11,28-30]

Infection with *dupA*$^+$ strains is associated with increased interleukin-8 (IL-8) production from the gastric antral mucosa and the development of duodenal ulcers (DUs), in 3 different countries (Japan, Korea, and Colombia). Infection with the *dupA*$^+$ strain is also a potential protective factor against atrophic gastritis and GC.[28] However, subsequent studies in various geographic areas were unable to confirm the close association between *dupA* and clinical outcomes.[31-42] Moreover, the findings of previous Japanese studies have been inconsistent.[28,39,40]

The association between the presence of *dupA* and other virulent factors of *H. pylori* remains largely unexamined. Although some previous studies have discussed the association between the statuses of *dupA* and *cagA*,[31-35,37,42] the genetic diversity of *cagA* (i.e., the East Asian-type or the Western-type) was not considered. Similarly, the genetic structure of dupA is not well known. Most previous studies analyzed the limited nucleotide sequences corresponding to *jhp0917* and *jhp0918*,[28,31,33,34,36,38,39,41,43,44] but only a few have investigated the surrounding *dupA* regions, especially the upstream ones, which may regulate *dupA* expression.[30,45,46] In this study, we aimed to examine whether (i) *dupA* could be a disease-specific risk determinant in Japan; (ii) *dupA* could explain the lower risk of GC in Okinawa; and (iii) *dupA* and *cagA* statuses were associated, even when the genotypes of *cagA* (i.e., the East Asian or Western genotype) were considered. We also discuss whether the genetic diversity of the *dupA* upstream region exists among the groups of clinical isolates in Japan.

Materials and Methods

H. pylori *strains, culture, and DNA extraction*

A total of 248 clinical *H. pylori* isolates were obtained from Japanese patients (111 patients in Fukui and 137 in Okinawa) during upper gastrointestinal endoscopy performed at the University of Fukui Hospital, Fukui, and Okinawa Chubu Hospital, Okinawa. The patient group in Fukui comprised 20 patients with DU, 20 with gastric ulcer (GU), 39 with chronic gastritis, and 32 with GC. The patient group in Okinawa comprised 26 patients with DU, 18 with GU, 60 with chronic gastritis, and 33 with GC. This study was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from each patient and the institutional ethics committee approved the study protocol. The presence of *H. pylori* in a patient was confirmed through positive results of the rapid urease test (RUT), the C13 urea breath test (UBT), or a histological analysis. The clinical diagnosis was established by endoscopic findings. In addition, the diagnosis of GC was histologically confirmed in all cases. *H. pylori* culture and DNA extractions were conducted as described previously.[21-25] Patients from whom *H. pylori* could not be cultured were excluded from the study.

H. pylori *sequence analysis*

The primers used in this study are shown in **table 1** and **figure 1**. The presence of *dupA* was defined by both dot-blot hybridization and polymerase chain reaction (PCR) as described below.

Dot-blot hybridization was performed using Hybond-N$^+$ nylon membranes (GE Healthcare Lifesciences, Uppsala, Sweden) containing 10-20 ng aliquots of genomic DNA per spot from each strain of interest and hybridization probes labeled using the enhanced chemiluminescence kit (Amersham™ ECL™ Direct Nucleic Acid Labeling and Detection System; GE Healthcare Lifesciences), according to the manufacturer's instructions. Probes for the 2 *dupA* segments (*jhp0917* and *jhp0918*) were generated by PCR from J99 genomic DNA with primers JHP0917(+) and JHP0917(-)(307 bp product) and JHP0918-1F and JHP0918-1R (377 bp product). The PCR-amplified DNAs were purified using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) prior to ECL labeling for use in hybridization according to the manufacturer's instructions. A probe for *ureI* (a housekeeping gene of *H. pylori*) was used as a positive control for hybridization.

Following the dot-blot hybridization, PCR was performed using the DupAF113/R1083 and DupAFb/Rb primer pairs. Specific PCR and DNA sequencing was conducted as described previously.[21-27] The PCR products were purified on Centricon-100 Concentrator columns (Amicon, Beverly, MA, USA) and then sequenced directly using the BigDye Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Cycle sequencing reactions were performed for both strands. DNA sequencing editing and analysis were performed with GENETYX-MAC software version 14.0.1 (GENETYX Corporation, Tokyo, Japan).

For the dot-blot hybridization, the tested strains were considered *dupA*$^+$ or *dupA*$^-$ if the *jhp0917* and *jhp0918* genes were both present or absent, respectively. When a strain was positive for only one of these genes, the *dupA* status was defined in accordance with the PCR results. For the PCR assay, only strains showing both DupAF113/R1083 and DupAFb/Rb positivity were considered *dupA*$^+$, while all the remaining strains were defined as *dupA*$^-$. Both assays were performed for all strains. If a strain showed discrepant results between the 2 tests, the PCR result was adopted to define its *dupA* status.

The *cagA* detection and genotyping of all 248 isolates were performed by PCR tests and subsequent direct sequencing as described previously.[23-27]

Nucleotide sequence accession numbers

The DNA sequences of the *dupA* region of the 14 representative Japanese strains used in this study were deposited in the DDBJ database under accession numbers AB617832-AB617845; their characteristics are listed in **table 2**. In this study, we refer to the released sequences of 12 *H. pylori* strains whose complete genomes were deposited in GenBank under the following accession numbers: AE001439 for J99, CP001072 for Shi470,

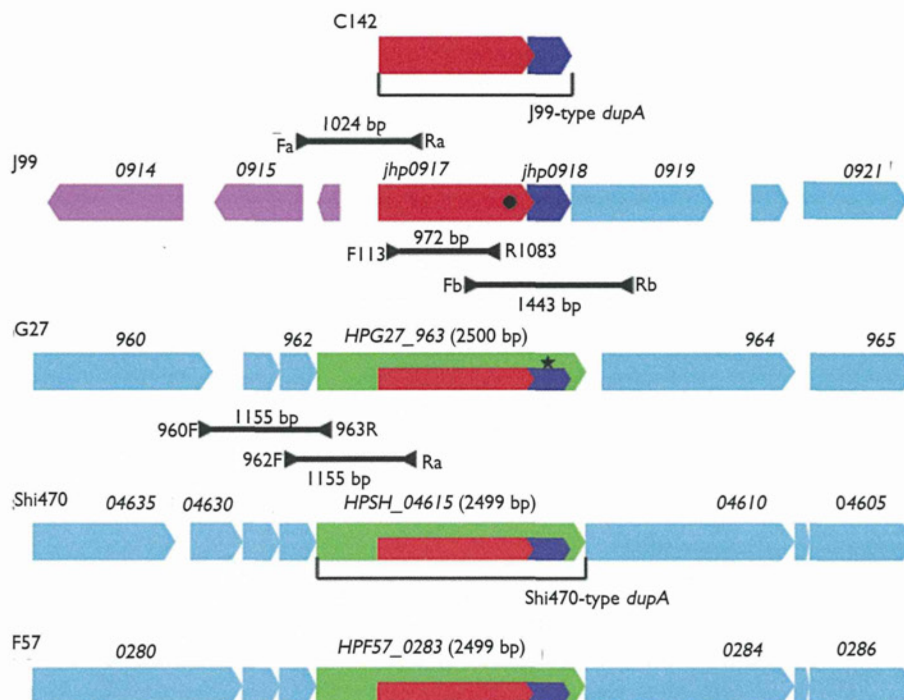

Figure 1. Comparison of the genomic regions surrounding duodenal ulcer-promoting gene A (*dupA*) in 4 published *Helicobacter pylori* (*H. pylori*) genomes and strain C142. The positions of the primers used in this study are also shown.
Sequences of *dupA* (corresponding to *jhp0917* and *jhp0918*) and the surrounding region in the published full-genome sequences of 4 *dupA*⁺ *H. pylori* strains—J99 from the United States, G27 from Italy, Shi470 from a remote Amazonian village in Peru, and F57 from Japan—and strain C142 from Colombia are shown. Initially, *dupA* was reported in J99 as a 1839 bp gene consisting of *jhp0917* and *jhp0918* genes and was found to contain a 1 bp insertion at the 3′ end of *jhp0917*, which J99 lacked (indicated by a black dot). Lu et al. found *dupA* of C142 to be a representative gene.[27] In strains Shi470 and F57, the sequences corresponding to dupA (*jhp0917* and *jhp0918*) were parts of continuous 2499 bp genes, which were highly homologous to each other (*HP_04615* and *HPF57_0283*, respectively). *HPG27_963*, a 2500 bp sequence in strain G27, was homologous to both *HP_04615* and *HPF57_0283* but contained a stop codon created by a 1-bp insertion of a "T" after position 1176, leadings to truncation of the gene product (indicated by a black star). In this study, *jhp0917* and *jhp0918* with the described 1bp insertion was defined as the J99-type *dupA* (indicated by red and blue boxes) and *HPSH_04615* as the Shi470-type *dupA* (indicated by a green box). In J99, the upstream region of *dupA* was completely different from those of the 3 other strains with a full published genome. The nucleotide sequences surrounding Shi470-type *dupA* of Shi470, F57, and G27 were homologous. In the present study, 5 primer pairs were designed to confirm the positivity of *dupA* and consequently classify the genotype of *dupA* as either the J99 or Shi470 type.

CP001173 for G27, CP002076 for Cuz20, CP002184 for HP 908, CP002332 for Gambia 94/24, CP002336 for SouthAfrica7, AP011945 for F57, CP002571 for HP 2017, CP002572 for HP 2018, CP002982 for Puno135, and CP002983 for SNT49. We also refer to the dupA sequence of strains C142 (GenBank accession number AB196363; **Fig. 1**), and AB21 (GenBank accession number GU932735).

Statistical analysis
The associations of *dupA* prevalence with clinical diagnosis, geographic locations, and *cagA* genotypes were examined using the Fisher's exact probability test for univariate analysis, and multivariable logistic regression for multivariable analysis. In the multivariable logistic regression, *dupA* positivity was used as a dependent variable and the other 3 as independent variables. All tests were 2-sided, and a probability level (P) of <0.05 was considered significant. All statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA).

Results

Association between dupA *and clinical outcomes or* cagA *status*
Among the 111 tested strains from Fukui, 24 (21.6%) were positive for both *jhp0917* and *jhp0918* on the dot-blot hybridization, while 22 (19.8%) were positive for both DupAF113/R1083 and DupAFb/Rb according to the PCR test. Accordingly, these strains were defined as *dupA*⁺ on each test. For the dot-blot hybridization, 2 strains were positive for both *jhp0917* and *jhp0918*, but were finally revealed to be *dupA*⁻ by the PCR. One strain was considered *dupA*⁻ because it was *jhp0917*⁺ but *jhp0918*⁻ on the dot-blot hybridization and was confirmed as *dupA*⁻ based on the PCR results. In Okinawa, no strains showed opposite results between the dot-blot hybridization and the PCR tests. The findings were consistent between these 2 tests, with 38 (27.7%) of the 137 tested Okinawan strains defined as *dupA*⁺. The overall agreement between the dot-blot hybridization and the PCR results was 98.2% (109/111) in Fukui and 100% (137/137) in Okinawa, respectively. Combining the results of the

Table 1 Oligonucleotide primers used in this study

Primer	Primer sequence (5'→3')	Reference
DupAF113	GACGATTGAGCGATGGGAATAT	Argent et al. (2007)
DupAR1083	CTGAGAAGCCTTATTATCTTGTTGG	Argent et al. (2007)
DupAFa	TCCCAAAATGCTTTGCCTGCG	Present study
DupARa	GCGCTCTGTCAGTAGAAACC	Present study
DupAFb	TGAGCGTGGTAGCTCTTGAC	Present study
DupARb	TCGCTTACAATCCTACCTAGCC	Present study
DupA960F	CCTGAGCCTGTGAATAAACCTG	Present study
DupA963R	ATATGGCGTGGTGCTATTGGTG	Present study
DupA962F	GTGTAGCCACCTCTTCTTTATC	Present study
JHP0917 (+)	TGGTTTCTACTGACAGAGCGC	Lu et al. (2005)
JHP0917 (-)	AACACGCTGACAGGACAATCTCCC	Lu et al. (2005)
JHP0918-1F	CTCAAGCTAGAAAGATCAACGG	Present study
JHP0918-1R	ACTCTTCCTTATAAGTTTCTTGG	Present study

Table 2 Characteristics of the sequenced strains

H. pylori strain	Origin	Disease	*cagA* genotype	*dupA* genotype	*dupA* size (bp)	*dupA* null mutation
F51	Fukui	DU	East Asian	Shi470	2499	–
F57	Fukui	GC	East Asian	Shi470	2499	–
F58	Fukui	Gastritis	East Asian	Shi470	2499	–
F64	Fukui	DU	East Asian	Shi470	2499	–
F77	Fukui	GU	East Asian	Shi470	2499	–
F80	Fukui	Gastritis	Hybrid	J99	1840	+
F228	Fukui	GC	East Asian	Shi470	2499	–
OK99	Okinawa	DU	East Asian	Shi470	2499	–
OK108	Okinawa	GU	East Asian	Shi470	2499	+
OK165	Okinawa	Gastritis	East Asian	Shi470	2499	–
OK169	Okinawa	DU	East Asian	Shi470	2499	–
OK203	Okinawa	Gastritis	Western	Shi470	2499	–
OK303	Okinawa	GC	East Asian	Shi470	2499	–
OK309	Okinawa	GC	East Asian	Shi470	2499	–
OK317	Okinawa	Gastritis	East Asian	J99	1840	+

H. pylori, Helicobacter pylori; *cagA*, cytotoxin-associated gene A; *dupA*, duodenal ulcer-promoting gene A; DU, duodenal ulcer; GC, gastric cancer; GU, gastric ulcer.

dot-blot hybridization and the PCR, the *dupA* status of Fukui and Okinawa were determined.

Twenty-two (19.8%) *dupA*$^+$ strains were found in Fukui. The frequency was higher in strains from patients with DU (7/20, 35.0%) than in those from patients with GU (3/20, 15.0%), chronic gastritis (6/39, 15.4%), or GC (6/32, 18.8%). The *dupA* gene was positive in 38 strains (27.7%) isolated in Okinawa, and the prevalence was greater among strains from patients with GC (14/33, 42.4%) than in those with DU (5/26, 19.2%), GU (5/18, 27.8%), or chronic gastritis (14/60, 23.3%). However, no statistical significance between the *dupA* status and clinical outcomes was observed in both areas (**Table 3**). Further, no association was observed between the dupA status and GC histology, with the intestinal and diffuse types observed in both areas (data not shown).

We also determined the *cagA* genotypes. Two strains of F80 (*dupA*$^+$) and OK204 (*dupA*$^-$) carried the East Asian-type CagA motif (AB'DD and ABD, respectively) but were assigned as Western in origin on the basis of our previous phylogenetic analysis of *cagA* and were considered a hybrid type. Both F80 and OK 204 were isolated from DU patients. Another strain (OK181) isolated from an Okinawan host with chronic gastritis could not be classified owing to the lack of a CagA C/D motif, but was assigned as a Western type by phylogenetic analysis. Consistent with our previous reports,[23] a variety of genotypes was observed for *cagA* among the 137 *H. pylori* strains in Okinawa, including 110 (80.3%) strains with East Asian-type *cagA*, 20 (14.6%) with Western-type *cagA*, 6 (4.4%) *cagA*$^-$ strains, and 1 (0.7%) with hybrid-type *cagA*. In contrast, almost all strains in Fukui (109/111, 98.2%) possessed East Asian-type *cagA*, only 1 (0.9%) carried Western-type *cagA*, and none were *cagA*$^-$. One (0.9%) strain with hybrid-type *cagA* was also detected in Fukui. This observed high *cagA* prevalence in Japan was also consistent with previous reports.[47,48]