let-7b (m7b) diminished its inhibitory effect on HCV expression and resulted in a slight inhibition of HCV reporter virus luciferase activity ($p < 0.05$). Together, these data implicate that inhibition of HCV RNA expression accounts for the suppressive effect of let-7b on HCV infection.

Let-7b physically interacts with the HCV genome

Argonaute 2 is the core component of miRNA-induced silencing complex (miRISC), which binds the guide miRNA to silence target mRNAs [30]. To determine whether Ago2 together with let-7b and HCV RNA form a miRISC complex, HCV RNA was co-transfected with let-7b into Huh7.5 cells followed by immunoprecipitation using the anti-Ago2 antibody (Ago2-IP). Because the "site 1" sequence (Table 1) is the most important HCV genome sequence for miR-122 binding and for regulation of HCV by miR-122, "site 1" mutation S1-p34 m (miR-122-mut) [11, 12] was introduced into the HCV subgenome to minimize the interference from the endogenous miR-122. The amount of Ago2-IP-associated HCV RNA was quantified by real-time RT-PCR. As shown in Fig. 4a, both HCV RNA subgenomes with wild-type or mutant miR-122 binding site were found to associate with the miRNP complex, while let-7b promoted HCV-miRNP interactions when miR-122 binding site was mutated. The total amount of let-7b associating with the Ago2-IP fraction was unchanged (Fig. 4b). Furthermore, knockdown of endogenous let-7b in Huh7.5 cells followed by Ago2-IP revealed that both the amounts of HCV RNA (Fig. 4c) and let-7b (Fig. 4d) in the Ago2-IP fraction were dramatically decreased. These data thereby indicate that let-7b physically interacts with HCV RNA in the Ago2-containing miRNP complex.

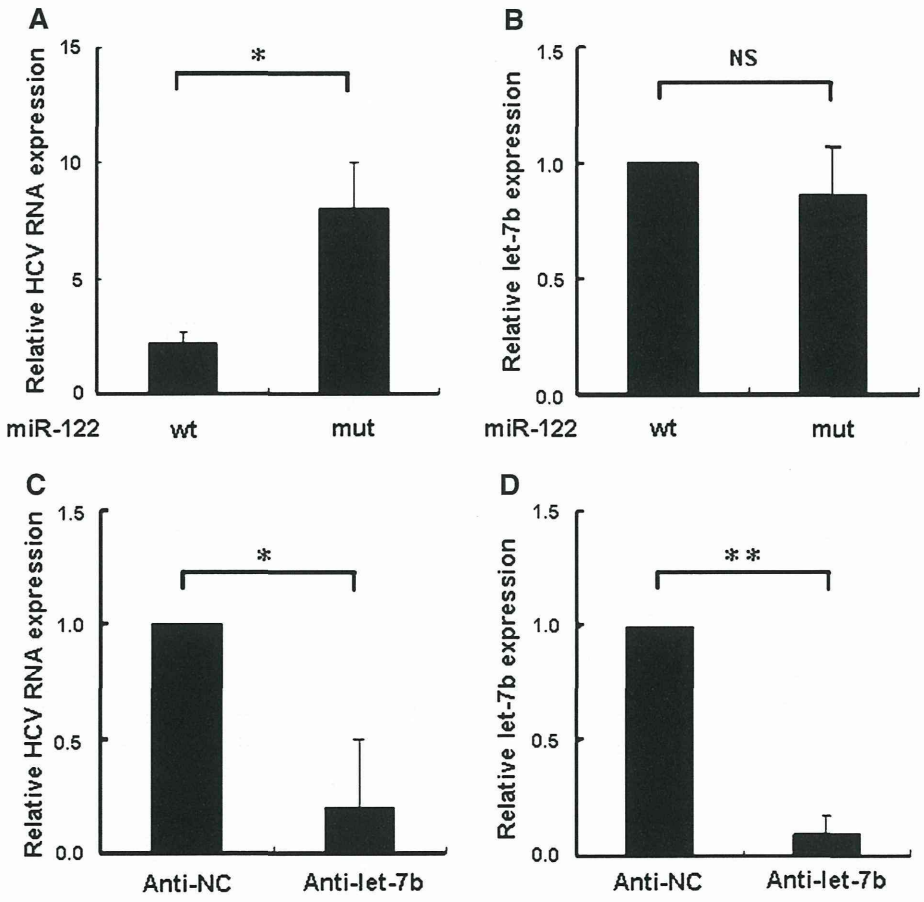

Fig. 4 Let-7b is associated with HCV genome in miRNP complex. **a**, **b** Huh7.5 cells were transfected with 100 pmol of miRNA along with 10 μg of either wild-type (miR-122-wt) or mutant (miR-122-mut) HCV subgenome RNA. The cell extracts were collected to perform co-immunoprecipitation with the anti-Ago2 antibody (Ago2-IP). HCV replicon RNA (*panel* **a**) and let-7b (*panel* **b**) were measured by real-time RT-PCR using total RNA sample from the Ago2-IP fraction. **c** ,**d** For knockdown of endogenous let-7b, Huh7.5 cells were transfected with 100 pmol of the indicated miRNA inhibitors (Anti-NC and Anti-let-7b) for 24 h followed by electroporation of the cells with 10 μg of miR-122-mut and 100 pmol of the indicated miRNA inhibitors. The cell extracts were collected to perform Ago2-IP and the HCV replicon RNA (*panel* **c**) and let-7b (*panel* **d**) were measured by real-time RT-PCR. The relative levels for HCV RNA and let-7b in the Ago2-IP complexes were shown. The data represented the mean ± SD ($n = 3$; $*p < 0.05$, $**p < 0.01$)

Identification of let-7b-responsive elements on the HCV genome

To identify MRE for let-7b, HCV-N genomic sequences (genotype 1b) were subject to bioinformatic prediction using miRanda and RNAhybrid. Several putative MREs for let-7b were revealed (Fig. 5a; Supplementary Tables 3, 4). It is noted that MRE2 (nt 8,745–8,766) and MRE3 (nt 8,977–8,995) that located within the HCV NS5B coding region had the highest prediction score and were selected for analysis. Moreover, the MRE1 (nt 338–365) and MRE4 (nt 9,566–9,603) at the non-coding region that had the lowest minimum free energies (MFE) in NCR region (Table 2) were also subject to further analysis.

To determine whether any of these sequences is the authentic MREs for let-7b, luciferase reporter plasmids with the reported let-7b target sequence (pluc-let-7b) and the putative MREs in HCV genome were constructed (pluc-MRE1, pluc-MRE2, pluc-MRE3, and pluc-MRE4). After co-transfection with let-7b or a negative control miRNA into 293T cells, the luciferase activities for each individual reporter plasmid were measured. Our data revealed that let-7b decreased pluc-MRE1, pluc-MRE2, and pluc-MRE3 luciferase activity by 28, 31, and 37%, respectively (Fig. 5b). No effect was found for pluc-MRE4. These data indicate that the MRE1, MRE2, and MRE3 are the potential let-7b binding sites on HCV genome.

Mutations of the putative let-7b MREs were introduced into the HCV subgenome to exam whether these MREs are responsible for the suppressive effect of let-7b. Silent mutations of MRE2 and MRE3 were designed to avoid amino acid changes while a six-nucleotide substitution mutation was introduced into HCV Rep-Feo subgenome (wild-type) to generate mMRE1, mMRE2, mMRE3, and mMRE2,3, respectively. Structural analysis of these mutations revealed that most of the HCV RNA genome structures were maintained except that mMRE1 appeared

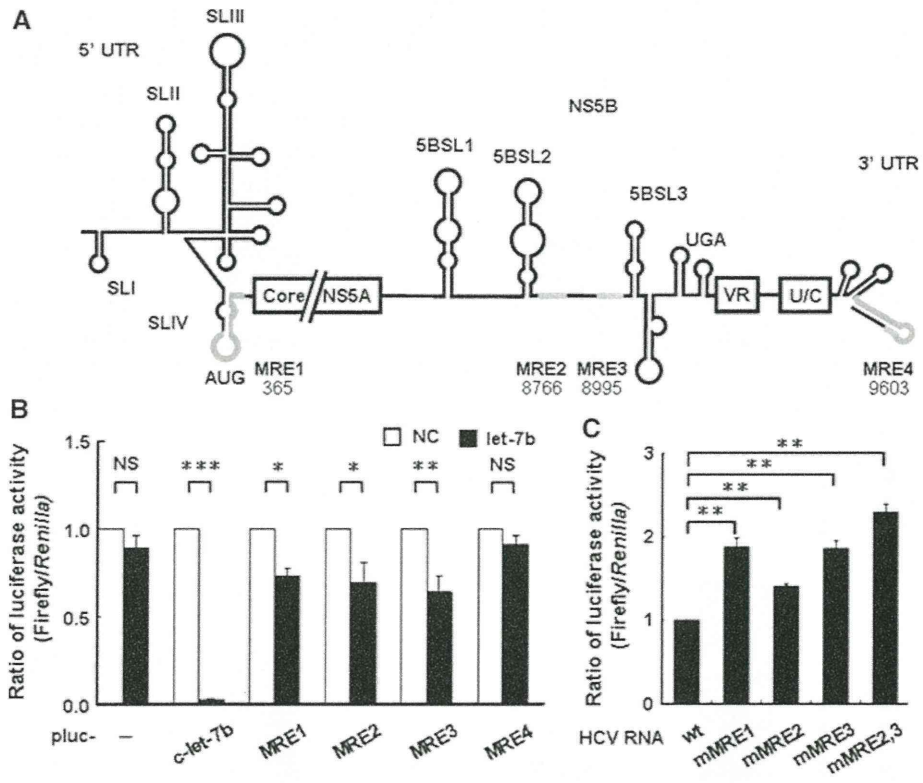

Fig. 5 The MREs of let-7b are located at the NS5B coding sequences and 5′-UTR of HCV genome. **a** Schematic representation for the predicted MREs of let-7b on HCV genome. The *number* corresponds to the first nucleotide of the predictive seed region. **b** The precursor of let-7b or negative control miRNA (NC) was co-transfected with the indicated luciferase reporter plasmid and pRL-TK into 293T cells for 24 h. The luciferase activities were measured and the relative firefly versus Renilla luciferase activity was shown. The plasmid containing perfect complementary sequence of let-7b (c-let-7b) and the vector control reporter plasmid (luciferase activity arbitrarily denoted as one) was used as the positive and negative control, respectively. The data represented the mean ± SD ($n = 3$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *NS* no significance). **c** The RNAs for wild-type (wt) HCV genome and the genome with mutations at the indicated MREs regions were obtained by in vitro transcription and were transfected individually into Huh7.5 cells along with let-7b precursor or negative control miRNA (NC) by electroporation. The luciferase activity was determined at 96 h post-transfection. The luciferase activity generated by wild-type HCV genome was arbitrarily denoted as 1 and the luciferase activity derived from each mutant HCV genome normalized by luciferase activity from wild-type was shown. The data represented the mean ± SD ($n = 3$; $**p < 0.01$)

Table 2 Characterization of let-7b predicted binding sites on HCV genome

MRE	Secondary structure	MFE[a]	Score[a]	Position (HCV-N)
MRE1	miRNA 3'-UUGG----UGUGUU--GGAUGAUGGAGU-5' Target 5'-CACCAUGAGCACGAAUCCUAA-ACCUCAA-3'	-26	84	338-365
MRE2	miRNA 3'-UUGGUGUGUUGGAUGAUGGAGU-5' Target 5'-GGCAAAAGGGUGUACUACCUCA-3'	-16.7	156	8745-8766
MRE3	miRNA 3'-UUGGUGUGUUGGAUGAUGGAGU-5' Target 5'-AGCCACUUGAC---CUACCUCA-3'	-21.1	152	8977-8995
MRE4	miRNA 3'-UUGGUGUGUUGG----------AUGA-UGGAGU-5' Target 5'GAGCCGCAUGACUGCAGAGAGUGCUGAUACUGGCCUCU-3'	-28.5	93	9566-9603

[a] MFE was calculated by RNAhybrid while Score was calculated by miRanda

to generate a small stem-loop structure (Supplementary Fig. S1). These mutated HCV RNAs were obtained by in vitro transcription and, together with let-7b, electroporated into Huh7.5 followed by analysis of luciferase activity. Our data revealed that the luciferase activities for HCV subgenome with mMRE1, mMRE2, and mMRE3 were 87, 40, and 86% higher than the wild-type HCV subgenome, respectively (Fig. 5c), implicating that let-7b-mediated suppression of HCV replicon activity is abrogated by mutating the target sequences on HCV genome. Moreover, HCV subgenome with double mutations of MRE2 and MRE3 synergistically enhanced luciferase activity when compared to the single mutant for these two MREs. The RNA structure did not contribute to the loss of let-7b responsiveness because Mfold analysis demonstrated that the wild-type and mutant HCV subgenome had similar RNA structure (Supplement Fig. S1). These data thereby indicate that 5′-UTR and NS5B coding sequences contain let-7b binding sites.

The antiviral effect of let-7b is independent of inhibition of HCV translation

It has been reported that miR-122 enhances HCV replication by stimulating internal ribosome entry site (IRES)-mediated translation in cultured cells [12]. Because MRE1 is located at domain IV of IRES [31], the possibility of let-7b modulating HCV replication through translation was also examined. The replication-deficient J6/JFH (p7-Rlu2A) GNN HCV mutant RNA was transfected along with a capped and polyadenylated FLuc mRNA as an internal control for transfection and translation. The ratio of Renilla luciferase (RLuc) to firefly luciferase (FLuc) activity was used to measure the IRES-directed translation activity. As shown in Fig. 6, miR-122 enhanced HCV IRES activity for approximate threefold, while miR-122 inhibitor resulted in approximate 50% decrease of the activity. However, let-7b or its inhibitor had no effects on HCV translation (Fig. 6). These data indicate that let-7b regulates HCV RNA replication through a mechanism independent of HCV translational regulation.

Let-7b and INFα-2a elicit synergistic anti-HCV activity

We examined further whether there is a synergistic effect between let-7b and IFNα-2a. The Huh7/Rep-Feo cells were treated with different concentrations of IFNα-2a and the luciferase activity for HCV subgenome was measured to determine the optimized dosage of IFNα-2a for synergistic study. As shown in Fig. 7a, the luciferase activity was suppressed by IFNα-2a in a dose-dependent manner with the IC50 equivalent to 1.39 ng/ml. When Huh7/Rep-Feo cells were transfected with let-7b followed by treatment with IFNα-2a, a 60 and 70% decrease in luciferase activity was observed in relative to let-7b or IFNα-2a alone, respectively (Fig. 7b, $p < 0.01$). These data thereby indicate that let-7b and IFNα-2a elicit synergistic inhibitory effect on HCV expression.

Discussion

The interplays between viral infection and miRNA have been demonstrated since the first report unfolding miR-32 as the negative regulator of primate foamy virus RNA accumulation [32]. Subsequently a number of miRNAs

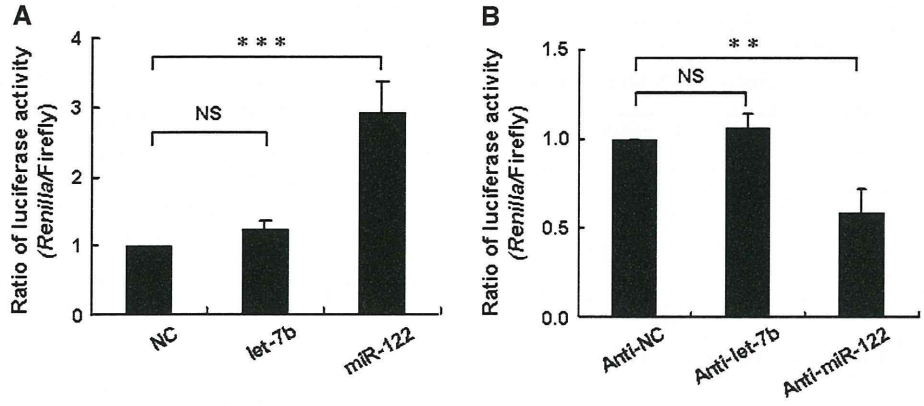

Fig. 6 Let-7b decreases HCV RNA expression independent on translation inhibition. **a, b** Huh7.5 cells were transfected with the indicated miRNAs (*panel* **a**) or miRNA inhibitors (*panel* **b**). Twenty-four hour later, HCV RNAs carrying GND mutation and Renilla luciferase coding sequence were transfected with a capped and polyadenylated firefly luciferase mRNA. At 4 h after transfection, the cell lysates were subject to dual luciferase activity assays. The relative firefly versus Renilla luciferase activity is shown. The data represented the mean ± SD ($n = 3$; $^{**}p < 0.01$; $^{***}p < 0.001$; *NS* no significance)

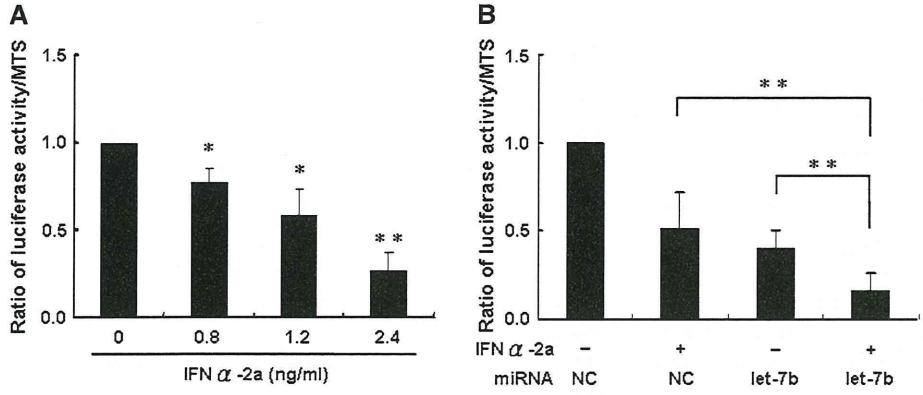

Fig. 7 Let-7b and IFNα-2a elicit synergistic inhibitory effects on HCV RNA accumulation. **a** Huh7/Rep-Feo cells (3×10^4) were treated with the indicated doses of IFN-2α for 72 h and the luciferase activity and cell viability were determined. **b** Huh7/Rep-Feo cells were transfected with 100 nM of let-7b or negative control miRNA (NC) by RNAiMAX for 4 h followed by treatment with INF-2α or medium control for additional 72 h. The luciferase activity and cell viability were determined. The data represented the mean ± SD ($n = 3$) with the luciferase activity normalized by the cell viability. ($^{*}p < 0.05$; $^{**}p < 0.01$)

were found to elicit anti-HCV activity [11, 13, 14]. In this study, bioinformatic tools and virological analyses are employed to unveil novel cellular miRNAs associated with HCV infection. We demonstrate that, in addition to miR-122 that has been reported to augment HCV infection, let-7b targets the HCV genome leading to a decrease in HCV RNA accumulation and viral production. This study thereby represents the first report to identify let-7b as a negative regulator of HCV infection.

Let-7b is the first known human miRNA [33] that is closely associated with the status of cellular differentiation and is usually down-regulated in cancers [34, 35]. Experimental evidence we present in this study unveils the role of let-7b in the control of HCV pathogenesis. Let-7b expression is irreversibly correlated with HCV infectivity in the cell-based systems; the cell lines bearing replicated HCV genome that are permissive for HCV replication (such as Huh7.5 and Con1) usually have low levels of let-7b expression. Moreover, let-7b is associated with HCV genome in Ago2 miRNP complex. Cellular study further reveals that let-7b diminishes luciferase reporter gene expression in Huh7/Rep-Feo subgenome replicon (1b genotype), the viral protein expression in ConI replicon (1b genotype) and HCV RNA accumulation and viral production upon HCVcc infection (2a genotype). These findings not only indicate that let-7b plays a role in the host antiviral response, but also reveal the universal effects of let-7b on different HCV genotypes.

The molecular basis for the anti-HCV effect of let-7b is also elucidated in this study. Our data support the notion that let-7b directly interacts with the HCV genome and modulates virus production. Although a number of miRNAs have been reported to regulate HCV replication and pathogenesis, only miR-122 and miR199a* were

demonstrated to directly target on HCV genome [11, 13]. Let-7b thereby represents the third cellular miRNA that elicits a direct effect on HCV genome and modulates HCV replication. In contrast to miR-122 and miR-199a*, of which the target sequences mapped to 5′-UTR [11, 13], one of the unique features for let-7b is that two of the let-7b target sequences, MRE2 and MRE3, are located within the coding region of NS5B. Although it is not common, miRNA has been shown to affect gene expression by interacting with mRNA coding regions [36, 37]. For example, miR-148 and miR-24 repress DNA methyltransferase 3b and p16 expression, respectively, primarily through the coding region recognition site [38, 39]. Let-7b targets the coding sequence of Dicer and establishes a miRNA/Dicer autoregulatory negative feedback loop. While the advantages for let-7b targeting the HCV coding sequence remain to be elucidated, let-7b symbolizes the first cellular miRNA with recognition sequences in the coding region of the HCV genome.

Although our data indicate that let-7b acts on the HCV genome leading to a decrease in HCV expression, we cannot rule out that the host factors regulated by let-7b may also play a role in the regulation of HCV expression. Several host factors are down-regulated by let-7b, including HMGA2 [40–42]. Moreover, TargetScan prediction reveals at least 79 cellular target genes regulated by let-7b have some associations with HCV infection. Despite that knockdown of HMGA2 does not have any effect of HCV protein expression, whether the other host factors mediating let-7b effects on HCV expression remains to be investigated.

While only let-7b meets our preset selection criteria, bioinformatic prediction data reveal that three other family members of let-7, including let-7a, let-7c, and let-7f, are also liver-abundant (Supplementary Table 1). Because let-7 family members differ by only one to a few nucleotides [43], let-7a, let-7c, and let-7f were also tested for their potential effects on HCV expression. As shown in Supplementary Fig. S2, let-7a and let-7f were expressed at a lower level in HCV-sensitive cell lines while let-7c was expressed at a higher level. In addition, these three miRNAs can also reduce HCV activity on subgenome replicon cells and reporter virus. However, let-7b exhibits more prominent suppressive effects than the others. Hence, it is likely that let-7 family members may act on HCV in a similar way to let-7b with various regulations.

In addition to controlling multiple cellular events, miRNA has been proposed as a therapeutic regimen for various diseases [44, 45]. Recently, a locked nucleic acid-modified oligonucleotide complementary to miR-122 exhibits a long-lasting suppression of HCV viremia in chronically infected chimpanzees [46]. Small-molecule inhibitors and activators of miR-122 have been developed to reduce HCV viral replication [47]. In this study, we found that let-7b plays a role in host defense to combat HCV infection and reduces HCV infectivity. The synergistically inhibitory effect of let-7b and IFNα-2a on HCV replication further implies that let-7b is a good candidate for developing an adjuvant regimen for IFNα-2a in a clinical setting.

In conclusion, we demonstrate for the first time that let-7b inhibits HCV expression and replication by targeting the conserved HCV 5′UTR and coding region. Furthermore, let-7b and IFNα-2a elicit synergistically inhibitory effect on HCV infection. This study thereby contributes to our understanding for let-7b on the control of HCV pathogenesis and offers new insight for developing novel anti-HCV therapeutic approaches.

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Conflict of interest None.

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Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFNα-2b/RBV and quantified expressions of viral sensors (*RIG-I, MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFNλ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈3.3-fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈2.6-fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. IPS-1 down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of IPS-1 protein cleavage was associated with the variable treatment response. *Conclusion:* Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFNα/RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.[1] Pegylated interferon α (PEG-IFNα) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFNα/RBV combination therapy.[2] In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ-GTP, γ-glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; IPS-1, IFNβ promoter stimulator 1; ISG15, interferon-stimulated gene 15; MDA5, melanoma differentiation associated gene 5; NVR; nonvirological responders; PEG-IFNα, pegylated interferonα; SNP, single nucleotide polymorphism; RIG-I, retinoic acid-inducible gene I; RBV, ribavirin; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; USP18, ubiquitin-specific protease 18; VR, virological responder.

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(*IL28B*) that encodes for type III IFNλ3 were shown to be strongly associated with a virological response to PEG-IFNα/RBV combination therapy.[3-5] In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFNα/RBV.[3] However, mechanisms involving resistance to PEG-IFNα/RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.[6] The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.[7,8] The IFNβ promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFNβ, mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in *IFNβ* gene activation.[9-12] RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,[8,13] a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.[14] Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[15] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.[16,17] Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.[9,18] Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFNα/RBV combination therapy.[19] We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFNα/RBV combination therapy.[19] However, association of gene expression involving innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFNα/RBV combination therapy in CH-C patients.

Patients and Methods

Patients. Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.[19] Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment Protocol. The patients were administered subcutaneous injections of PEG-IFNα-2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5 μg kg^{-1} week^{-1} for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFNα-2b was reduced to 0.75 μg kg^{-1} week^{-1} when either neutrophil count was less than 750/mm^3 or platelet count was less than 80 $\times$ 10^3/mm^3. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

Measurement of Hepatic Gene Expression. Liver biopsy was performed immediately before initiating

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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), $\times 10^3$/μL	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

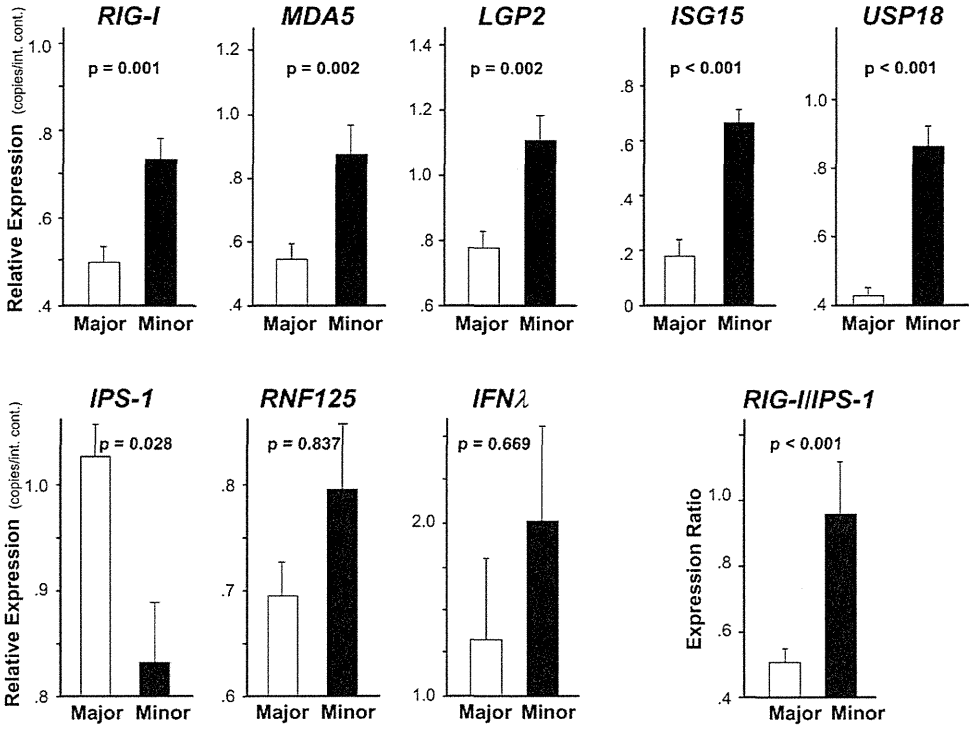

Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), 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

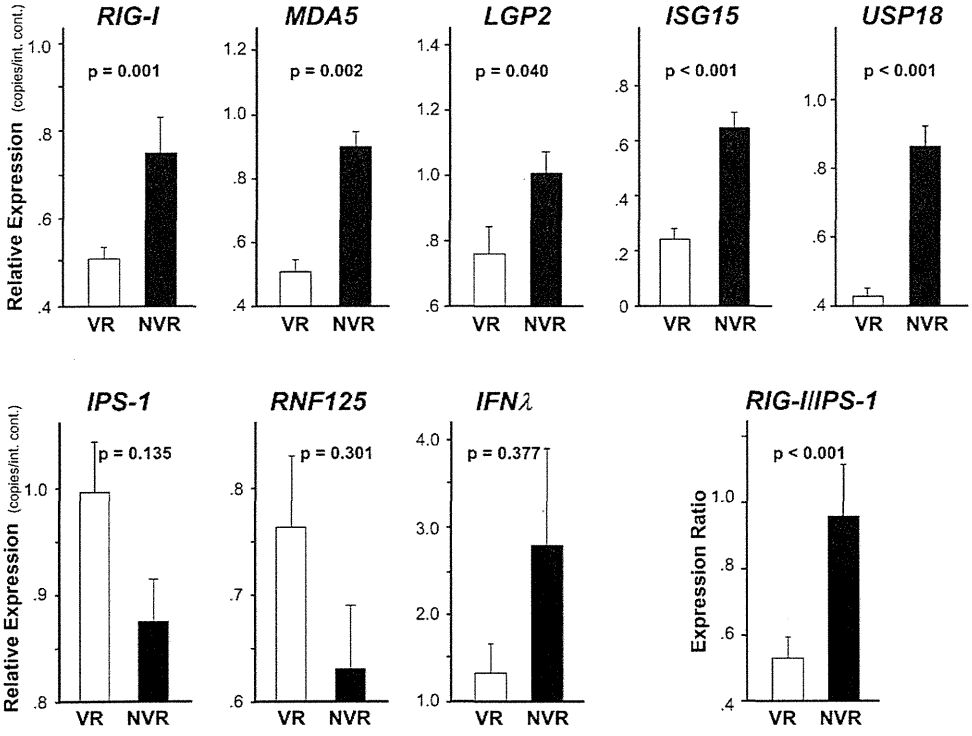

Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) 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.

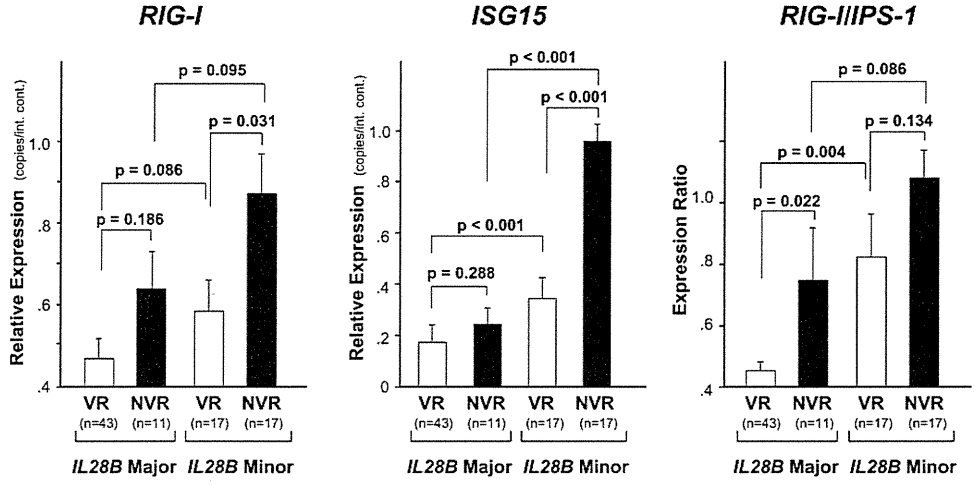

Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* 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

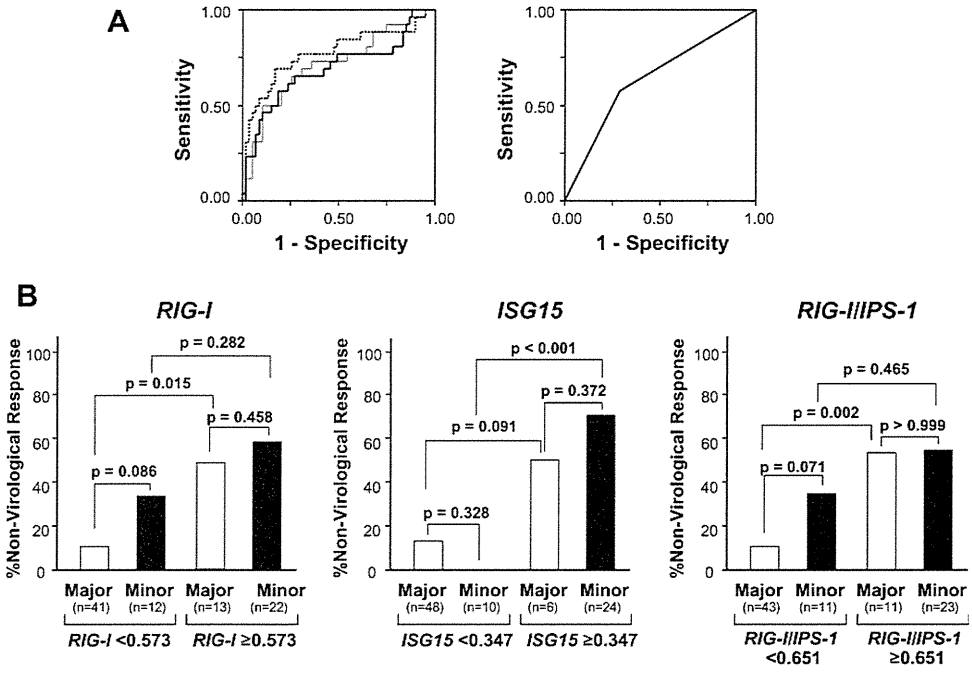

Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological 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.

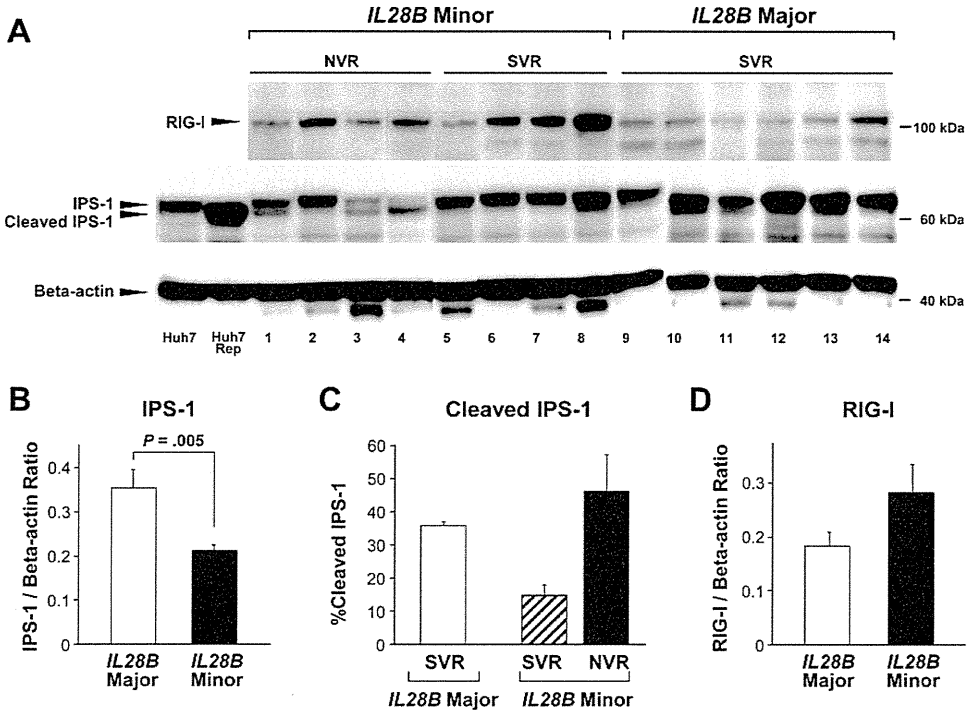

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes 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

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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

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

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

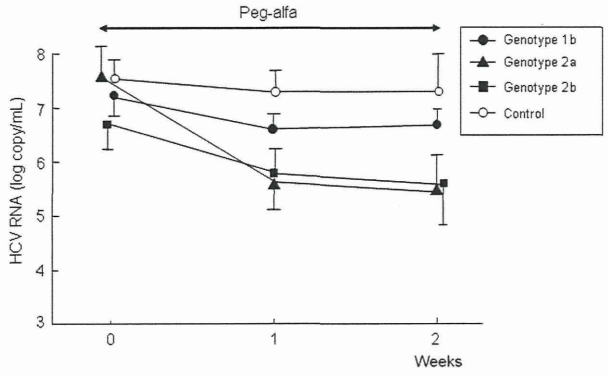

Figure 1 Antiviral effects of Peg-alfa treatment in mice. Mice were infected with hepatitis C virus (HCV) genotypes 1b (n=3), 2a (n=4) or 2b (n=4), then treated with 10 μg/kg of Peg-alfa twice per week for 2 weeks. HCV-infected mice without treatment (n=3) were also analysed (control). Mice serum HCV 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

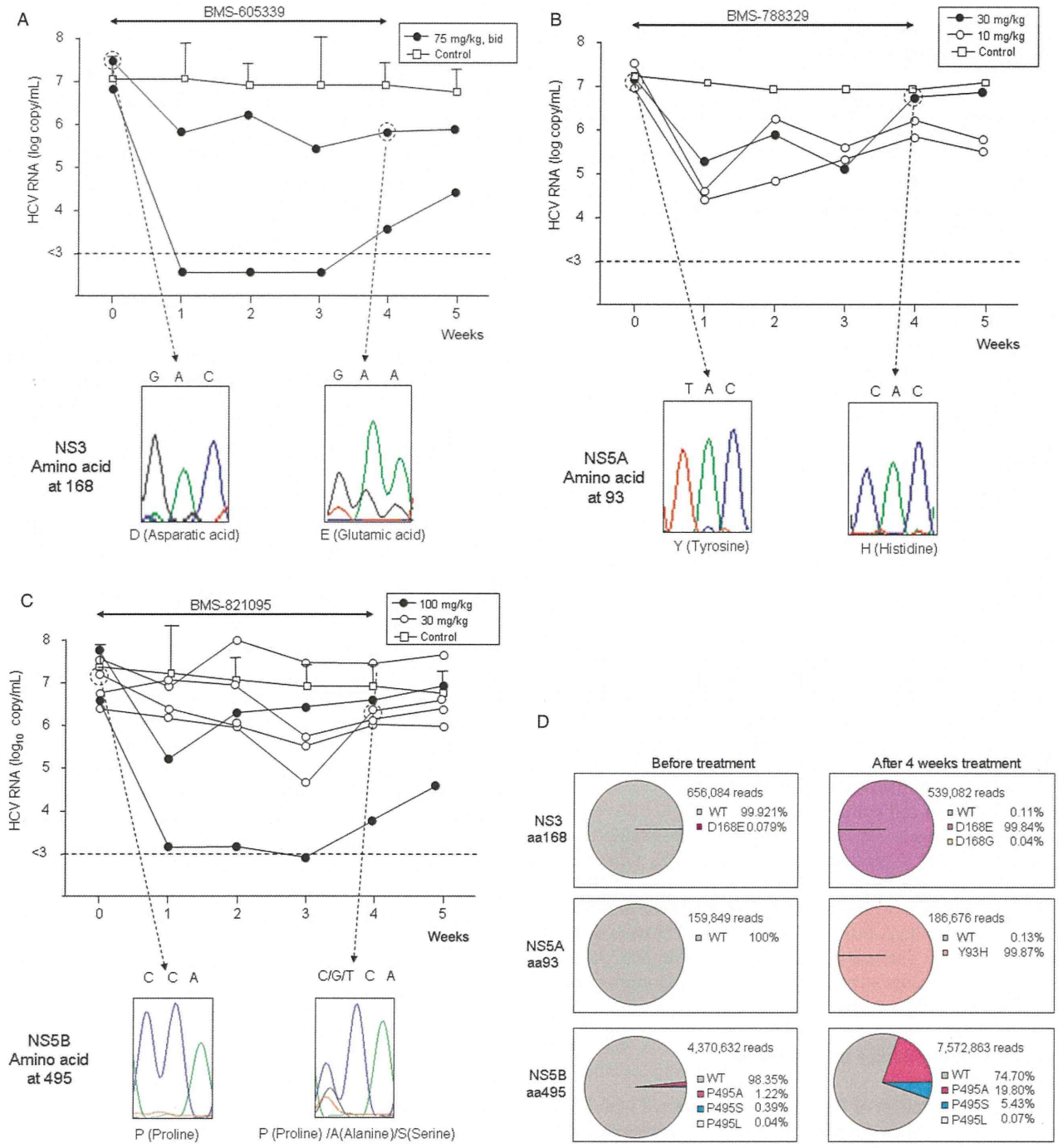

Figure 2 Antiviral effects of BMS-605339, BMS-788329 or BMS-821095 monotherapy in mice infected with hepatitis C virus (HCV) genotype 1b. Mice were injected intravenously with 10^5 copies of HCV genotype 1b. Eight weeks after HCV infection, mice were treated with the indicated concentrations of BMS-605339 (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).