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Interleukin-29 Suppresses Hepatitis A and C Viral Internal Ribosomal Entry Site-Mediated Translation

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Abstract

Our aim was to investigate the effects of interferons (IFNs)-λ (interleukin-29 [IL-29], IL-28A, and IL-28B) on hepatitis C virus (HCV) and hepatitis A virus (HAV) internal ribosomal entry site (IRES)-mediated translation. The effects of these IFNs on HCV/HAV translation from HAV/HCV IRES were investigated using bicistronic reporter constructs. We transfected HCV/HAV IRES constructs into these IFN-expressing cell lines. IL-29 showed stronger inhibition of their IRES-mediated translation. Combining IL-29 with IFN-α or amantadine resulted in stronger inhibition of HAV IRES activity. Our findings demonstrated a novel antiviral effect of IFNs-λ against HAV and HCV through the suppression of IRES-mediated translation.

Introduction

Hepatitis A virus (HAV), a nonenveloped RNA virus of the Picornaviridae family, is the major causative agent of acute viral hepatitis, and occasionally leads to acute liver failure including fulminant hepatitis (17,31,32). The HAV genome is approximately 7600 nt in length and consists of a 5′ nontranslated region (5′ NTR), a single open reading frame that encodes both structural (VP4, VP2, VP3, and VP1) and nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D), and a 3′ NTR with a polyadenylation signal (polyA) tail. The HAV genome also has an internal ribosomal entry site (IRES) that can promote 5′-end-independent initiation of RNA translation (2,11,12,15,19,20,45).

Hepatitis C virus (HCV), an enveloped RNA virus of the Flaviviridae family, causes a spectrum of diseases ranging from an asymptomatic carrier state to end-stage liver disease, including cirrhosis and hepatocellular carcinoma (HCC) (1,14,16). HCV has a 5′ NTR, a long open reading frame, and a 3′ NTR. The HCV genome is approximately 9600 nt in size and encodes a polyprotein precursor of about 3000 amino acids, which is cleaved by both viral and host proteases into structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. HCV also has IRES containing a 5′ NTR and part of the core coding region which forms a secondary structure, and supports translation initiation of an HCV genome in a cap-independent manner (18,24).

IFNs-λ are the most recently defined members of the class III cytokine family, consisting of IL-28A (IFN-λ2), IL-28B (IFN-λ3), and IL-29 (IFN-λ1), and a component of their receptor, IL28-Rα. IL-28 and IL-29 represent an evolutionary link between type I IFNs and the IL-10 family (39). This receptor-ligand system might contribute to antiviral or other defenses by a mechanism similar to, but independent of, type I IFN (25). Additional study is necessary to determine whether IFN-λ can synergize with IFN-α in viral infections, or whether it plays an independent primary role in the antiviral system (41).

There are several reports that IFN-λ exerts antiviral activity against HBV (36), HCV (36), West Nile virus (WNV) (29), influenza A virus, influenza B virus, respiratory syncytial virus, human metapneumovirus, and severe acute respiratory syndrome (SARS) coronavirus (34,41). Human hepatocytes express the IFN-λ receptor complex and IFNs-λ induce signal transducer and activator of transcription 1 (STAT1) phosphorylation (5). Activation of STAT1 is an important factor for the eradication of HCV after antiviral treatments (33). In addition, cellular proteins known as IRES trans-acting factors (ITAFs) are also required for efficient IRES-mediated translation (23,26). The subset of ITAFs that regulates translation initiation appears to be specific for each IRES element (23,26). IFNs including IFNs-λ affect host factors (21,27).

We reported that HAV IRES is an attractive target of anti-HAV drugs because IRES is located in the 5′ NTR, the most

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conserved region among different HAV strains (19,20,45), and is well conserved among clinical isolates (15,17). It has also been reported that HCV IRES is an attractive target of anti-HCV drugs because IRES is located in the 5′ NTR, the most conserved region among different HCV strains (18,24). We focused on the IRES as an antiviral target of these viruses because IRES activity seems to be correlated with translation of viral protein, which is important for viral replication, although there are contrary opinions. Recently, several groups reported that IL-28B SNP predicts hepatitis C treatment-induced viral clearance and natural clearance (10,16,36,42). In the present study, we examined the inhibitory effects of IFN-λ against HAV and HCV IRES-mediated translation.

Materials and Methods

Cells and virus

Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. The plasmids pcDNA3.1-IL28A, pcDNA3.1-IL28B, and pcDNA3.1-IL29 [kindly provided by Prof. T. Betakova, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic (41)] were plasmids expressing IL-28A, IL-28B, and IL-29, respectively. Huh7 cells were transfected with the expression plasmids pcDNA3.1-IL28A, pcDNA3.1-IL28B, pcDNA3.1-IL29, or pcDNA3.1 in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 (Promega, Madison, WI) was added at 1000 μg/mL for selection of Huh7-IL28A, Huh7-IL28B, Huh7-IL29, or Huh7-pcDNA3.1. After 3 wk, to avoid monoclonal selection, all cells were collected for further analysis.

Cell culture-grown HCV JFH1 (genotype 2a) was used in Huh7-derived cell lines (14,43). Tissue culture-adapted HAV strain KRM003 (genotype IIIB) was used in African green monkey kidney GL37 cells (22,37). A HAV DNA-based subgenomic replicon [kindly provided by Prof. V. Gauss-Muller, Institute of Virology, University of Luebeck, Luebeck, Germany (9)] was also used in HuhT7 cells that stably express T7-RNA polymerase in cytoplasm (9,38).

Reagents

The chemicals used were human recombinant IFN-α (Sigma-Aldrich, St. Louis, MO), human recombinant IL29 (IFN-λ1; Acris Antibodies GmbH, Herford, Germany), and amantadine hydrochloride (Sigma-Aldrich).

Bicistronic reporter plasmids

The bicistronic plasmid pSV40-HAV IRES-luc, encoding *Renilla reniformis* luciferase (Rluc) and firefly luciferase (Fluc), was separated by HAV IRES derived from pHM175 (kindly provided by Prof. S.U. Emerson, U.S. National Institutes of Health) under the control of SV40 promoter, with a polyadenylation signal (polyA; Fig. 1A) (19). The bicistronic plasmid pSV40-HCV IRES-luc (kindly provided by Prof. M. Kruger, Medizinische Hochschule Hannover, Hannover, Germany), carries the Rluc gene, the HCV IRES, including the full-length HCV core, and the Fluc gene under control of the SV40 promoter, with a polyadenylation signal (polyA; Fig. 1B) (18,24).

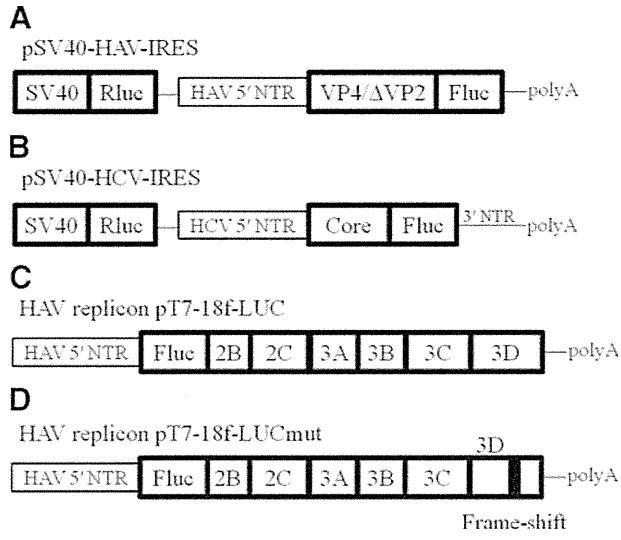


FIG. 1. Structure of HAV/HCV reporter constructs used in this study. (**A**) pSV40-HAV IRES-luc encodes the *Renilla reniformis* luciferase (Rluc), the internal ribosomal entry site (IRES) of HAV HM175, and firefly luciferase (Fluc) under the control of SV40 promoter, with a polyadenylation signal (polyA) (19). (**B**) pSV40-HCV IRES-luc encodes Rluc, the HCV IRES, including the full-length HCV core, and the Fluc gene under control of SV40 promoter, with a polyadenylation signal (polyA) (18,24). (**C**) Replication-competent HAV replicon pT7-18f-LUC contains an open-reading frame of Fluc flanked by the first four amino acids of HAV polyprotein, and by 12 C-terminal amino acids of VP1. This segment is followed by P2 and P3 domains of HAV strain HM175 18f (9). (**D**) Replication-incompetent HAV replicon pT7-18f-LUCmut contains a frameshift mutation in the polymerase 3D (9) (5′ NTR, 5′ nontranslated region; 3′ NTR, 3′ nontranslated region).

Transfection and in vitro *reporter assays*

Approximately 1.0×10^5 cells per well were placed in a six-well plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. The cells were transfected with 0.4 μg of pSV40-HAV (HCV) IRES-luc using Effectene Transfection Reagent (Qiagen) following the manufacturer's protocol. Forty-eight or 72 h after transfection, the cells were harvested using reporter lysis buffer (Toyo Ink, Tokyo, Japan), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO, Tokyo, Japan) (44). To control for variations in transcription, IRES activity was assessed by measuring the ratio of Rluc and Fluc activities. The relative ratio of Fluc activity to Rluc activity (Fluc:Rluc) was defined as 100% in the untreated condition. We accept more than 10^2 of Fluc/Rluc as being positive for IRES activity. All samples were run in triplicate.

RNA extraction, cDNA synthesis, and RT-PCR

The cells were seeded into 6-well plates, and total cellular RNA was extracted 48 h later with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were then stored at −80°C until use. RNA quality was examined using the A_{280}:A_{260} ratio (Pharmacia Biotech, Bedford, MA). cDNA synthesis was performed with a random hexamer using Prime Script reverse transcriptase

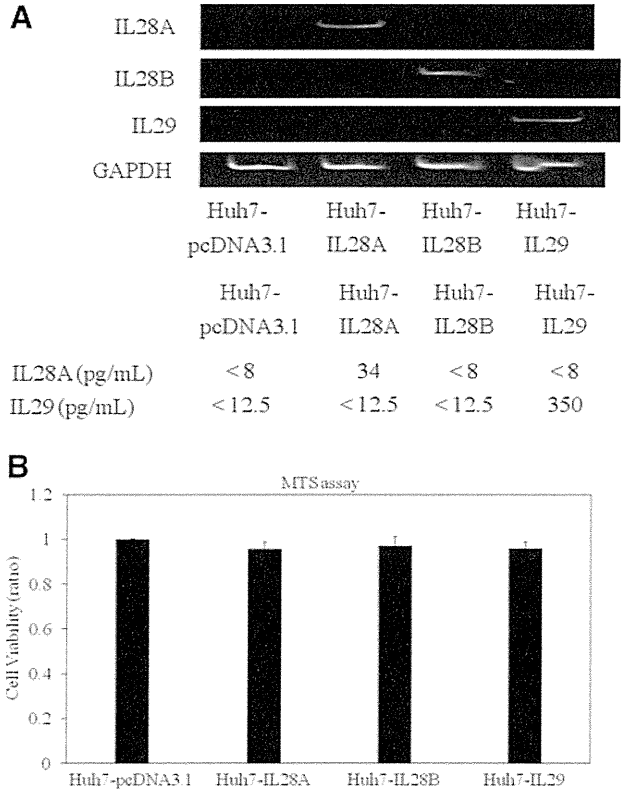


FIG. 2. Overexpression of IL-28A, IL-28B, or IL-29 in human hepatoma cell line Huh7. (**A**) Expression of IFNs-λ mRNA in Huh7-pcDNA3.1, Huh7-IL28A, Huh7-IL28B, and Huh7-IL29 cells (upper panel). RT-PCR was performed using each specific primer. ELISA results for IL-28A and IL-29 are shown in the lower panel. The sensitivities for human IL-28A and IL-29 by these ELISA kits were 8 pg/mL and 12.5 pg/mL, respectively. (**B**) IL-28A, IL-28B, or IL-29 expression does not inhibit cell growth and viability. Huh7-derived cells were plated at a density of 0.5×10^6, and MTS assay was performed at 24 h. The value of Huh7-pcDNA3.1 was set at 1. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of four independent experiments.

(Takara Bio Inc., Otsu, Shiga, Japan). For detection of ectopic expression of IL-28A, IL-28B, and IL-29, RT-PCR was performed with a Thermal Cycler (TP3000; Takara Bio Inc.) using PrimeSTAR HS DNA polymerase (Takara Bio Inc.) with primers as previously described (41), together with primers for GAPDH (44).

Enzyme-linked immunosorbent assay (ELISA) for IL-28A and IL-29

Cell culture fluid was analyzed for human IL-28A and for IL-29 by ELISAs (R&D Systems, Minneapolis, MN and eBioscience, San Diego, CA, respectively), following the manufacturers' protocols. Briefly, cell culture fluid samples were incubated in plates at 37°C overnight, followed by incubation with biotinylated monoclonal antibodies. Avidin-conjugated peroxidase was added to the plates, and enzyme activity was detected with a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA) (44). The sensitivities of human IL-28A and IL-29 by these ELISA kits were 8 pg/mL and 12.5 pg/mL, respectively.

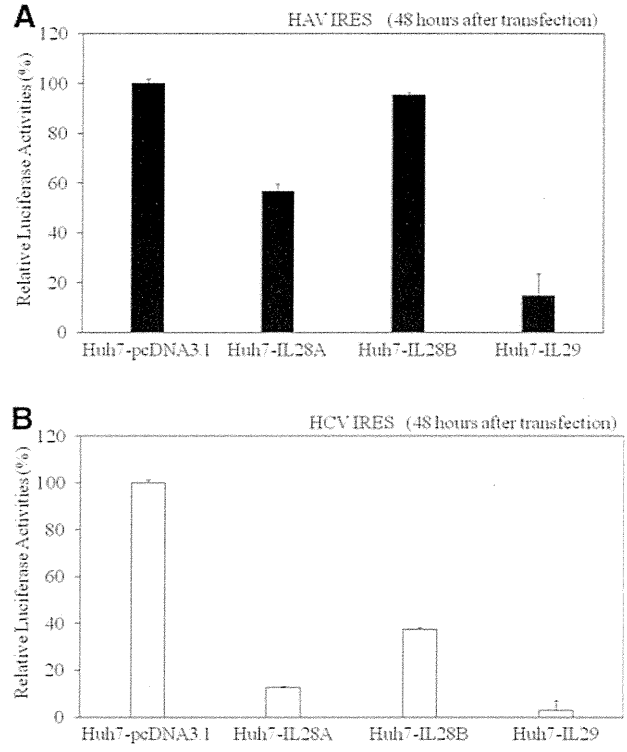


FIG. 3. Interferon-λ inhibits hepatitis A virus (HAV) (**A**) and HCV (**B**) internal ribosomal entry site (IRES)-mediated translation in human hepatoma cells. Huh7-IL28A, Huh7-IL28B, Huh7-IL29, and Huh7-pcDNA3.1, were transfected with pSV40-HAV IRES reporter vector (19) (**A**), and pSV40-HCV IRES reporter vector (18,24) (**B**), and 48 h later, luciferase activity was measured and IRES activity was determined. Relative luciferase activity (Fluc/Rluc) in Huh7-pcDNA3.1 was set at 100%. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of three independent experiments.

MTS assay

To evaluate cell growth and cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed with the CellTiter 96 Aqueous One-Solution cell proliferation assay (Promega).

Statistical analysis

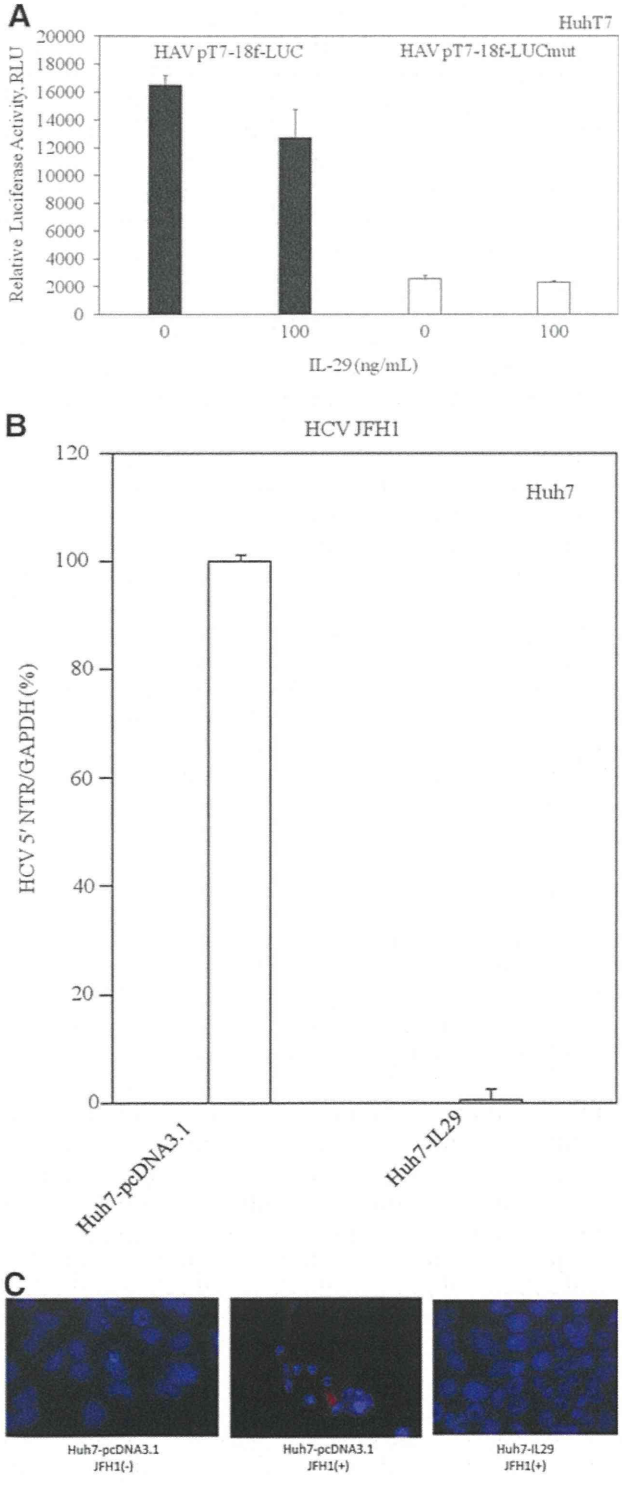
Data were expressed as mean ± SD. Statistical analysis was done by Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

Overexpression of IL-28A, IL-28B, or IL-29, in Huh7 cells

We used three protein plasmid vectors under control of the CMV promoter: pcDNA3.1-IL28A, pcDNA3.1-IL28B, and pcDNA3.1-IL29 (41). We established three IFN-λ-overexpressing Huh7 cells, designated as Huh7-IL28A, Huh7-IL28B, and Huh7-IL29. We also used pcDNA3.1 for the establishment of a control cell, Huh7-pcDNA3.1. For the generation of stable cell lines, Huh7 cells were transfected

with these vectors and treated with G418. Antibiotic-resistant colonies were expanded for further analysis. To test the ability of these cells to express IL-28A and IL-29, we detected these mRNAs by RT-PCR and measured these cytokines by ELISA (Fig. 2A, upper and lower panels). IL-28A or IL-29 mRNAs were detected only in Huh7-IL28A or Huh7-IL29 cells, respectively. IL-28A or IL-29, respectively, could be measured in each cell culture fluid of Huh7-IL28A or Huh7-IL29. We confirmed the expression of IL-28B mRNA in the cellular RNA of Huh7-IL28B since we could not use ELISA for IL-28B at this time (Fig. 2A, upper panel). We next examined whether overexpression of IFN-λ had any effect on cell proliferation. Equal numbers of control Huh7-pcDNA3.1 and IFN-λ-overexpressing Huh7 cells (Huh7-IL28A, Huh7-IL28B, and Huh7-IL29) were plated, and cell viability was counted at 24 h by MTS assay (Fig. 2B). There were no differences in cell viabilities among these cell lines.



HAV IRES activity in Huh7-IL28A, Huh7-IL28B, and Huh7-IL29

Next, we examined the effects of these IFNs-λ on HAV IRES-mediated translations using a luciferase reporter assay. Huh7-IL28A, Huh7-IL28B, Huh7-IL29, and Huh7-pcDNA3.1 were transfected with pSV40-HAV IRES reporter vector encoding SV40 promoter driven-Rluc and Fluc, separated by HAV IRES (19), and 48 h later, luciferase activity was measured and IRES activity was determined (Fig. 3A). HAV IRES activity was inhibited in Huh7-IL28A (56.7%; n=3, $p<0.0001$), Huh7-IL28B (95.3%, n=3, $p=0.0021$), and Huh7-IL29 (14.9%, n=3, $p<0.0001$), compared to that in control Huh7-pcDNA3.1 (n=3, 100%). IL-28A and IL-28B demonstrated inhibitory effects on HAV IRES activity, but they seemed less efficient than IL-29 (Fig. 3A).

HCV IRES activities in Huh7-IL28A, Huh7-IL28B, and Huh7-IL29

It is known that HCV also has IRES structures and plays an important role in the translation of HCV proteins (18,24). In order to compare the effects of IFNs-λ on HCV IRES-mediated translation with those on HCV, we next tested their effects on HCV IRES-mediated translation using a luciferase reporter assay. Huh7-IL28A, Huh7-IL28B, Huh7-IL29, and Huh7-pcDNA3.1 were transfected with pSV40-HCV IRES reporter vector encoding SV40 promoter driven-Rluc and Fluc, separated by HCV IRES (24), and 48 h later, luciferase activity was measured and IRES activity was determined (Fig. 3B). HCV IRES activity was inhibited in Huh7-IL28A (12.5%, n=3, $p<0.0001$), Huh7-IL28B (37.5%, n=3, $p<0.0001$), and Huh7-IL29 (2.7%, n=3, $p<0.0001$), compared to that in control Huh7-pcDNA3.1 (n=3100%). Similarly to HAV IRES, IL-28A and IL-28B demonstrated inhibitory effects on HCV

FIG. 4. IL29 suppresses hepatitis A virus (HAV) (**A**) and HCV (**B** and **C**) replication. (**A**) HuhT7 cells were transfected with replication-competent HAV replicon pT7-18f-LUC or replication-incompetent HAV replicon pT7-18f-LUCmut (9). At 60 h post-transfection, the cells were treated with 0 or 100 ng/mL IL-29. At 72 h post-transfection, reporter assays were performed to evaluate HAV subgenomic replication. (**B**) Huh7-IL29 and Huh7-pcDNA3.1 were infected with HCV JFH1 (genotype 2a) (14,43), and 72 h later, HCV RNA was measured by real-time RT-PCR and HCV 5'NTR/GAPDH ratios were measured by ddCt methods. (**C**) After 72 h of infection, HCV was detected by immunofluoresence using antibody to the core protein. Shown are representative photomicrographs of JFH1 virus production in Huh7-IL29 and Huh7-pcDNA3.1 cells. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of three independent experiments.

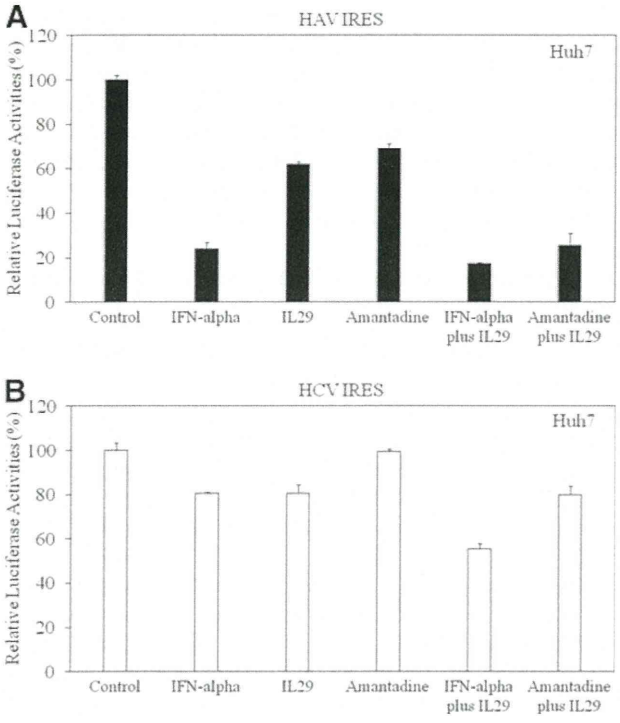


FIG. 5. Effects of IL-29 with or without interferon (IFN)-α or amantadine on hepatitis A virus (HAV) (**A**) and HCV (**B**) internal ribosomal entry site (IRES)-mediated translation in Huh7 cells. Huh7 cells were transfected with pSV40-HAV IRES reporter vector (19) (**A**), or pSV40-HCV IRES reporter vector (18,24) (**B**), and 48 h later, the cells were treated with 50 ng/mL IL-29 with or without 50 μg/mL amantadine or 100 IU/mL IFN-α, and 24 h later luciferase activity was measured and IRES activity was determined. Relative luciferase activity (Fluc/Rluc) without treatment was set at 100%. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of three independent experiments.

IRES activity, but they seemed less efficient than IL-29 (Fig. 3B).

IL-29 inhibits both HAV and HCV replication

Next we investigated the effect of IL-29 on HAV subgenomic replication in HuhT7 cells (9). IL-29 at 100 ng/mL led to 22.8% (n = 3, $p = 0.038$) inhibition of HAV replication, but we observed no reduction of HAV mut replicon replication (Fig. 4A). We also examined whether IL-29 inhibits HAV strain KRM003 propagation in GL37 cells, but at 50 ng/mL of IL-29 we could not observe any effect on the inhibition of HAV propagation. Two-hundred and fifty and 500 ng/mL of IL-29 showed a tendency to inhibit HAV propagation without cell damage. However, it was difficult to obtain a stable reaction. Further study will be needed.

We also examined whether IL-29 inhibits HCV replication in Huh7-derived cell lines. Huh7-IL29 and Huh7-pcDNA3.1 were infected with HCV JFH1 (genotype 2a) (14,43), and 72 h later, HCV RNA was detected less in Huh7-IL29 (0.6%; n = 3, $p < 0.0001$) than in Huh7-pcDNA3.1 (100%; n = 3, $p < 0.0001$; Fig. 4B). HCV core protein expression was also less observed in Huh-IL29 than in Huh7-pcDNA3.1 (Fig. 4C).

Exogenous IL-29 with or without IFN-α or amantadine inhibits HAV IRES activity in Huh7

As Huh7-IL29 cells had the strongest inhibitory effect on HAV IRES-mediated translation (Fig. 3A), we investigated whether exogenous IL-29 had similar effects on HAV IRES-mediated translation using a luciferase reporter assay (Fig. 5A). Huh7 cells were transfected with pSV40-HAV IRES reporter vector (19), and 48 h later, cells were treated with IL-29 with or without amantadine or IFN-α, and 24 h after this, luciferase activity was measured and IRES activity was determined (Fig. 5A).

We previously reported that amantadine with or without IFN-α inhibits HAV IRES-mediated translation in human hepatoma cells (19,45). HAV IRES activity was significantly inhibited, to 24.1% (n = 3, $p < 0.0001$), 62.1% (n = 3, $p < 0.0001$), and 69.1% (n = 3, $p < 0.0001$), by 100 IU/mL IFN-α, 50 ng/mL IL-29, and 50 μg/mL amantadine, respectively (Fig. 5A). The combination of IL-29 with IFN-α or amantadine led to 82.7% (n = 3, $p < 0.0001$), or 74.6% (n = 3, $p < 0.0001$) inhibition of HAV IRES activity, respectively, with these combinations demonstrating stronger effects than IL-29 alone (Fig. 5A).

In order to compare the effects of exogenous IL-29 on HAV IRES-mediated translation with those on HCV, we next tested the effects of IL-29 with or without amantadine or IFN-α on HCV IRES-mediated translation using a luciferase reporter assay. Huh7 cells were transfected with pSV40-HCV IRES reporter vector (24), and 48 h later, the cells were treated with IL-29 with or without amantadine or IFN-α, and 24 h after this, luciferase activity was measured and IRES activity was determined (Fig. 5B). HCV IRES activity demonstrated significant inhibition, to 80.6% (n = 3, $p = 0.00061$) and 80.6% (n = 3, $p = 0.00027$) by IFN-α and IL-29, respectively, but showed no inhibition by amantadine only (99.3%, n = 3; Fig. 5B). The combination of IL-29 with IFN-α led to a 44.5% (n = 3, $p < 0.0001$) inhibition of HCV IRES activity, with this combination demonstrating stronger effects than IL-29 alone. However, the combination of IL-29 with amantadine resulted in only 20% inhibition (n = 3, $p = 0.00027$) of HCV IRES activity, similarly to the effect of IL-29 alone (Fig. 5B).

Discussion

We demonstrated that IL-29 inhibited HAV as well as HCV IRES activity in human hepatoma cell line Huh7, and that Huh7-IL29 had stronger effects than Huh7-IL28A, Huh7-IL28B, and Huh7-pcDNA3.1. The combination of IL-29 with IFN-α or amantadine seemed to have stronger inhibitory effects on HAV IRES activity than IL-29 alone.

IFNs-λ modulate innate and adaptive immune responses to environmental pathogens and protect the host against diseases such as cancer. Expression of IFNs-λ is tightly regulated by viral infection, including hepatitis viral infection (6,7). IFNs-λ utilize a receptor complex different from IFN-α, but both types of IFN induce STAT1 and STAT2, as well as STAT3 activation (33,48). Binding of IFN to the IFN receptor leads to the activation of receptor-associated Janus tyrosine protein kinase (Jak1). IFN stimulation results in tyrosine phosphorylation, dimerization, and nuclear import of STATs (3). STATs and the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex move into the nucleus and bind to IFN-stimulated response elements (ISRE) in the promoters of the

IFN-stimulated genes (ISGs). ISGs inhibit viral replication and activate numerous downstream cellular responses.

In contrast to IFN-α, IFNs-λ bind to a heterodimeric receptor consisting of IL-28Rα subunit and IL10Rβ receptor subunit, that is also shared by IL-10, IL-22, and IL-26. Because the IFN-λ receptor is different from that of IFN-α, their uses as alternative therapies for viral hepatitis need to be examined (6). Although the biological activities of IFNs-λ could overlap with IFN-α, the expression of IL-28Rα receptor is limited in contrast to the ubiquitously expressed IL10Rβ, and IFNs-λ might have fewer adverse events than type I IFN (28). We also found that the combination of IL-29 with IFN-α or amantadine demonstrated stronger inhibitory effects on HAV IRES activity. The combination of IL-29 with amantadine may also be useful in some HAV patients.

We did not observe any differences in HAV 5′ NTR or HCV 5′ NTR RNA detection by RT-PCR among Huh7-pcDNA3.1, Huh7-IL28A, Huh7-IL28B, and Huh7-IL29 at 72 h after transfection of pSV40-HAV IRES-luc or pSV40-HCV IRES-luc (data not shown), although we could not completely exclude the destruction of IRES mRNAs, because IFNs-λ as well as IFN-α activate double-stranded protein kinase PKR and 2′,5′-oligo A (2–5A) synthetases (21). Several noncanonical translation initiation factors such as La protein and polypyrimidine tract binding protein (PTB) have been implicated in translation from HAV and HCV IRESes (4,13,40,46). The effects of IL-29 on these proteins should be examined in future studies.

We previously demonstrated that siRNAs targeted against HAV IRES, amantadine, and IFN-α, inhibited HAV IRES-mediated translation and HAV replication (15,19,33,45). In the present study, we planned to examine the effects of IFN-λ on HAV IRES-mediated translation. IFNs are proteins induced by lymphocytes and other cells including hepatocytes in response to viruses such as HAV. Our study also supports the notion that IFNs-λ might inhibit HAV IRES-mediated translation as one of the host defense mechanisms against HAV infection.

It has been reported that genetic variations in IL-28B SNPs predict hepatitis C treatment-induced viral clearance and natural clearance (10,16,33,42). Tanaka *et al.* (42) reported that IL-28B minor SNP was associated with a null virological response in the treatment of Japanese patients infected with HCV genotype 1. Yu *et al.* (47) also reported that the IL-28B rs8099917 TT genotype is significantly independently predictive of RVR, which is the single best predictor of SVR, in Asian HCV genotype 2 patients. Pegylated IL-29 induces antiviral gene expression and represses hepatitis B and C replication *in vitro* (6), and HCV replication *in vivo* (35). Among IFNs-λ, it was reported that IL-28A inhibits HCV IRES-mediated translation and suppresses HCV replication (49). Kato *et al.* (21) reported that IFN-α, as well as IFN-β, specifically suppress the translation from HCV IRES. We also demonstrated that siRNAs targeted against HCV IRES were potent inhibitors of HCV IRES-mediated translation and HCV replication (18). In the present study, we demonstrated that IL-29, as well as IFN-α, inhibited HCV IRES-mediated translation, although amantadine did not inhibit HCV IRES-mediated translation in our experimental condition.

In conclusion, we demonstrated that IL-29 suppressed HAV as well as HCV IRES-mediated translation. Viral IRES activity may influence the level of replication (8,15,18), although it was reported that the preponderance of host factors might determine the clinical presentation (30). To inhibit HAV or HCV IRES-mediated translation, the combination IL-29 with IFN-α or amantadine has a stronger inhibitory effect. IFNs-λ might also play an important role in host defense mechanisms and in HAV pathogenesis.

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Author Disclosure Statement

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Article

Roles of ITPA and IL28B Genotypes in Chronic Hepatitis C Patients Treated with Peginterferon Plus Ribavirin

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Abstract: It has been reported that inosine triphosphatase (ITPA) gene variants protect against ribavirin-induced anemia in patients treated for chronic hepatitis C. IL28B variants also influence the treatment response of peginterferon plus ribavirin treatment in these patients. In the present study, we examined how ITPA and IL28B genotypes have clinical impacts on treatment-induced hematotoxicities and treatment response in HCV-infected patients treated with peginterferon plus ribavirin. ITPA genotypes (rs1127354 and rs6051702) and IL28B genotype (rs8099917) were determined by TaqMan SNP assay. We compared clinical background, treatment course and treatment response in terms of these genotypes. Only IL28B rs8099917 major type could predict sustained virological response. ITPA rs1127354 major type leads to significantly greater ribavirin-induced anemia than ITPA rs1127354 minor type between days 0 and 84. We noticed that IL28B rs8099917 minor genotype was associated with higher reduction of neutrophils and platelets. ITPA rs1127354 is useful for the prediction of ribavirin-induced anemia in the early phase after the commencement of peginterferon plus ribavirin treatment and IL28B rs8099917 is useful for the prediction of sustained virological response. Use of the combination of these two genotypes could lead to safe and effective treatment of chronic hepatitis C patients.

Keywords: anemia; HCV; IL28B; ITPA; SNP; sustained virological response

1. Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and a leading cause of end-stage liver disease worldwide [1]. The current standard therapy is based on a combination of peginterferon and ribavirin, but this treatment leads to only about 50% sustained virological response (SVR) in patients with HCV genotype 1 and high viral loads, who were mostly null-responders or relapsers [2]. Recently, the direct-acting antiviral (DAA) agents boceprevir and telaprevir were licensed for the treatment of HCV infection [3], and these drugs might be more powerful tools for HCV-infected patients.

Interleukin 28B (IL28B) variants influence the treatment response of peginterferon plus ribavirin treatment in HCV-infected patients [4–8]. Genome-wide association study has revealed a strong relationship between single-nucleotide polymorphisms (SNPs) near IL28B on chromosome 19 and null virological response in the treatment of patients with HCV genotype 1 in Australian [4], Japanese [5] and other populations [6]. Baseline plasma interferon-gamma inducible protein 10 kDa (IP-10 or CXCL10) is significantly associated with IL28B-related SNPs, and augments the level of predictiveness of the first-phase decline in HCV RNA, rapid virological response (RVR) and final treatment outcome [9,10]. Further studies will be needed to reveal the mechanism concerning IL28B and the response to interferon.

It has also been reported that inosine triphosphatase (ITPA) gene variants protect against ribavirin-induced hemolytic anemia in chronic hepatitis C patients [11]. Proposed mechanisms of action for ribavirin against HCV include (1) direct effect against HCV RNA-dependent RNA polymerase [12], (2) induction of misincorporation of nucleotides leading to lethal mutagenesis [13,14], (3) depletion of intracellular pools via inhibition of inosine monophosphate dehydrogenase [15], (4) alteration in the cytokine balance from a Th2 profile (anti-inflammatory) to a Th1 profile (pro-inflammatory) [16], and (5) potentiating the effect of interferon via up-regulation of genes involved in interferon signaling [17,18]. Clinical studies provide strong evidence for the benefit of ribavirin in combination with DAAs for both interferon containing and sparing regimens [18].

In the present study, we examined how ITPA and IL28B genotypes clinically contribute to treatment-induced hematotoxicities and treatment response in HCV-infected patients treated with peginterferon plus ribavirin. We found that IL28B rs8099917 minor genotype was associated with greater reduction of neutrophils and platelets. Use of a combination of these genotypes could lead to a safe and effective treatment for chronic hepatitis C patients. It is conceivable that these variants may modulate treatment responses as well as treatment pathways, and the result of this study might show the way of the future direction of these gene variants in treatment or drug development.

2. Results

2.1. Patient Characteristics According to IL28B and ITPA Genotypes

First, we genotyped IL28B rs8099917, and ITPA rs1127354 and rs6051702 in 97 HCV-infected patients (Table 1). Sixty and 37 patients possessed IL28B rs8099917 major and minor genotypes, respectively. Seventy-four and 23 patients possessed ITPA rs1127354 major and minor genotypes, respectively, and 59 and 38 possessed ITPA rs6051702 major and minor genotypes, respectively.

Table 1. Background of study population at enrollment.

Study variables	Total (n = 97)
Age (years)	55.1 ± 10.8
Gender (male/female)	44/53
SNP genotype	
IL28B rs8099917 TT/TG/GG	60/35/2
ITPA rs1127354 CC/CA/AA	74/21/2
ITPA rs6051702 AA/AC/CC	59/32/6
Response to previous therapy	
Naïve/relapse/null response	67/17/13
HCV RNA (H/L)	95/2
HCV genotype (G1/G2)	81/16
AST (IU/L)	56.0 ± 49.4
ALT (IU/L)	67.9 ± 62.4
γGTP (IU/L)	53.5 ± 73.2
WBC (/mm^3)	5,410 ± 1,640
Hemoglobin (g/dL)	14.0 ± 1.1
Platelets (×10^4/mm^3)	17.5 ± 5.1
History of diabetes mellitus (+/−)	15/82
US (CLD/cirrhosis/unknown)	83/12/2
Treatment Response	
RVR (+/−/unknown)	14/82/1
EVR (+/−)	52/45
SVR (+/relapser/null/unknown)	40/27/22/8

H, high viral load (≥5 log IU/mL); L, low viral load (<5 log IU/mL); G1, genotype 1; G2, genotype 2; WBC, white blood cell count; US, ultrasound finding; CLD, chronic liver disease.

IL28B rs8099917 major-type patients included more interferon treatment-naïve patients than minor-type patients. Lower γGTP levels were seen in IL28B rs8099917 major-type patients (Table 2). ITPA rs1127354 major-type patients were older than ITPA rs1127354 minor-type patients and tended to be female-dominant in the present study (Table 2).

2.2. Treatment Response According to IL28B and ITPA Genotypes

Next, we compared the treatment response among patients according to IL28B and ITPA genotypes (Table 3). IL28B rs8099917 could predict SVR, as previously reported [4–9], while both ITPA genotypes did not in the present study. We reconfirmed that IL28B rs8099917 is one of the predictive values for treatment response in interferon-included regimens.

Table 2. Baseline characteristics of patients grouped according to *IL28B* and *ITPA* genetic variations.

Study variables	*IL28B rs8099917*			*ITPA rs1127354*			*ITPA rs6051702*		
	TT	**TG/GG**	***P-value***	**CC**	**CA/AA**	***P-value***	**AA**	**AC/CC**	***P-value***
No. of patients	60	37		74	23		59	38	
Age (years)	55.7 ± 11.2	54.7 ± 10.1	N.S.	56.8 ± 9.7	49.6 ± 12.2	0.0043	55.6 ± 11.3	54.4 ± 9.9	N.S.
Gender (male/female)	25/35	19/18	N.S.	29/45	15/8	0.0511	29/30	15/23	N.S.
Response to previous therapy (naïve/relapse/null response)	46/10/4	21/7/9	0.029	48/17/9	19/0/4	N.S.	40/12/7	27/5/6	N.S.
HCV RNA (H/L)	58/2	37/0	N.S.	73/1	22/1	N.S.	58/1	37/1	N.S.
HCV genotype (G1/G2)	49/11	32/5	N.S.	63/11	18/5	N.S.	48/11	33/5	N.S.
AST (IU/L)	53.3 ± 56.2	60.3 ± 36.0	N.S.	52.8 ± 31.9	66.2 ± 84.4	N.S.	51.6 ± 30.1	62.8 ± 69.5	N.S.
ALT (IU/L)	62.4 ± 65.3	76.9 ± 57.0	N.S.	62.3 ± 48.5	85.7 ± 93.5	N.S.	62.4 ± 47.5	76.4 ± 80.2	N.S.
γGTP (IU/L)	35.5 ± 34.5	82.8 ± 104	0.0016	55.1 ± 80.9	48.7 ± 40.1	N.S.	51.6 ± 72.1	56.5 ± 75.6	N.S.
WBC (/mm^3)	5580 ± 1820	5140 ± 1260	N.S.	5390 ± 1630	5470 ± 1680	N.S.	5570 ± 1690	5160 ± 1540	N.S.
Hb (g/dL)	13.9 ± 1.1	14.3 ± 1.1	N.S.	13.9 ± 1.0	14.3 ± 1.2	N.S.	14.0 ± 1.1	14.0 ± 1.1	N.S.
Platelets (×10^4/mm^3)	17.9 ± 5.3	16.8 ± 5.0	N.S.	17.4 ± 5.4	17.7 ± 4.3	N.S.	17.8 ± 5.4	17.0 ± 4.7	N.S.
History of diabetes mellitus (+/−)	9/51	7/30	N.S.	11/63	4/19	N.S.	8/51	7/31	N.S.
US (CLD/cirrhosis/unknown)	51/8/1	32/4/1	N.S.	62/10/2	21/2	N.S.	50/8/1	33/4/1	N.S.

H, high viral load (≥5 log IU/mL); L, low viral load (<5 log IU/mL); G1, genotype 1; G2, genotype 2; WBC, white blood cell count; US, ultrasound finding; CLD, chronic liver disease.

Table 3. Treatment response in patients grouped according to *IL28B* and *ITPA* genetic variations.

Study variables	*IL28B rs8099917*			*ITPA rs1127354*			*ITPA rs6051702*		
	TT	**TG/GG**	***P-value***	**CC**	**CA/AA**	***P-value***	**AA**	**AC/CC**	***P-value***
No. of patients	60	37		74	23		59	38	
RVR (+/−/unknown)	12/47/1	2/35/0	0.085	10/63/1	4/19/0	N.S.	10/49/0	4/33/1	N.S.
EVR (+/−)	43/17	9/28	0.000014	36/38	16/17	N.S.	31/28	21/17	N.S.
SVR (+/Relapser/Null/unknown)	29/6/18/7	11/16/9/1	0.042	28/17/22/7	12/5/5/1	N.S.	27/13/16/3	13/9/11/5	N.S.

RVR, rapid virological response; EVR, early virological response; SVR, sustained virological response.

2.3. Ribavirin-Induced Anemia According to IL28B and ITPA Genotypes

Next, we examined ribavirin-induced anemia among patients according to IL28B and ITPA genotypes (Figure 1). IL28B rs8099917 did not influence ribavirin-induced anemia (Figure 1A–D), nor did ITPA rs6051702 (Figure 1I-1L). ITPA rs1127354 major type led to significantly greater ribavirin-induced anemia than ITPA rs1127354 minor type in Japanese patients during peginterferon plus ribavirin treatment (Figure 1E-1H).

Figure 1. Ribavirin-induced reduction of hemoglobin according to IL28B and ITPA genotypes. (**A**)–(**D**), IL28B rs8099917; (**E**)–(**H**), ITPA rs1127354; (**I**)–(**L**), ITPA rs6051702. (**A**), (**E**) and (**I**) show the changes of hemoglobin (Hb) between days 0 and 14, (**B**), (**F**) and (**J**) between days 0 and 28, (**C**), (**G**) and (**K**) between days 0 and 54, and (**D**), (**H**) and (**L**) between days 0 and 84.

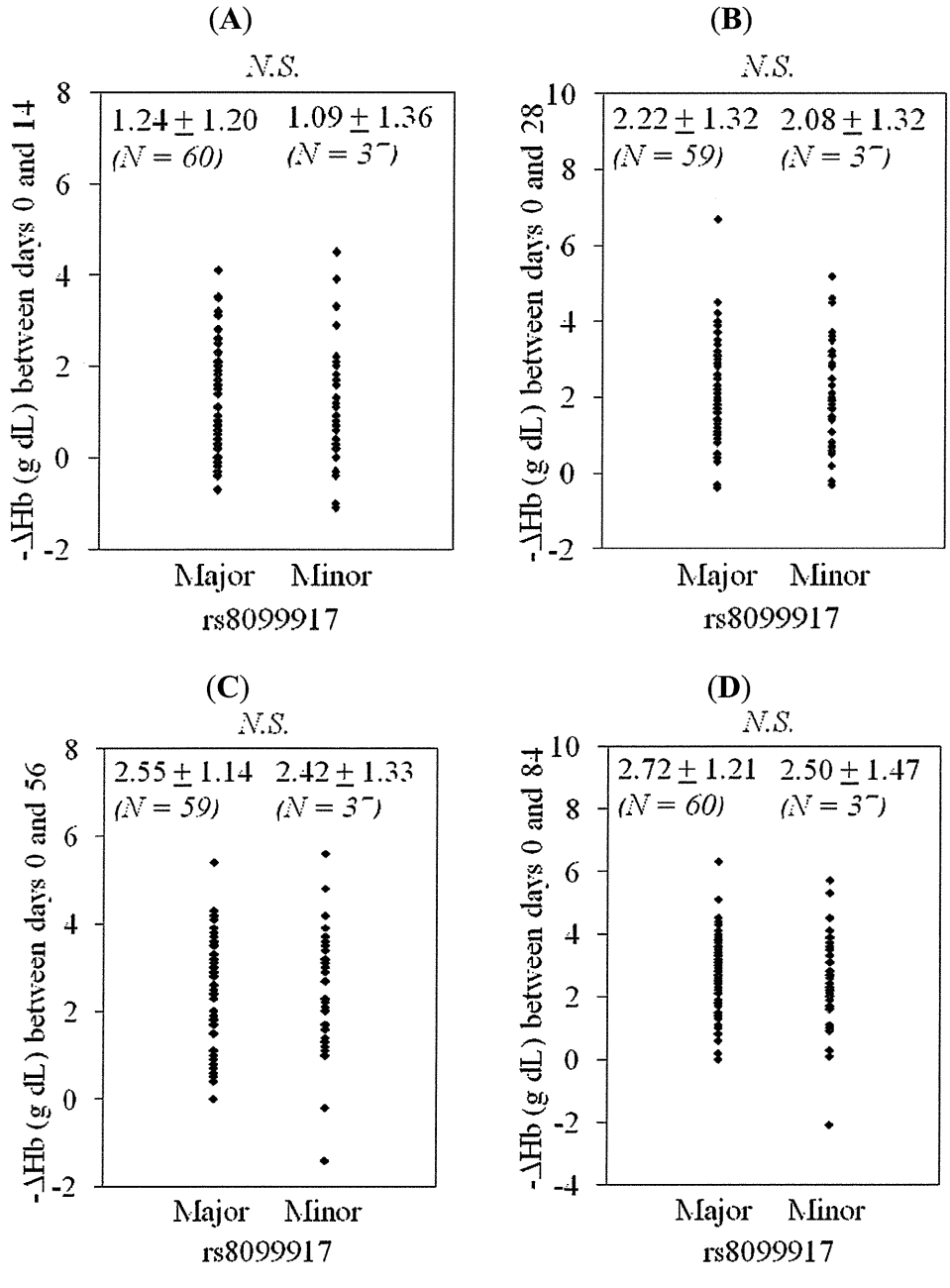


Figure 1. *Cont.*

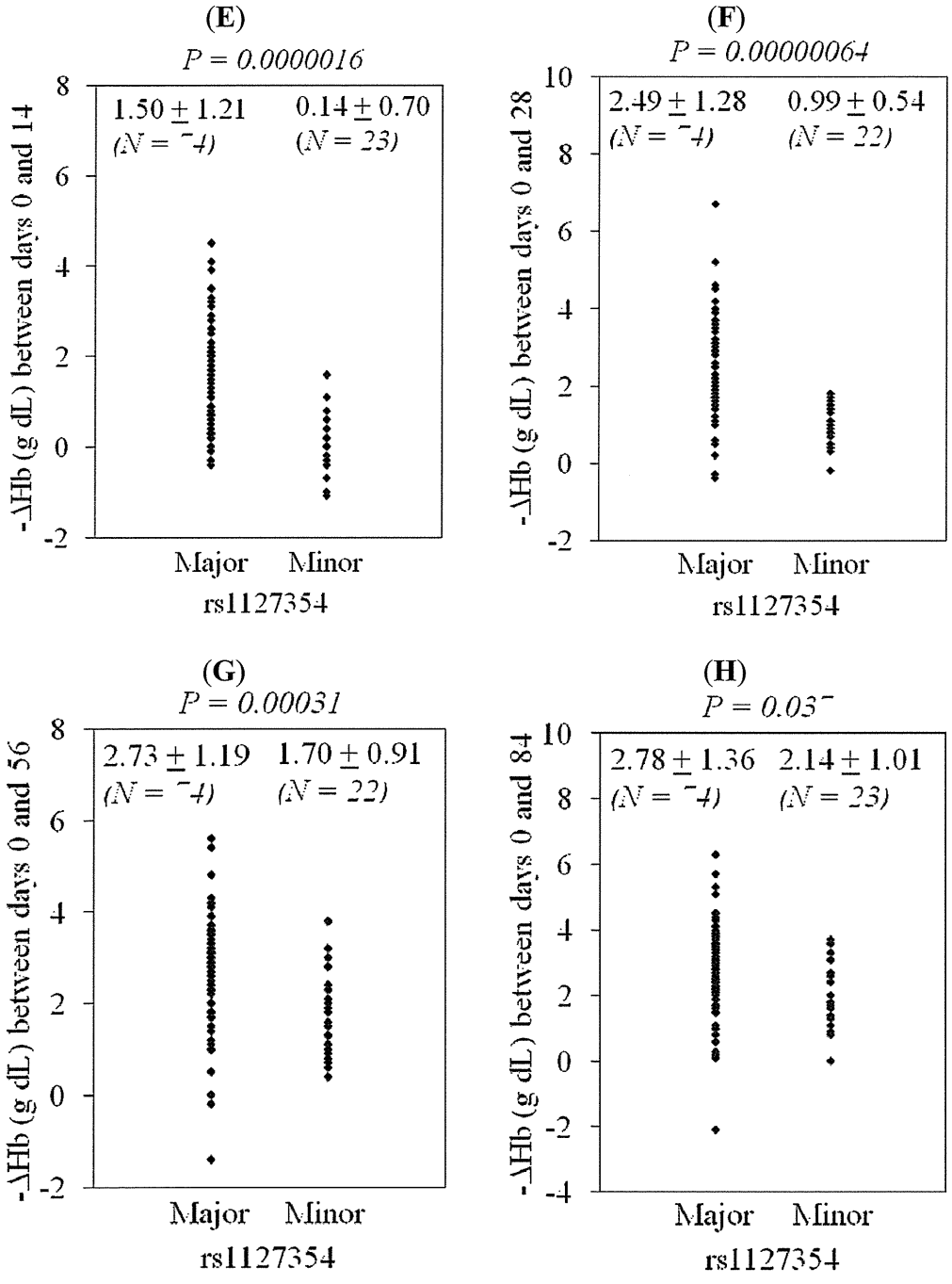
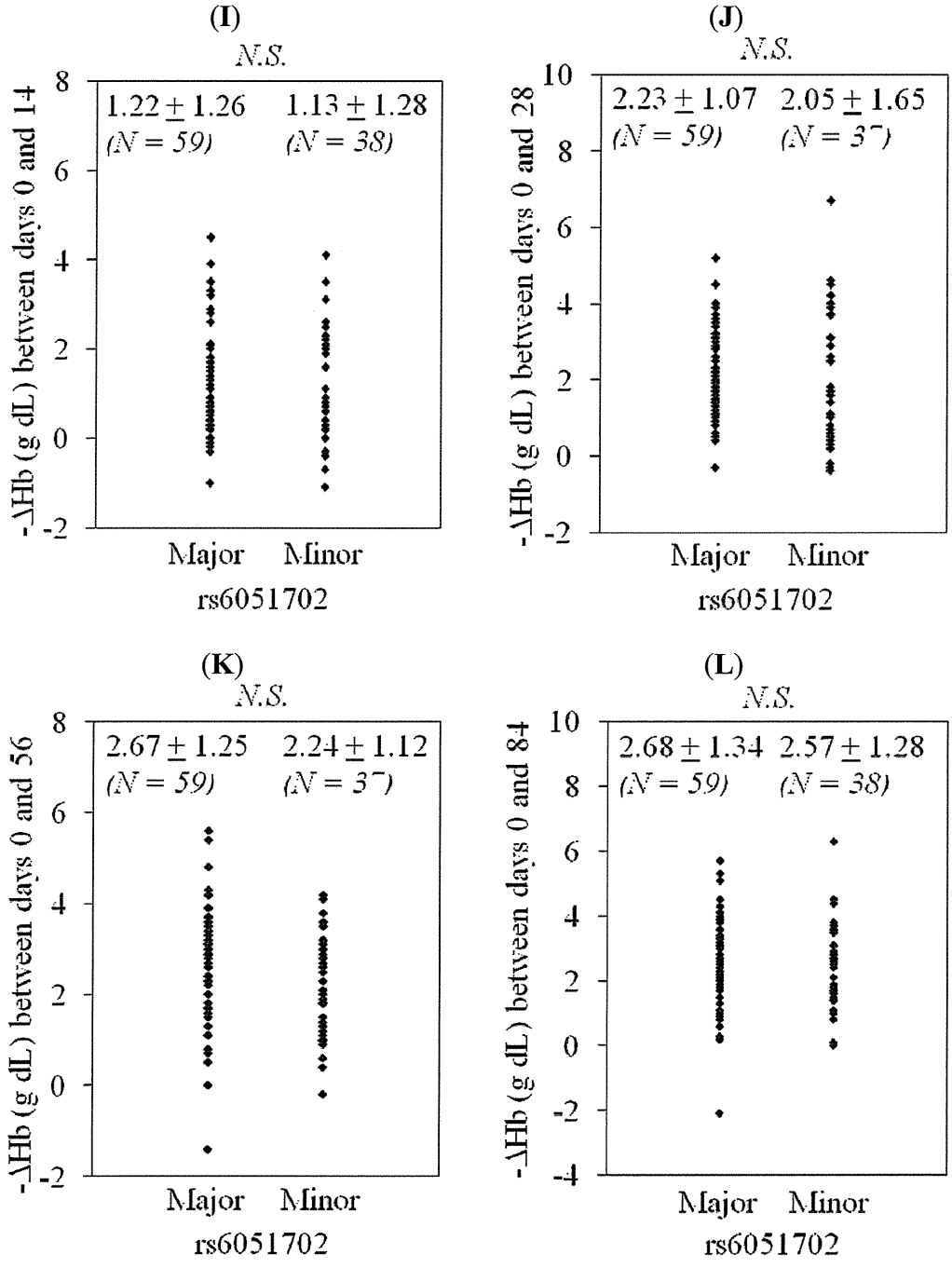


Figure 1. *Cont.*



2.4. Association Between ITPA rs1127354 Genotype and Dose Reduction of Drugs During Treatment

Next, we investigated the association between ITPA rs1127354 genotype and dose reduction of drugs at day 28 (Table 4). ITPA rs1127354 genotype could not predict the dose reduction of peginterferon (Table 4A), but ITPA rs1127354 major type could predict the dose reduction of ribavirin (Table 4B). We also examined the association between ITPA rs1127354 genotype and dose reduction of drugs at day 84 (data not shown). In patients with reduced ribavirin and/or peginterferon with null response, and in patients relapsed to the treatment, the proportion of patients with ITPA rs1127354

major type was greater among the patients with reduced ribavirin doses than among those with reduced peginterferon doses (20/20, 100% *vs.* 12/15, 80%; $P = 0.036$).

Table 4. Association between ITPA rs1127354 genotype and dose reduction of drugs at day 28. (A) Pegylated interferon (N = 74, no statistically significant difference); (B) Ribavirin (N = 74, $P = 0.0071$)

Study variables	*ITPA rs1127354* major type	*ITPA rs1127354* minor type
A		
Dose reduction (+)	17	4
Dose reduction (−)	57	19
B		
Dose reduction (+)	22	0
Dose reduction (−)	52	23

2.5. Effects of IL28B and ITPA Genotypes on the Reduction of White Blood Cell/Neutrophil Count

Next, we investigated the association between IL28B and ITPA genotypes, and other hematotoxicities between days 0 and 14, 28, 56 and 84 (data not shown). IL28B rs8099917 minor type induced higher reduction of white blood cell count ($P = 0.043$) as well as neutrophil count between days 0 and 14 ($P = 0.034$). We also analyzed the neutropenia, adjusting for background difference, and we confirmed these data. ITPA rs1127354 major type induced higher reduction of white blood cell count ($P = 0.035$) as well as higher reduction of neutrophil count between days 0 and 28 ($P = 0.020$). These genotypes had no effects on the reduction of white blood cell and neutrophil counts at any other time points, and ITPA rs6051702 had no effects on these reductions at any of the time points.

2.6. Effects of IL28B and ITPA Genotypes on the Reduction of Platelet Count

IL28B rs8099917 minor type induced higher reduction of platelet count between days 0 and 14 ($P = 0.013$) as well as between days 0 and 84 ($P = 0.032$) (data not shown). We also analyzed the thrombocytopenia, adjusting for the background difference, and we confirmed these data. ITPA rs1127354 minor-type induced higher reduction of platelet count between days 0 and 28 ($P = 0.026$) (data not shown). At any other time point these genotypes had no effects on the reduction of platelet count, and ITPA rs6051702 had no effects on this reduction at any time point.

3. Experimental Section

3.1. Patients

Between February 2010 and January 2011, blood samples were obtained from 97 chronic hepatitis C patients at the Department of Gastroenterology, Chiba University Medical School Hospital. Some of these patients had already been included in previous reports [7,8]. Written informed consent was obtained from each patient participating in this study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics review committee of Chiba

University, Graduate School of Medicine. Baseline characteristics are listed in Table 1. Sixty-seven and 30 patients were treatment-naïve and previously treated with interferon therapy, respectively. Previous relapse was defined as undetectable HCV RNA by the end of therapy [2], but then its reappearance after the end of therapy, and the definition of null response was less than 2 $\log_{10}$ decrease in HCV RNA from baseline after 12 weeks of therapy [2]. In 17 relapsers, 7, 3, 2, 2 and 3 received standard interferon monotherapy, standard interferon plus ribavirin, peginterferon monotherapy, peginterferon plus ribavirin and unknown, respectively. In 13 null-responders, 10, 1 and 2 received standard interferon monotherapy, standard interferon plus ribavirin and peginterferon plus ribavirin, respectively. Most patients were infected with HCV genotype 1 (83.5%) with high viral load (>5 log IU/mL) (97.9%). Ultrasound (US) findings showed cirrhosis of the liver in 12 cases (Table 1), 3 of which were also biopsy-proven.

3.2. Treatment

All 97 patients were treated with peginterferon-alfa once weekly and 400–1,000 mg of ribavirin daily [19–21]. Some of them stopped treatment at 12–16 weeks according to the early stopping rule.

3.3. HCV RNA Quantification

HCV RNA was determined by Amplicor HCV monitor assay, version 2.0 (range: 0.5–850 KIU/mL) (Roche Diagnostics, Tokyo, Japan), Amplicor HCV assay (Roche) or COBAS TaqMan HCV test (Roche) (range: 1.2–7.8 log IU/mL). The detection limit of this qualitative assay was 50 IU/mL, corresponding to 1.7 log IU/mL by COBAS TaqMan PCR assay [19]. We defined HCV RNA >5 log IU/mL and <5 log IU/mL as high and low viral titers of HCV RNA, respectively.

3.4. HCV Genotyping

HCV genotype was determined using the antibody-serotyping assay of Tsukiyama-Kohara *et al.* [22]. In this assay, HCV serotypes 1 and 2 correspond to genotypes 1a/1b and 2a/2b, respectively, according to Simmonds' classification [23].

3.5. Classification of Treatment Outcome

Patients were classified as having achieved RVR and early virological response (EVR) if HCV RNA was undetectable (<50 IU/mL) in serum at treatment week 4 and week 12, respectively, and as having SVR if HCV RNA was undetectable in serum 24 weeks after the completion of therapy.

3.6. DNA Extraction and TaqMan SNP Assay

To prepare the DNA sample from blood cells, we used DNA Extract All Lysis Reagents (Applied Biosystems Inc., Foster City, CA, USA). A specific TaqMan genotyping assay was performed for rs1127354, rs6051702 and rs8099917. Primers were manufactured by Applied Biosystems. Thermal cycling was performed with the ABI Step One real-time PCR system according to the manufacturer's protocol. Activation of TaqMan GTXpress Master Mix (Applied Biosystems) and the initial denaturation cycle was at 95 °C for 20 seconds, followed by 40 cycles at 95 °C for 3 seconds and 60 °C

for 20 seconds. We analyzed IL28B rs8099917 TT as major type and TG/GG as minor type, ITPA rs1127354 CC as major type and CA/AA as minor type, and ITPA rs6051702 AA as major type and AC/CC as minor type in the present study.

3.7. Statistical Analysis

Data were expressed as mean ± standard deviation. We used univariate analyses to compare patient characteristics and outcomes, applying Student's t-test or Chi-square test as appropriate. $P < 0.05$ was considered statistically significant.

4. Discussion and Conclusion

In the present study, we also observed that IL28B rs8099917 major genotype was useful for the prediction of treatment response, as in previous studies [4–9], which reported the association between IL28B genotypes and HCV eradication with peginterferon plus ribavirin therapies in chronic hepatitis C patients. SVR was strongly associated with IL28B major genotype (rs8099917 TT). Serum γGTP levels were significantly higher in IL28B rs8099917 minor-type patients, as we reported previously [8].

Previous studies [21,24] showed that HCV-infected patients who can be maintained on >80% of peginterferon and ribavirin dosage for the duration of treatment exhibit enhanced SVR rates. Adherence to therapy decreased over time with both antiviral medications, but more so with ribavirin [25]. Ribavirin could be associated with clinically significant hemolytic anemia, resulting in its necessary dose reduction or discontinuation [26,27]. However, we did not observe any association between ITPA genotypes and SVR.

We also observed that ribavirin-induced anemia is highly dependent on the ITPA rs1127354 genotypes between days 0 and 84, and ITPA rs1127354 major type has been reported to be associated with a reduction in hemoglobin between weeks 0 and 4 [28,29]. In the present study, we observed a difference in age between ITPA rs1127354 major and minor types (Table 2), albeit with a rather limited number of the latter patients. In this respect, further study will be needed, although our previous study showed that the SVR rate of patients aged ≤65 years was similar to that of patients aged >65 years [21]. Genetic variation of ITPA causing an accumulation of inosine triphosphate (ITP) could result in ribavirin-induced anemia. ITP confers protection against ribavirin-induced adenosine triphosphate (ATP) reduction by substituting for erythrocyte GTP, which is depleted by ribavirin, in the biosynthesis of ATP [30]. It is possible that ribavirin-induced anemia is due primarily to the effect of the drug on GTP and consequently ATP levels in erythrocytes [30].

Interestingly, we found that IL28B rs8099917 minor genotype was associated with greater reductions of neutrophils and platelets, although it was reported that IL28B polymorphisms were not associated with interferon-related cytopenia [31]. Our data support the previous reports that patients with ITPA rs1127354 major type had a higher degree of reactive increase in platelet count [32,33]. Further studies will be needed to investigate the potential underlying mechanism and to examine whether there is a synergistic effect of IL28B and ITPA. In the not-too-distant future, HCV therapy will likely move away from interferon-based regimens with increasing numbers of potent antiviral agents being approved, meaning that IL28B and/or ITPA genotyping would not play any additional role and be useful in clinical practice [34–36].

Recent studies revealed that IL28B is associated with hepatic interferon-stimulated gene (ISG) expression [10], hepatic STAT1-nuclear localization [9], hepatic suppressor of cytokine signal 3 (SOCS3) [37] and plasma interferon-gamma inducible protein-10 (IP-10) levels in chronic HCV infection [8]. It is possible that IL28B genotypes affect virus-host interaction through the interaction with interferon signaling pathways. IL28B major type also reported to be associated with a lower prevalence of hepatic steatosis and a less pronounced lipid metabolism, as reflected both by serum lipoprotein levels and hepatic steatosis in HCV infection [38–41]. Insulin resistance is more common in IL28B minor genotype than in major type in treatment-naïve patients with chronic hepatitis C [42,43]. Although there are contrary opinions [44,45], IL28B genotypes influence the stage of liver fibrosis [46,47] and HCV-related hepatocarcinogenesis [48]. Thus, IL28B genotypes play important roles in not only eradication of HCV but also HCV-related pathology.

In HCV infection, patients who developed HCC had lower platelet counts [49]. It is well known that the platelet count decreased with stage advancement of liver diseases in chronic hepatitis C patients [2,49–52]. Chronic hepatitis C is associated with variable degrees of anemia, neutropenia, and/or thrombocytopenia [52]. Multiple factors, including ITPA genotypes, might be involved in this phenomenon.

Our study showed that about 60% of Japanese patients infected with HCV have the preferable allele of IL28B rs8099917, but about 70% of patients also have the undesirable allele of ITPA rs1127354. There seem different distributions between IL28B and ITPA genotypes in the world [6,11]. In conclusion, ITPA rs1127354 is useful for the prediction of ribavirin-induced anemia in the earlier phase of peginterferon plus ribavirin treatment, and IL28B rs8099917 is useful for the prediction of SVR. Use of a combination of these genotypes could lead to a safe and effective treatment for chronic hepatitis C patients.

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Conflict of Interest

The authors declare no conflict of interest.

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