

**Table 2.** Baseline and demographic characteristics of SVR- and non-SVR-retreated patients

	SVR	Non-SVR	P-value*
Number of patients	18	11	N.S.
Age (years)	60.0 ± 10.0	60.3 ± 6.3	N.S.
Gender (male/female)	8/10	7/3	N.S.
Body mass index (kg/m <sup>2</sup> )	26.0 ± 3.4	26.5 ± 4.0	N.S.
HCV RNA (log IU/mL)	5.5 ± 1.9	5.5 ± 2.1	N.S.
ALT (IU/L)	57.8 ± 50.7	55.6 ± 52.8	N.S.
γ-GTP (IU/L)	46.0 ± 40.7	30.4 ± 17.6	N.S.
AFP (ng/mL)	5.7 ± 3.4	6.2 ± 5.7	N.S.
Leukocyte count (/mm <sup>3</sup> )	4940 ± 1670	4670 ± 940	N.S.
Hemoglobin (g/dL)	14.0 ± 1.6	13.6 ± 2.0	N.S.
Platelet count (x10 <sup>4</sup> /mm <sup>3</sup> )	16.2 ± 5.2	12.6 ± 4.1	0.061
<i>Treatment response</i>			
Duration of treatment (~24/48/72 wks)	6/11/1	3/7/1	N.S.
RVR rates, %	44.4 (8/18)	18.1 (2/11)	N.S.
HCV RNA negativity at 8 wks	88.8 (16/18)	66.6 (6/9)	N.S.
EVR rates, %	88.8 (16/18)	88.8 (8/9)	N.S.
Adherence (≥80/≥80/≥80), yes	44.4 (8/18)	54.5 (6/11)	N.S.

Data are expressed as mean ± SD. \*P-value, between groups with and without SVR by Student's t-test or chi-square test; N.S., not statistically significant; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transferase; AFP, alpha-fetoprotein; RVR, rapid virological response; EVR, early virological response; SVR, sustained virological response; adherence was classified according to the previous report [19].

### Previous treatment response and SVR rates in HCV genotype 2 retreated patients

The relationship between previous treatment response and SVR rates of HCV genotype 2 retreated patients is shown in Table 3. In 1 patient previously treated with peginterferon plus ribavirin and non-response, treatment was discontinued due to side effects by ~8 weeks and SVR was not obtained. Of 13 patients previously treated with peginterferon plus ribavirin who had relapsed, 2 discontinued treatment due to side effects by ~8 weeks.

### Female cases retreated, in whose sera a single very low-titer fluctuation of HCV RNA from negative to positive was detected after HCV RNA had been undetected

Furthermore, we tried to determine the clinical features of non-SVR HCV genotype 2 patients retreated with peginterferon alfa-2a plus ribavirin. We noticed 3 females retreated with peginterferon alfa-2a plus ribavirin and resulting in non-SVR, in whose sera

a single very low-titer fluctuation of HCV RNA from negative to positive was detected after HCV RNA had been undetected (Figure 1). HCV RNA finally relapsed in all 3 cases. Treatment with SOC might need to be stopped in HCV genotype 2 female patients with these features.

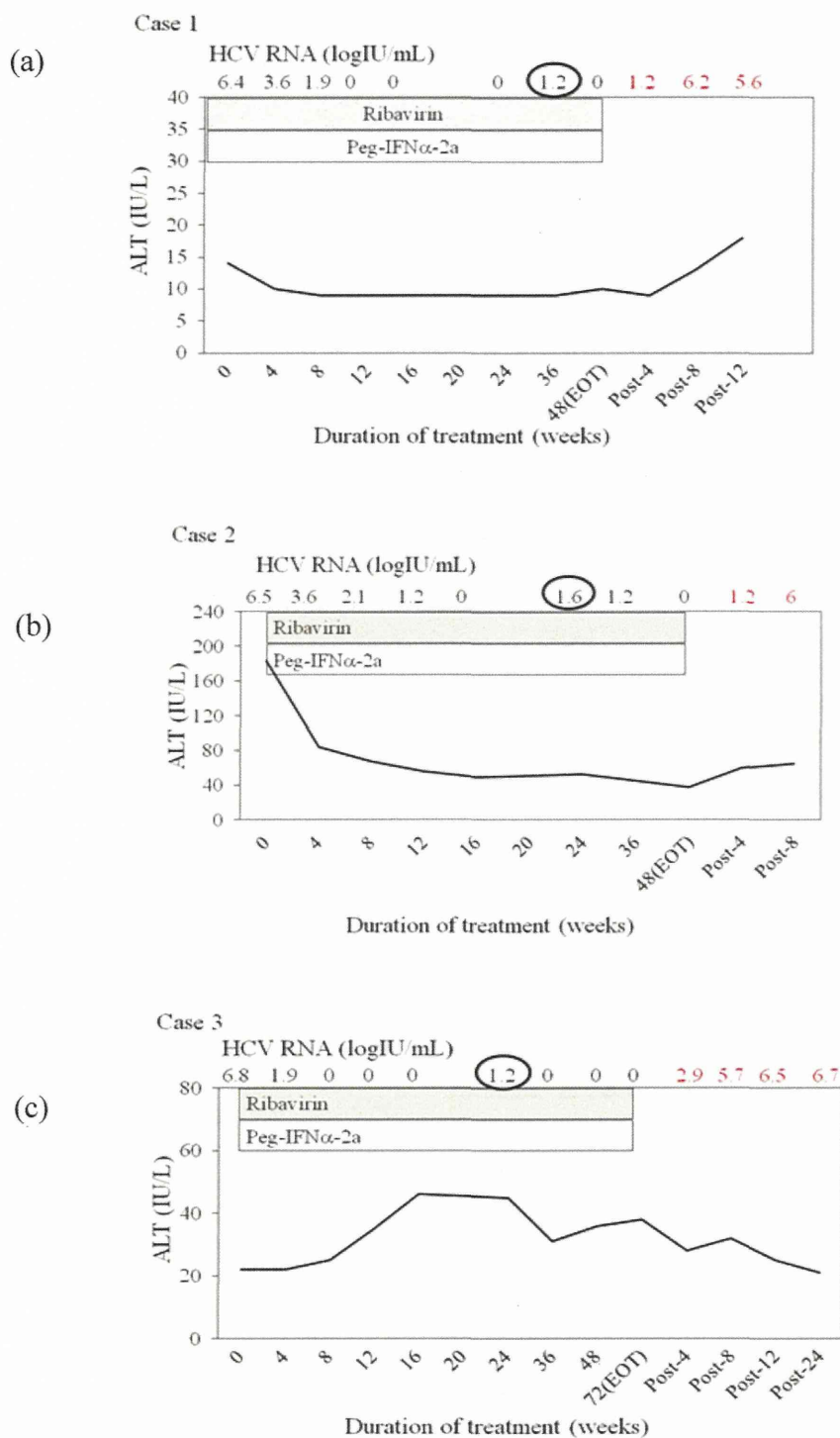
**Table 3.** Previous treatment response and SVR rates in 25 retreated patients

Number of patients	Previous treatment (Treatment response)	Formula of re-treatment	SVR rates (%)
6	Peginterferon alfa-2a (NR)	Peginterferon alfa-2a plus ribavirin (~24wks)	66.6
1	Peginterferon plus ribavirin (NR)	Peginterferon alfa-2a plus ribavirin (~24wks)	0
5	(Peg-)interferon (relapse)	Peginterferon alfa-2a plus ribavirin (~48wks)	60
13	Peginterferon plus ribavirin (relapse)	Peginterferon alfa-2a plus ribavirin (24~48wks)	69.9

NR, non-response

## DISCUSSION

In the present study, we focused on the virological response in HCV genotype 2-infected Japanese patients retreated with peginterferon alfa-2a plus ribavirin. We did not observe any differences in baseline background between SVR patients retreated and non-SVR patients retreated, although we must admit that the number of patients was small. However, during this study, we did find 3 females who did not obtain SVR by the retreatment and had unique features. That is, in their sera, a single very low-titer fluctuation of HCV RNA from negative to positive was detected after HCV RNA had been undetected (Figure 1). These 3 cases did not discontinue peginterferon alfa-2a or ribavirin. In Figure 1, cases 1 and 2 had reduced peginterferon alfa-2a but not reduced ribavirin. On the other hand, case 3 had reduced ribavirin due to anemia, but did not have a reduction of peginterferon alfa-2a. In cases 2 and 3, adherence (≥80/≥80/≥80) [19] based on the calculation at 48 weeks was not lower. These 3 cases were relapsers and seemed different from non-responders having anti-interferon-alfa neutralizing antibody [20]. We do not know the exact reasons at this time.



**Figure 1.** Three females retreated with peginterferon alpha plus ribavirin and resulting in non-sustained virological response, in whose sera a single very low-titer fluctuation of HCV RNA from negative to positive was detected after HCV RNA had been undetected. (a) Case 1, 68 years, female, IL28Brs8099917 TT. She was previously treated with peginterferon alpha-2a for 48 weeks, with details unknown. (b) Case 2, 58 years, female, IL28Brs8099977, not determined. She was previously treated with peginterferon alpha-2a for 48 weeks, with relapse. (c) Case 3, 58 years, female, IL28Brs8099917 TG. She was previously treated with peginterferon alpha-2b plus ribavirin, with details unknown. HCV RNA was determined by COBAS TaqMan HCV test (Roche), with levels ranging from 1.2 to 7.8 log IU/mL [16].

In the present study, 44% of patients had rapid virological response (RVR) and 89% of the patients had EVR (cEVR) in the retreated genotype 2 chronic hepatitis C patients with an SVR (Table 2). These results were concordant with previous studies. However, 89% of the non-SVR patients also had EVR (Table 2). Among the 8 non-SVR patients, 3 had lower adherence ( $\geq 80/\geq 80/\geq 80$ ) (data not shown). In the present study, the adherence rates were quite low (44% in patients with SVR, and 54% in patients without SVR). In certain cases, lower adherence may be one of the reasons for non-SVR.

For HCV genotype 1 patients, direct acting antivirals (DAAs) such as telaprevir and boceprevir have been available in clinical practice [7, 21-23]. The addition of telaprevir or boceprevir to peginterferon plus ribavirin resulted in significantly higher rates of SVR in previously treated patients with chronic HCV genotype 1 infection [7, 21-23]. It will require more time until the more frequent use of DAAs for the treatment of HCV genotype 2 patients will become possible [24, 25]. Until then, we have to retreat HCV genotype 2-infected patients with peginterferon alfa-2a plus ribavirin for 24-48 weeks.

Recently, it was reported by several groups that genetic variations in IL28B-SNP predict HCV genotype 1 treatment-induced viral clearance [7, 26-29]. Yu et al. [30] reported that rs8099917 TT genotype is significantly independently predictive of RVR, which is the single best predictor of SVR, in Asian HCV genotype 2 patients. Further study will be needed.

In conclusion, we showed that retreatment of HCV genotype 2-infected Japanese patients with peginterferon alfa-2a plus ribavirin for 24-48 weeks resulted in 60 to 66.6% SVR in patients previously treated with (peg-)interferon monotherapy and in 69.9% SVR in relapsers previously treated with peginterferon plus ribavirin, which supports the previous reports [12, 13]. Attention should be paid to certain patients with unique features. Selection of patients according to previous treatment could lead to optimal therapy in HCV genotype 2 treatment-experienced patients.

## ACKNOWLEDGEMENTS

We thank Dr Yutaka Yonemitsu, Dr Fumihiko Kanai, Dr Akinobu Tawada, Dr Nobuyuki Sugiura, Dr Rintaro Mikata, Dr Tetsuhiro Chiba, Dr Motohisa Tada, Dr Motohide Takashi, Dr Kenichi Fukai, Dr Yasushi Maru, Dr Takeshi Nihei, Dr Norio Kikuchi, Dr Noritomo Shimada, Dr Yasuo Hirai, Dr Shuuichi Saito, Dr Shinichi Hino, Dr Shousuke Iwama, Dr Masaaki Saito, Dr Hiroshige Kojima, Dr Michio Kimura, Dr Kazuhiko Kita, Dr Susumu Nakahori, Dr

Shinichi Sato, Dr Yutaka Natsuki, Dr Hidetaka Terabayashi, Dr Masahiko Sanada, Dr Noriaki Suzuki, Dr Ryosaku Azemoto, Dr Hideki Takanashi, Dr Katsumi Doai, Dr Shinnen Kin, Dr Akito Nozaki, Dr Satoru Kaneda, Dr Michikazu Abe, Dr Hikaru Nagahara, Dr Yoko Hoshino, Dr Kinki Rin and all other investigators for coordinating with this work. This study was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (TK, SN and TT), a grant from the Ministry of Health, Labour and Welfare of Japan (OY), and a Special Coordination Fund for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government (TK).

## CONFLICT OF INTEREST

Dr. Tatsuo Kanda reports receiving lecture fees from Chugai Pharmaceutical, MSD, and Ajinomoto, and Prof. Osamu Yokosuka receiving grant support from Chugai Pharmaceutical, Bayer, MSD, Daiichi-Sankyo, and Mitsubishi Tanabe Pharma.

## ABBREVIATIONS

cEVR: Complete early virological response; DAA: Direct-acting antiviral; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; IL28B: Interleukin-28B; RVR: Rapid virological response; SNP: Single nucleotide polymorphism; SD: Standard deviation; SOC: Standard of care; SVR: Sustained virological response.

## REFERENCES

- Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. *Hepatology*. 1997; 26 (3 Suppl 1): 34S-38S.
- Kanda T, Imazeki F, Mikami S, et al. Occurrence of hepatocellular carcinoma was not a rare event during and immediately after antiviral treatment in Japanese HCV-positive patients. *Oncology*. 2011; 80: 366-372.
- Simmonds P, Bukh J, Combet C, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology*. 2005; 42: 962-973.
- Takano S, Yokosuka O, Imazeki F, et al. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology*. 1995; 21: 650-655.
- Mangia A, Santoro R, Minerva N, et al. Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. *N Engl J Med*. 2005; 352: 2609-2617.
- Shiffman ML, Suter F, Bacon BR, et al. Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med*. 2005; 357: 124-134.
- Kanda T, Imazeki F, Yokosuka O. New antiviral therapies for chronic hepatitis C. *Hepatol Int*. 2010; 4: 548-561.
- Yu ML, Dai CY, Huang JF, et al. A randomized study of peginterferon and ribavirin for 16 versus 24 weeks in patients with genotype 2 chronic hepatitis C. *Gut*. 2007; 56: 553-559.
- Lagging M, Langeland N, Pedersen C, et al. Randomized comparison of 12 or 24 weeks of peginterferon alpha-2a and ribavirin in chronic hepatitis C virus genotype 2/3 infection. *Hepatology*. 2008; 47: 1837-1845.
- Yokosuka O, Iwama S, Suzuki N, et al. High sustained virologic response rate after interferon monotherapy in Japanese hepatitis C patients with a low HCV RNA titer and/or HCV genotype 2. A prospective study. *Intervirol*. 2004; 47: 328-334.

11. Omata M, Kanda T, Yu ML, et al. APASL consensus statements and management algorithms for hepatitis C virus infection. *Hepatology*. 2012; 6: 409-435.
12. Sherman M, Yoshida EM, Deschenes M, et al. Peginterferon alfa-2a (40KD) plus ribavirin in chronic hepatitis C patients who failed previous interferon therapy. *Gut*. 2006; 55: 1631-1638.
13. Poynard T, Colombo M, Bruix J, et al. Peginterferon alfa-2a and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. *Gastroenterology*. 2009; 136: 1618-1628.
14. Kanda T, Imazeki F, Azemoto R, et al. Response to peginterferon-alfa 2b and ribavirin in Japanese patients with chronic hepatitis C genotype 2. *Dig Dis Sci*. 2011; 56: 3335-3342.
15. Etoh R, Imazeki F, Kurihara T, et al. Pegylated interferon-alfa-2a monotherapy in patients infected with HCV genotype 2 and importance of rapid virological response. *BMC Res Notes*. 2011; 4: 316.
16. Kanda T, Imazeki F, Yonemitsu Y, et al. Quantification of hepatitis C virus in patients treated with peginterferon-alfa 2a plus ribavirin treatment by COBAS TaqMan HCV test. *J Viral Hepat*. 2011; 18:e292-297.
17. Tsukiyama-Kohara K, Yamaguchi K, Maki N, et al. Antigenicities of Group I and II hepatitis C virus polypeptides—molecular basis of diagnosis. *Virology*. 1993; 192:430-437.
18. Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. *Hepatology*. 1994; 19: 1347-1353.
19. McHutchison JG, Manns M, Patel K, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology*. 2002; 123: 1061-1069.
20. Matsuda F, Torii Y, Enomoto H, et al. Anti-interferon- $\alpha$  neutralizing antibody is associated with nonresponse to pegylated interferon- $\alpha$  plus ribavirin in chronic hepatitis C. *J Viral Hepat*. 2012; 19: 694-703.
21. McHutchison JG, Manns MP, Muir AJ, et al. Telaprevir for previously treated chronic HCV infection. *N Engl J Med*. 2010; 362: 1292-1303.
22. Zeuzem S, Andreone P, Pol S, et al. Telaprevir for retreatment of HCV infection. *N Engl J Med*. 2011; 364: 2417-2428.
23. Bacon BR, Gordon SC, Lawitz E, et al. Boceprevir for previously treated chronic HCV genotype 1 infection. *N Engl J Med*. 2011; 364: 1207-1217.
24. Foster GR, Hezode C, Bronowicki JP, et al. Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. *Gastroenterology*. 2011; 141:881-889.
25. Mangia A, Mottola L. What's new in HCV genotype 2 treatment. *Liver Int*. 2012; 32 Suppl 1: 135-140.
26. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*. 2009; 461: 399-401.
27. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet*. 2009; 41: 1105-1109.
28. Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet*. 2009; 41: 1100-1104.
29. Miyamura T, Kanda T, Nakamoto S, et al. Hepatic STAT1-nuclear translocation and interleukin 28B polymorphisms predict treatment outcomes in hepatitis C virus genotype 1-infected patients. *PLoS One*. 2011; 6: e28617.
30. Yu ML, Huang CF, Huang JF, et al. Role of interleukin-28B polymorphisms in the treatment of hepatitis C virus genotype 2 infection in Asian patients. *Hepatology*. 2011; 53: 7-13.

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

Tatsuo Kanda,<sup>1</sup> Shuang Wu,<sup>1</sup> Tomoko Kiyohara,<sup>3</sup> Shingo Nakamoto,<sup>1,2</sup> Xia Jiang,<sup>1</sup> Tatsuo Miyamura,<sup>1</sup> Fumio Imazeki,<sup>1</sup> Koji Ishii,<sup>3</sup> Takaji Wakita,<sup>3</sup> and Osamu Yokosuka<sup>1</sup>

## Abstract

Our aim was to investigate the effects of interferons (IFNs)- $\lambda$  (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- $\alpha$  or amantadine resulted in stronger inhibition of HAV IRES activity. Our findings demonstrated a novel antiviral effect of IFNs- $\lambda$  against HAV and HCV through the suppression of IRES-mediated translation.

## Introduction

**H**EPATITIS 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- $\lambda$  are the most recently defined members of the class III cytokine family, consisting of IL-28A (IFN- $\lambda$ 2), IL-28B (IFN- $\lambda$ 3), and IL-29 (IFN- $\lambda$ 1), and a component of their receptor, IL28-R $\alpha$ . 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- $\lambda$  can synergize with IFN- $\alpha$  in viral infections, or whether it plays an independent primary role in the antiviral system (41).

There are several reports that IFN- $\lambda$  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- $\lambda$  receptor complex and IFNs- $\lambda$  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- $\lambda$  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

<sup>1</sup>Department of Medicine and Clinical Oncology, and <sup>2</sup>Department of Molecular Virology, Chiba University Graduate School of Medicine, Chiba, Japan.

<sup>3</sup>Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

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- $\lambda$  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<sub>2</sub>. 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  $\mu$ g/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.

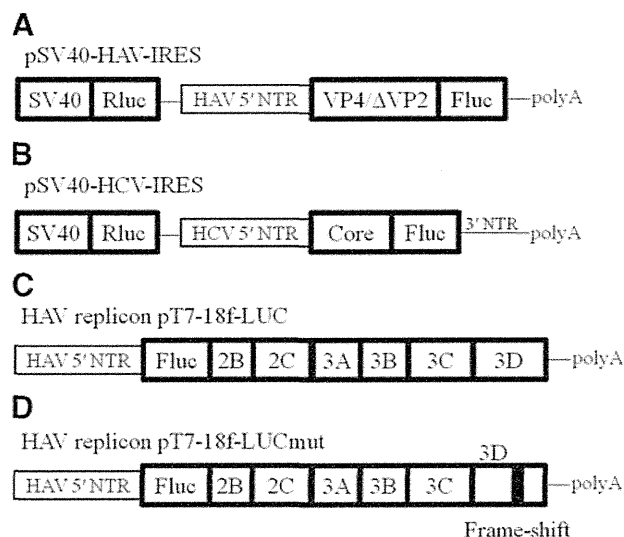
Cell culture-grown HCV JFH1 (genotype 2a) was used in Huh7-derived cell lines (14,43). Tissue culture-adapted HAV strain KRM003 (genotype IIIB) was used in African green monkey kidney GL37 cells (22,37). A HAV DNA-based subgenomic replicon [kindly provided by Prof. V. Gauss-Muller, Institute of Virology, 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- $\alpha$  (Sigma-Aldrich, St. Louis, MO), human recombinant IL29 (IFN- $\lambda$ 1; 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).



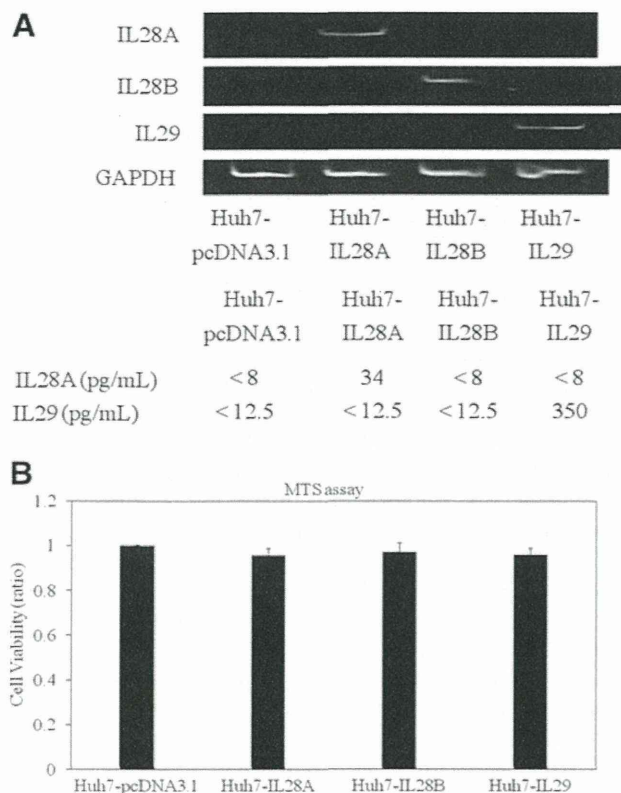
**FIG. 1.** Structure of HAV/HCV reporter constructs used in this study. (A) pSV40-HAV IRES-luc encodes the *Renilla reniformis* luciferase (Rluc), the internal ribosomal entry site (IRES) of HAV HM175, and firefly luciferase (Fluc) under the control of SV40 promoter, with a polyadenylation signal (polyA) (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  $1.0 \times 10^5$  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  $\mu$ g 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  $-80^\circ\text{C}$  until use. RNA quality was examined using the A<sub>280</sub>:A<sub>260</sub> ratio (Pharmacia Biotech, Bedford, MA). cDNA synthesis was performed with a random hexamer using Prime Script reverse transcriptase

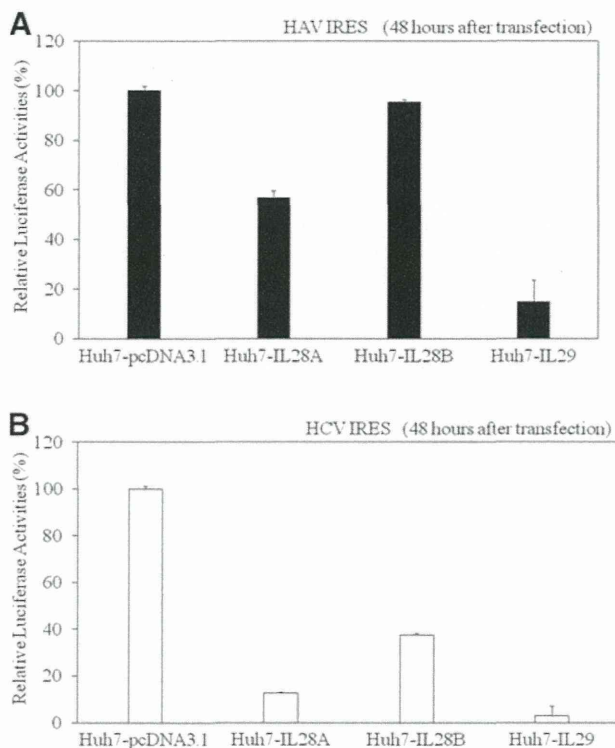


**FIG. 2.** Overexpression of IL-28A, IL-28B, or IL-29 in human hepatoma cell line Huh7. (A) Expression of IFNs- $\lambda$  mRNA in Huh7-pcDNA3.1, Huh7-IL28A, Huh7-IL28B, and Huh7-IL29 cells (upper panel). RT-PCR was performed using each specific primer. ELISA results for IL-28A and IL-29 are shown in 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  $0.5 \times 10^6$ , and MTS assay was performed at 24 h. The value of Huh7-pcDNA3.1 was set at 1. Data are expressed as mean  $\pm$  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- $\lambda$  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  $\pm$  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  $\pm$  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- $\lambda$ -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

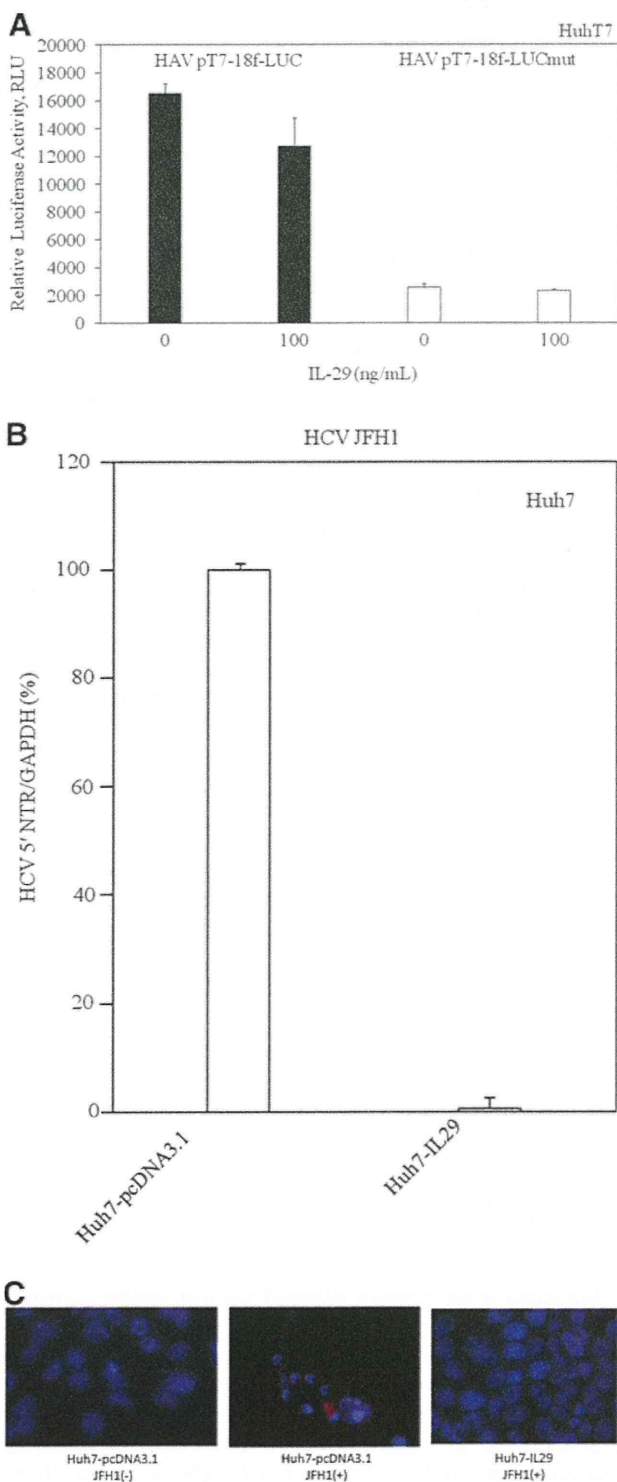
cellular RNA of Huh7-IL28B since we could not use ELISA for IL-28B at this time (Fig. 2A, upper panel). We next examined whether overexpression of IFN- $\lambda$  had any effect on cell proliferation. Equal numbers of control Huh7-pcDNA3.1 and IFN- $\lambda$ -overexpressing Huh7 cells (Huh7-IL28A, Huh7-IL28B, 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- $\lambda$  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).

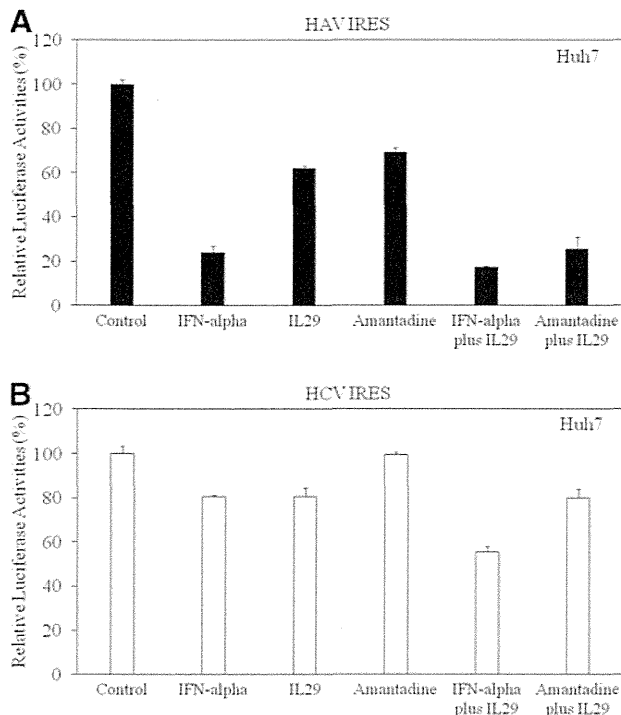
#### HCV IRES activities in Huh7-IL28A, Huh7-IL28B, and Huh7-IL29

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- $\lambda$  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 ( $n=3$ , 100%). 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) Huh7 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 immunofluorescence 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  $\pm$  SD of triplicate determinations from one experiment representative of three independent experiments.





**FIG. 5.** Effects of IL-29 with or without interferon (IFN)- $\alpha$  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  $\mu$ g/mL amantadine or 100 IU/mL IFN- $\alpha$ , 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  $\pm$  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 Huh7 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 mutant 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- $\alpha$ 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- $\alpha$ , 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- $\alpha$  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- $\alpha$ , 50 ng/mL IL-29, and 50  $\mu$ g/mL amantadine, respectively (Fig. 5A). The combination of IL-29 with IFN- $\alpha$  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- $\alpha$  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- $\alpha$ , 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- $\alpha$  and IL-29, respectively, but showed no inhibition by amantadine only (99.3%,  $n=3$ ; Fig. 5B). The combination of IL-29 with IFN- $\alpha$  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- $\alpha$  or amantadine seemed to have stronger inhibitory effects on HAV IRES activity than IL-29 alone.

IFNs- $\lambda$  modulate innate and adaptive immune responses to environmental pathogens and protect the host against diseases such as cancer. Expression of IFNs- $\lambda$  is tightly regulated by viral infection, including hepatitis viral infection (6,7). IFNs- $\lambda$  utilize a receptor complex different from IFN- $\alpha$ , 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- $\alpha$ , IFNs- $\lambda$  bind to a heterodimeric receptor consisting of IL-28R $\alpha$  subunit and IL10R $\beta$  receptor subunit, that is also shared by IL-10, IL-22, and IL-26. Because the IFN- $\lambda$  receptor is different from that of IFN- $\alpha$ , their uses as alternative therapies for viral hepatitis need to be examined (6). Although the biological activities of IFNs- $\lambda$  could overlap with IFN- $\alpha$ , the expression of IL-28R $\alpha$  receptor is limited in contrast to the ubiquitously expressed IL10R $\beta$ , and IFNs- $\lambda$  might have fewer adverse events than type I IFN (28). We also found that the combination of IL-29 with IFN- $\alpha$  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- $\lambda$  as well as IFN- $\alpha$  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- $\alpha$ , inhibited HAV IRES-mediated translation and HAV replication (15,19,33,45). In the present study, we planned to examine the effects of IFN- $\lambda$  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- $\lambda$  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- $\lambda$ , it was reported that IL-28A inhibits HCV IRES-mediated translation and suppresses HCV replication (49). Kato *et al.* (21) reported that IFN- $\alpha$ , as well as IFN- $\beta$ , 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- $\alpha$ , 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- $\alpha$  or amantadine has a stronger inhibitory effect. IFNs- $\lambda$  might also play an important role in host defense mechanisms and in HAV pathogenesis.

### Acknowledgments

The authors thank Prof. T. Betakova, Prof. M. Kruger, Prof. S.U. Emerson, Prof. V. Gauss-Muller, Prof. R.B. Ray, and Prof. S.M. Lemon for providing the plasmids and Huh7 cells. This work was supported by a grant from the Japan Society of Hepatology (T.K.), and a grant from the Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (T.K.), and a grant from the Ministry of Health, Labour and Welfare of Japan (O.Y.).

### Author Disclosure Statement

No competing financial interests exist.

### References

1. Bacon BR, and Khalid O: New therapies for hepatitis C virus infection. *Mo Med* 2011;108:255–259.
2. Balvay L, Soto Rifo R, Ricci EP, Decimo D, and Ohlmann T: Structural and functional diversity of viral IRESes. *Biochim Biophys Acta* 2009;1789:542–557.
3. Basu A, Meyer K, Lai KK, *et al.*: Microarray analyses and molecular profiling of Stat3 signaling pathway induced by hepatitis C virus core protein in human hepatocytes. *Virology* 2006;349:347–358.
4. Cordes S, Kusov Y, Heise T, and Gauss-Muller V: La autoantigen suppresses IRES-dependent translation of the hepatitis A virus. *Biochem Biophys Res Commun* 2008;368:1014–1019.
5. Diegelmann J, Beigel F, Zitzmann K, *et al.*: Comparative analysis of the lambda-interferons IL-28A and IL-29 regulating their transcriptome and their antiviral properties against hepatitis C virus. *PLoS One* 2010;5:e1522.
6. Doyle SE, Schreckhise H, Khuu-Duong K, *et al.*: Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* 2006;44:896–906.
7. Dunn C, Peppas D, Khanna P, *et al.*: Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. *Gastroenterology* 2009;137:1289–1300.
8. Fujiwara K, Kojima H, Yonemitsu Y, *et al.*: Phylogenetic analysis of hepatitis A virus in sera from patients with hepatitis A of various severities. *Liver Int* 2009;29:838–845.
9. Gauss-Muller V, and Kusov YY: Replication of a hepatitis A virus replicon detected by genetic recombination *in vivo*. *J Gen Virol* 2002;83:183–192.
10. Ge D, Fellay J, Thompson AJ, *et al.*: Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401.
11. Glass MJ, Jia XY, and Summers DF: Identification of the hepatitis A virus internal ribosome entry site: *in vivo* and *in vitro* analysis of bicistronic RNAs containing the HAV 5' noncoding region. *Virology* 1993;193:842–852.

12. Glass MJ, and Summers DF: Identification of a trans-acting activity from liver that stimulates hepatitis A virus translation *in vivo*. *Virology* 1993;193:1047–1050.
13. Ito T, Tahara SM, and Lai MM: The 3'-untranslated region of hepatitis C virus RNA enhances translation from an internal ribosomal entry site. *J Virol* 1998;72:8789–8796.
14. Kanda T, Basu A, Steele R, *et al.*: Generation of infectious hepatitis C virus in immortalized human hepatocytes. *J Virol* 2006;80:4633–4639.
15. Kanda T, Imazeki F, Nakamoto S, Okitsu K, Fujiwara K, and Yokosuka O: Internal ribosomal entry-site activities of clinical isolate-derived hepatitis A virus and inhibitory effects of amantadine. *Hepatol Res* 2010;40:415–423.
16. Kanda T, Imazeki F, and Yokosuka O: New antiviral therapies for chronic hepatitis C. *Hepatol Int* 2010;4:548–561.
17. Kanda T, Jeong SH, Imazeki F, Fujiwara K, and Yokosuka O: Analysis of 5' nontranslated region of hepatitis A viral RNA genotype I from South Korea: comparison with disease severities. *PLoS One* 2010;5:e15139.
18. Kanda T, Steele R, Ray R, and Ray RB: Small interfering RNA targeted to hepatitis C virus 5' nontranslated region exerts potent antiviral effect. *J Virol* 2007;81:669–676.
19. Kanda T, Yokosuka O, Imazeki F, Fujiwara K, Nagao K, and Saisho H: Amantadine inhibits hepatitis A virus internal ribosomal entry site-mediated translation in human hepatoma cells. *Biochem Biophys Res Commun* 2005;331:621–629.
20. Kanda T, Zhang B, Kusov Y, Yokosuka O, and Gaussmuller V: Suppression of hepatitis A virus genome translation and replication by siRNAs targeting the internal ribosomal entry site. *Biochem Biophys Res Commun* 2005;330:1217–1223.
21. Kato J, Kato N, Moriyama M, *et al.*: Interferons specifically suppress the translation from the internal ribosome entry site of hepatitis C virus through a double-stranded RNA-activated protein kinase-independent pathway. *J Infect Dis* 2002;186:155–163.
22. Kiyohara T, Totsuka A, Yoneyama T, Ishii K, Ito T, and Wakita T: Characterization of anti-idotypic antibodies mimicking antibody- and receptor-binding sites on hepatitis A virus. *Arch Virol* 2009;154:1263–1269.
23. Komar AA, and Hatzoglou M: Internal ribosome entry sites in cellular mRNAs: mystery of their existence. *J Biol Chem* 2005;280:23425–23428.
24. Korf M, Jarczak D, Beger C, Manns MP, and Kruger M: Inhibition of hepatitis C virus translation and subgenomic replication by siRNAs directed against highly conserved HCV sequence and cellular HCV cofactors. *J Hepatol* 2005;43:225–234.
25. Kotenko SV, Gallagher G, Baurin VV, *et al.*: IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003;4:69–77.
26. Licursi M, Komatsu Y, Pongnopparat T, and Hirasawa K: Promotion of viral IRES-mediated translation under amino acid starvation. *J Gen Virol* 2012;93:951–962.
27. Li J, Lin S, Chen Q, *et al.*: Inhibition of hepatitis B virus replication by MyD88 involves accelerated degradation of pregenomic RNA and nuclear retention of pre-S/S RNAs. *J Virol* 2010;84:6387–6399.
28. Li Q, Kawamura K, Ma G, *et al.*: Interferon-lambda induces G1 phase arrest or apoptosis in oesophageal carcinoma cells and produces anti-tumour effects in combination with anti-cancer agents. *Eur J Cancer* 2010;46:180–190.
29. Ma D, Jiang D, Qing M, *et al.*: Antiviral effect of interferon lambda against West Nile virus. *Antiviral Res* 2009;83:53–60.
30. Mackiewicz V, Cammas A, Desbois D, *et al.*: Nucleotide variability and translation efficiency of the 5' untranslated region of hepatitis A virus: update from clinical isolates associated with mild and severe hepatitis. *J Virol* 2010;84:10139–10147.
31. Martin A, and Lemon SM: Hepatitis A virus: from discovery to vaccines. *Hepatology* 2006;43:S164–S172.
32. Miyamura T, Ishii K, Kanda T, *et al.*: Possible widespread presence of hepatitis A virus subgenotype IIIA in Japan: Recent trend of hepatitis A causing acute liver failure. *Hepatol Res* 2011;42:248–253.
33. Miyamura T, Kanda T, Nakamoto S, *et al.*: Hepatic STAT1-nuclear translocation and interleukin 28B polymorphisms predict treatment outcomes in hepatitis C virus genotype 1-infected patients. *PLoS One* 2011;6:e28617.
34. Mordstein M, Neugebauer E, Ditt V, *et al.*: Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *J Virol* 2010;84:5670–5677.
35. Muir AJ, Shiffman ML, Zaman A, *et al.*: Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. *Hepatology* 2010;52:822–832.
36. Robek MD, Boyd BS, and Chisari FV: Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 2005;79:3851–3854.
37. Robertson BH, Jansen RW, Khanna B, *et al.*: Genetic relatedness of hepatitis A strains recovered from different geographical regions. *J Gen Virol* 1992;73:1365–1377.
38. Schultz DE, Honda M, Whetter LE, McKnight KL, and Lemon SM: Mutations within the 5' nontranslated RNA of cell culture-adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey kidney cells. *J Virol* 1996;70:1041–1049.
39. Sheppard P, Kindsvogel W, Xu W, *et al.*: IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003;4:63–68.
40. Shimazaki T, Honda M, Kaneko S, and Kobayashi K: Inhibition of internal ribosomal entry site-directed translation of HCV by recombinant IFN-alpha correlates with a reduced La protein. *Hepatology* 2002;35:199–208.
41. Svetlikova D, Kabat P, Ohradanova A, Pastorek J, and Betakova T: Influenza A virus replication is inhibited in IFN-lambda2 and IFN-lambda3 transfected or stimulated cells. *Antiviral Res* 2010;88:329–333.
42. Tanaka Y, Nishida N, Sugiyama M, *et al.*: Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;41:1105–1109.
43. Wakita T, Pietschmann T, Kato T, *et al.*: Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791–796.
44. Wu S, Kanda T, Imazeki F, *et al.*: Hepatitis B virus e antigen downregulates cytokine production in human hepatoma cell lines. *Viral Immunol* 2010;23:467–476.
45. Yang L, Kiyohara T, Kanda T, *et al.*: Inhibitory effects on HAV IRES-mediated translation and replication by a combination of amantadine and interferon-alpha. *Virol J* 2010;7:212.
46. Yi M, Schultz DE, and Lemon SM: Functional significance of the interaction of hepatitis A virus RNA with glyceraldehyde 3-phosphate dehydrogenase (GAPDH): opposing effects of GAPDH and polypyrimidine tract binding protein

- on internal ribosome entry site function. *J Virol* 2000;74:6459–6468.
47. Yu ML, Huang CF, Huang JF, *et al.*: Role of interleukin-28B polymorphisms in the treatment of hepatitis C virus genotype 2 infection in Asian patients. *Hepatology* 2011;53:7–13.
  48. Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, and Hartmann R: Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J Virol* 2007;81:7749–7758.
  49. Zhu H, Butera M, Nelson DR, and Liu C: Novel type I interferon IL-28A suppresses hepatitis C viral RNA replication. *Virology* 2005;2:80.

Address correspondence to:  
Tatsuo Kanda, MD, PhD  
Associate Professor, Department of Medicine  
and Clinical Oncology  
Graduate School of Medicine  
Chiba University  
1-8-1 Inohana  
Chuo-ku, Chiba 260-8677, Japan  
E-mail: kandat-cib@umin.ac.jp

Received February 16, 2012; accepted June 10, 2012.

Article

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

Tatsuo Miyamura<sup>1</sup>, Tatsuo Kanda<sup>1,\*</sup>, Shingo Nakamoto<sup>1,2</sup>, Shuang Wu<sup>1</sup>, Xia Jiang<sup>1</sup>, Makoto Arai<sup>1</sup>, Keiichi Fujiwara<sup>1</sup>, Fumio Imazeki<sup>1</sup> and Osamu Yokosuka<sup>1</sup>

<sup>1</sup> Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan; E-Mails: miyamura\_\_ta@hotmail.com (T.M.); nakamotoer@yahoo.co.jp (S.N.); wushuang@graduate.chiba-u.jp (S.W.); jxia925@yahoo.co.jp (X.J.); araim-cib@umin.ac.jp (M.A.); fujiwara-cib@umin.ac.jp (K.F.); imazekif@faculty.chiba-u.jp (F.I.); yokosukao@faculty.chiba-u.jp (O.Y.)

<sup>2</sup> Department of Molecular Virology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan

\* Author to whom correspondence should be addressed; E-Mail: kandat-cib@umin.ac.jp; Tel.: +81-43-226-2086; Fax: +81-43-226-2088.

Received: 12 June 2012; in revised form: 27 July 2012 / Accepted: 6 August 2012 /

Published: 14 August 2012

---

**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
<b>SNP genotype</b>	
IL28B rs8099917 TT/TG/GG	60/35/2
ITPA rs1127354 CC/CA/AA	74/21/2
ITPA rs6051702 AA/AC/CC	59/32/6
<b>Response to previous therapy</b>	
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 <sup>3</sup> )	5,410 ± 1,640
Hemoglobin (g/dL)	14.0 ± 1.1
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> )	17.5 ± 5.1
History of diabetes mellitus (+/-)	15/82
US (CLD/cirrhosis/unknown)	83/12/2
<b>Treatment Response</b>	
RVR (+/-/unknown)	14/82/1
EVR (+/-)	52/45
SVR (+/relapser/null/unknown)	40/27/22/8

H, high viral load ( $\geq 5$  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  $\gamma$ GTP 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	<i>IL28B rs8099917</i>			<i>ITPA rs1127354</i>			<i>ITPA rs6051702</i>		
	TT	TG/GG	<i>P</i> -value	CC	CA/AA	<i>P</i> -value	AA	AC/CC	<i>P</i> -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.
<i>Response to previous therapy</i> (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 <sup>3</sup> )	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 <sup>4</sup> /mm <sup>3</sup> )	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 ( $\geq 5$  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	<i>IL28B rs8099917</i>			<i>ITPA rs1127354</i>			<i>ITPA rs6051702</i>		
	TT	TG/GG	<i>P</i> -value	CC	CA/AA	<i>P</i> -value	AA	AC/CC	<i>P</i> -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–L). 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–H).

**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 56, and (D), (H) and (L) between days 0 and 84.

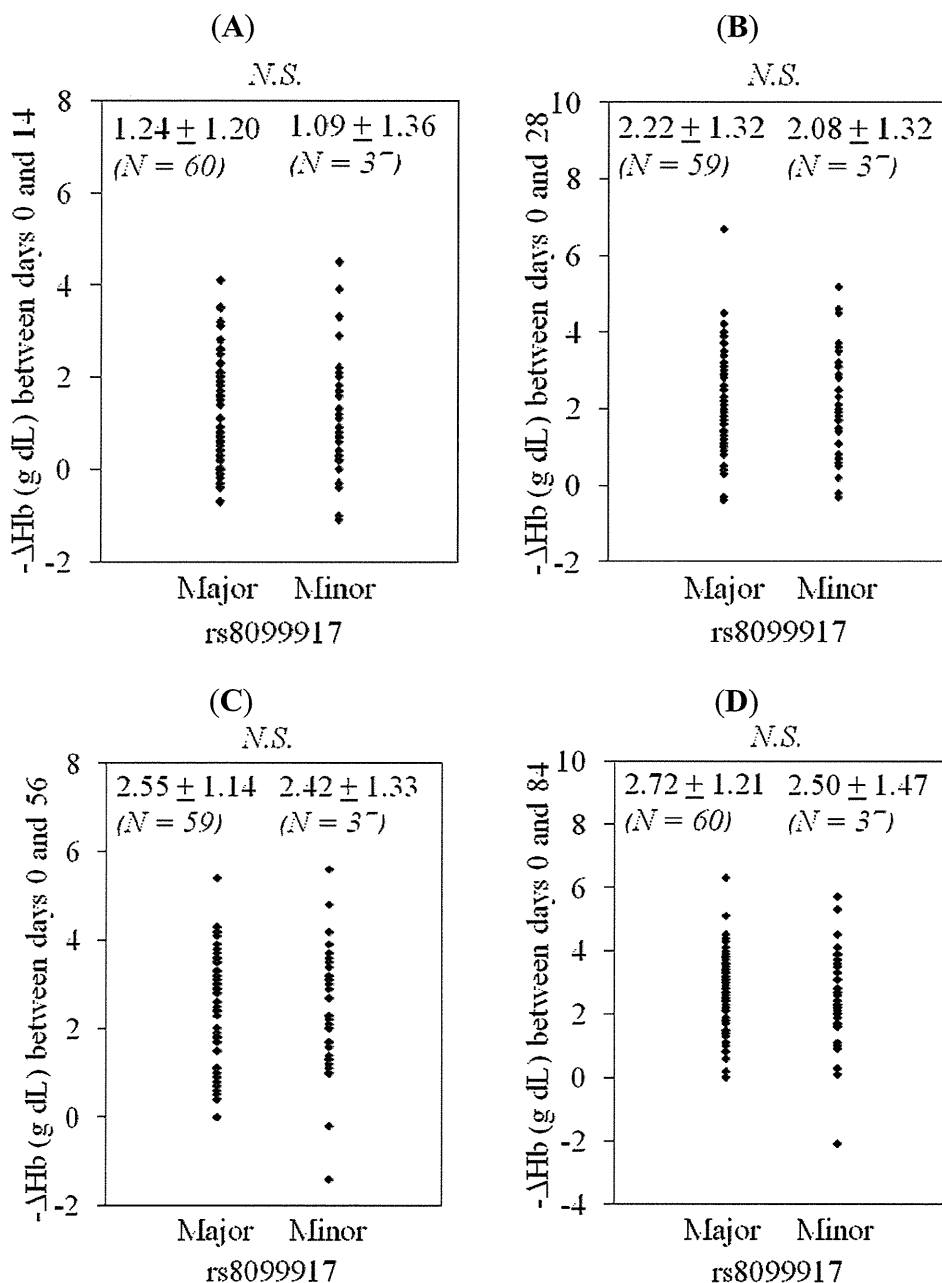


Figure 1. Cont.

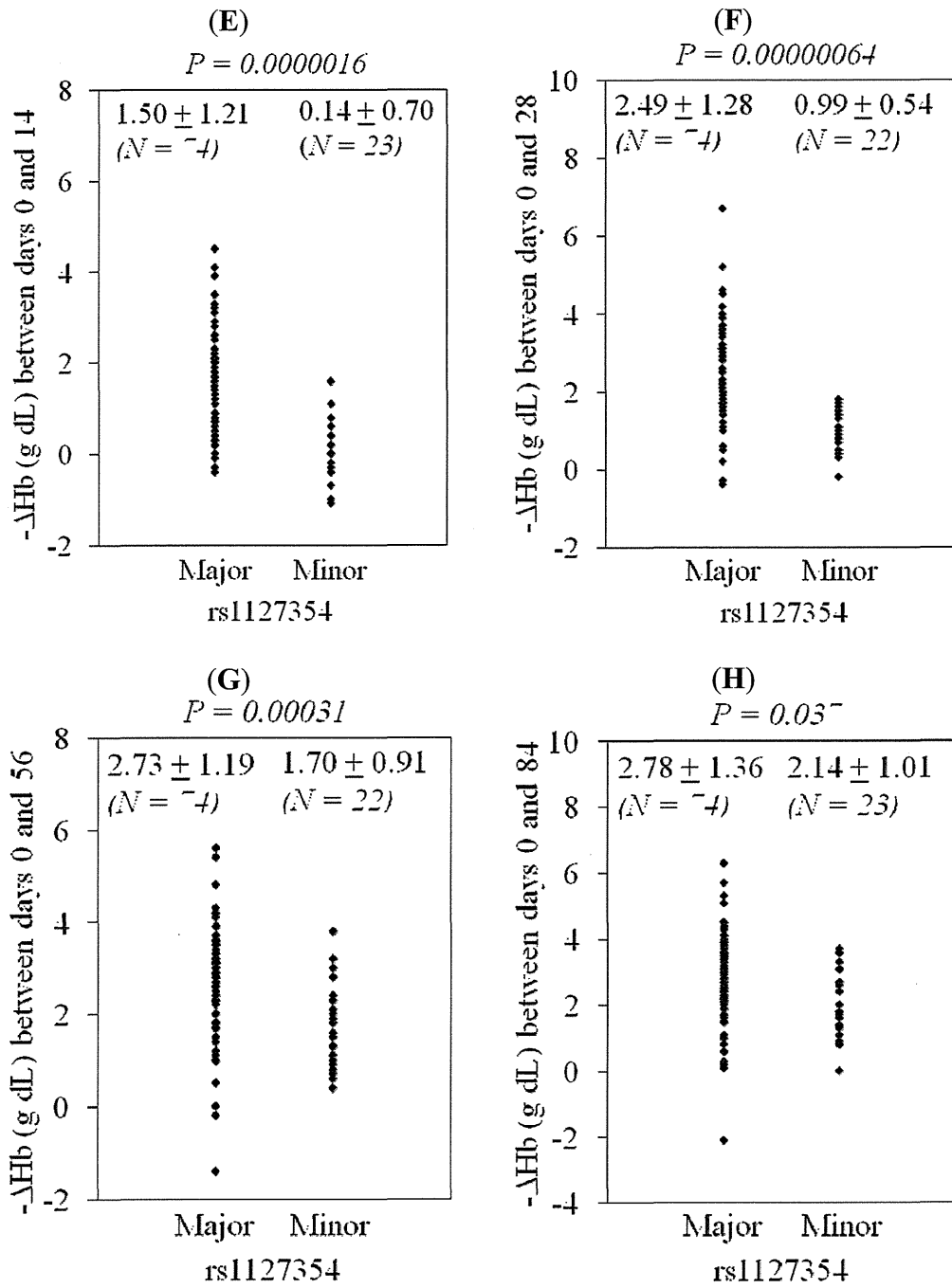
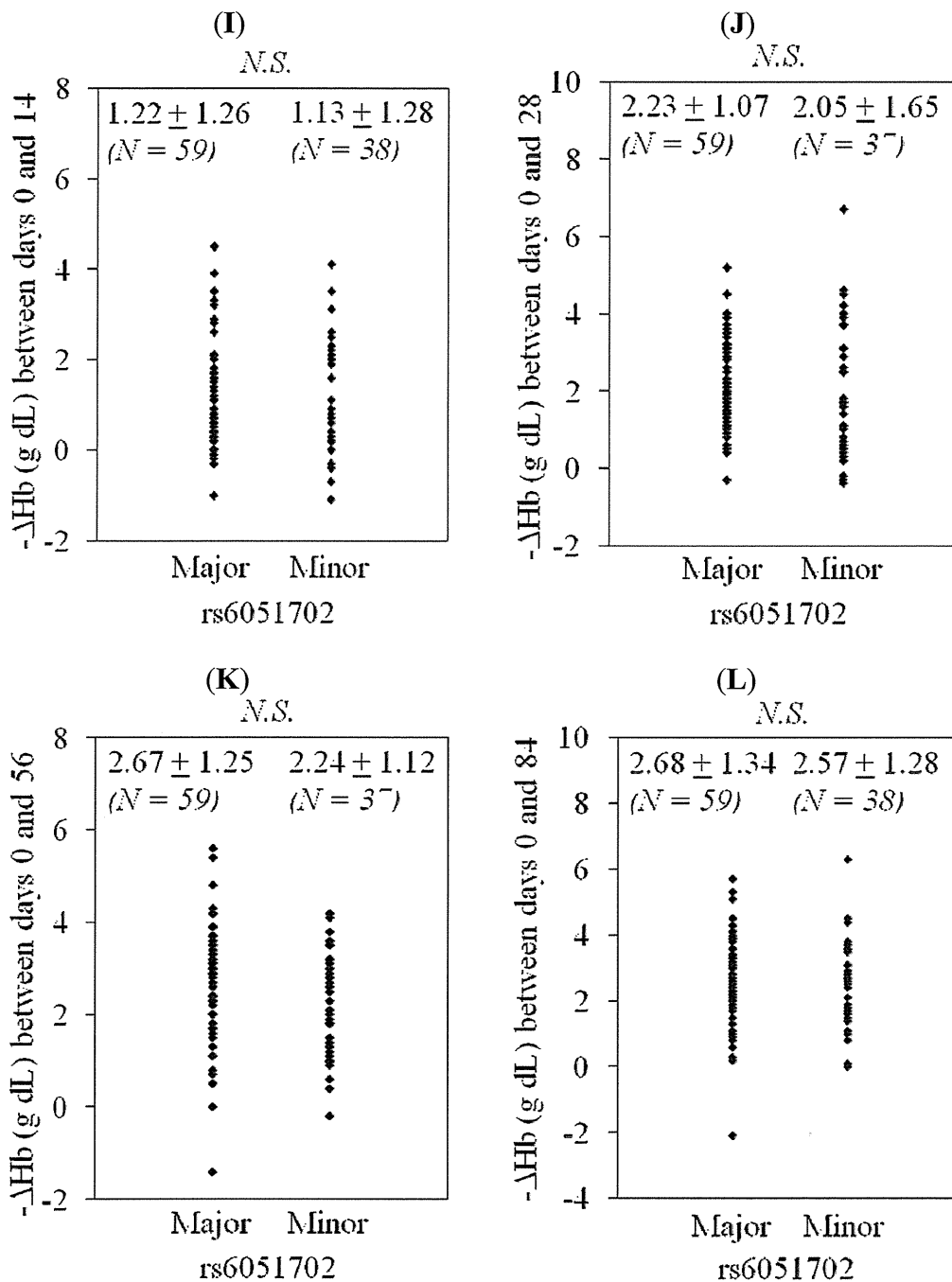


Figure 1. Cont.



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
<b>A</b>		
Dose reduction (+)	17	4
Dose reduction (−)	57	19
<b>B</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