Disclosure Statement

The authors declare no conflicts of interest.

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The JAK2 inhibitor AZD1480 inhibits hepatitis A virus replication in Huh7 cells

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ABSTRACT

The JAK2 inhibitor AZD1480 has been reported to inhibit La protein expression. We previously demonstrated that the inhibition of La expression could inhibit hepatitis A virus (HAV) internal ribosomal entry-site (IRES)-mediated translation and HAV replication *in vitro*. In this study, we analyzed the effects of AZD1480 on HAV IRES-mediated translation and replication. HAV IRES-mediated translation in COS7-HAV-IRES cells was inhibited by 0.1–1 μM AZD1480, a dosage that did not affect cell viability. Results showed a significant reduction in intracellular HAV HA11-1299 genotype IIIA RNA levels in Huh7 cells treated with AZD1480. Furthermore, AZD1480 inhibited the expression of phosphorylated-(Tyr-705)-signal transducer and activator of transcription 3 (STAT3) and La in Huh7 cells. Therefore, we propose that AZD1480 can inhibit HAV IRES activity and HAV replication through the inhibition of the La protein.

1. Introduction

Hepatitis A virus (HAV) infection is a major cause of acute hepatitis in both developing and developed countries [1–7]. In developed countries, persons hospitalized for hepatitis A tend to be older and are more likely to have other liver diseases and/or other comorbid medical conditions [7,8]. HAV belongs to the *Picornaviridae* family and possesses an internal ribosomal entry-site (IRES) that is a responsible for its cap-independent translation initiation. Among picornaviruses, only HAV and poliovirus can be controlled with vaccinations [9]. However, the costs are relatively expensive, and vaccinations are not universal in some countries, including Japan [10]. Despite the availability of efficient HAV vaccines, anti-HAV drugs are required to treat severe cases such as acute liver failure, outbreak cases, and vaccine-escape variants [11].

Recently, we reported that the Janus kinase (JAK) inhibitors SD1029 and AG490 reduced La expression and inhibited HAV IRES activities and HAV replication [12]. In the present study, two different antiviral assays were used: (i) inhibition of HAV IRES activity assay using COS7 cells stably expressing the HAV IRES reporter, and (ii) inhibition of HAV genotype IIIA replication in the human hepatoma cell line Huh7. We examined whether another JAK2 inhibitor (AZD1480) could inhibit HAV IRES activity and HAV replication. We also examined the effects of AZD1480 on the expression of phosphorylated-(Tyr-705)-signal transducer and activator of transcription 3 (STAT3) and La.

2. Materials and methods

2.1. Cell lines and reagents

The African green monkey kidney cell line COS7 and the human hepatoma cell line Huh7 were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich, St. Louis, MO, USA) under 5% CO_2 at 37 °C. The cultures were supplemented with AG490 (Calbiochem, Billerica, MA, USA), SD1029 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), AZD1480 (Selleck

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Chemicals, Houston, TX), interferon α-2a (Sigma–Aldrich), and amantadine (Sigma–Aldrich) where indicated.

2.2. RNA extraction and quantification of HAV RNA

Total cellular RNA was extracted from harvested cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 0.5 μg of total RNA using the PrimeScript RT reagent (Perfect Real Time; Takara, Otsu, Japan). Reverse transcription was performed at 37 °C for 15 min, followed by 95 °C for 5 s. For HAV RNA quantification, the following primer set was used: sense primer, 5′-AGGCTACGGGTGAAACCTCTTA-3′ and antisense primer, 5′-GCCGCTGTTACCCTATCCAA-3′ [13]. The primer set for the quantification of GAPDH mRNA was previously described [12]. Real-time PCR was performed with SyBr Green I on a StepOne Real-Time PCR system (Applied Biosystems). The PCR reaction was performed as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data analysis was based on the ΔΔCt method. Specificity was validated using melting curve analysis.

2.3. Western blot

The cells were lysed using sodium dodecyl sulfate lysis buffer. The proteins were subjected to electrophoresis on a 5–20% polyacrylamide gel and transferred onto a nitrocellulose membrane (ATTO, Tokyo, Japan). The membrane was probed with an antibody against phosphorylated-(Tyr-705)-STAT3, STAT3 (Cell Signaling Technology, Danvers, MA, USA), La or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were visualized using an enhanced chemiluminescent ECL Western blot substrate (GE Healthcare, Tokyo, Japan).

2.4. Infection of Huh7 cells with HAV

Huh7 cells were seeded 24 h before infection at a density of 1×10^5 cells/well in 12-well plates (AGC Techno Glass, Shizuoka, Japan). The cells were washed twice with PBS and infected with the HAV HA11-1299 genotype IIIA strain at a multiplicity of infection (MOI) of 0.1 in DMEM containing 2% FBS [12]. After 24 h of incubation, the cells were washed three times with PBS, followed by the addition of 1 mL of DMEM containing 2% FBS. After 72 or 96 h of incubation, the levels of HAV RNA in the inoculated cells were determined using real-time RT-PCR.

2.5. Luciferase assay

The SV40-HAV-IRES plasmid was constructed to analyze HAV IRES-mediated translation efficacy [14]. This HAV IRES was derived from pHM175 (kindly provided by Professor Suzanne U. Emerson, National Institutes of Health, MD, USA). Briefly, a plasmid expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HAV IRES-mediated translation initiation, and the pCXN2 vector (kindly provided by Professor Junichi Miyazaki, Osaka University, Japan) harboring a neomycin-resistant gene [15] were introduced by electroporation (850 μF and 250 V) into 5×10^6 COS7 cells using the Bio-Rad Gene Pulser Xcell system (Hercules, CA, USA). After 2 weeks of treatment with 1000 μg/mL G418 (Promega, Madison, WI, USA), COS7-HAV-IRES cells were cloned and established.

For the detection of HAV IRES activity, 10,000 COS7-HAV-IRES cells/well were seeded into a 96-well plate with or without various reagents as indicated. Forty-eight hours later, the cells were harvested using reporter lysis buffer (Toyo Ink, Tokyo, Japan) and luciferase activities were determined using a luminometer (Luminescencer-JNR II AB-2300, ATTO, Tokyo, Japan). All samples were run in triplicate.

2.6. MTS assays

For the evaluation of cell growth and cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using the CellTiter 96 Aqueous One-Solution cell proliferation assay (Promega). Enzyme activity was measured with a Bio-Rad iMark microplate reader (Bio-Rad) at the 490 nm wavelength.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviations (SD). Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of JAK2 inhibitors on COS7-HAV-IRES cell viability

To evaluate the effect of JAK inhibitors AZD1480, SD1029 and AG490 on HAV IRES activity, 5000 COS7-HAV-IRES cells per well were incubated with the inhibitors for 48 h (Fig. 1A–C). Interferon α-2a and amantadine were used as positive controls (Fig. 1D and E). The cytotoxicity of the drugs against COS7-HAV-IRES cells was determined using the MTS assay. We observed that the cell viabilities were not affected by supplementation with 0.1–1 μM AZD1480, 0.01–1 μM SD1029, 0.01–10 μM AG490, 1000–10,000 U/mL interferon α-2a and 0.5–50 μg/mL amantadine (Fig. 1A–E). These results showed that AZD1480 concentrations equal to or below 1 μM were safely tolerated by the cells.

3.2. Inhibitory effects of JAK2 inhibitors on HAV IRES activity in COS7-HAV-IRES cells

We previously reported that SD1029 and AG490 could inhibit HAV IRES activity and HAV replication in the African green monkey kidney cell line GL37 [12,16]. In the present study, we examined whether AZD1480 could inhibit HAV IRES activity in COS7-HAV-IRES cells. In COS7-HAV-IRES cells treated with 0.1 and 1 μM AZD1480 for 48 h, HAV IRES activities were reduced to 52.2% and 44.6% of the untreated control (Fig. 1A). Similarly, in COS7-HAV-IRES cells treated with 0.01, 0.1 and 1 μM SD1029 or 0.01, 0.1, 1 and 10 μM AG490 for 48 h, HAV IRES activities were reduced to 83.1%, 83.6% and 76.5%, or 88.8%, 86.4%, 78.8% and 77.1% of the untreated control, respectively (Fig. 1B and C). In COS7-HAV-IRES cells treated with 1000 and 10,000 U/mL interferon α-2a or 0.5, 5 and 50 μg/mL amantadine for 48 h, HAV IRES activities were reduced to 55.2% and 49.4% or 54.3%, 55.0% and 50.5% of the untreated control, respectively (Fig. 1D and E). These results indicated that AZD1480 could inhibit HAV IRES-mediated translation.

3.3. Inhibition of the replication of the HAV HA11-1299 genotype IIIA strain by AZD1480

To verify whether AZD1480 could also interfere with full-length HAV replication, Huh7 cells were infected with the HAV HA11-1299 genotype IIIA strain at an MOI of 0.1 24 h after treatment with 0.1 μM or 1 μM of AZD1480. At 96 h post-infection, intracellular HAV RNA levels were reduced to 86.1 ± 7% ($n = 3$, $p = 0.050$) or 83.6 ± 5.6% ($n = 3$, $p = 0.030$) of the untreated control, respectively

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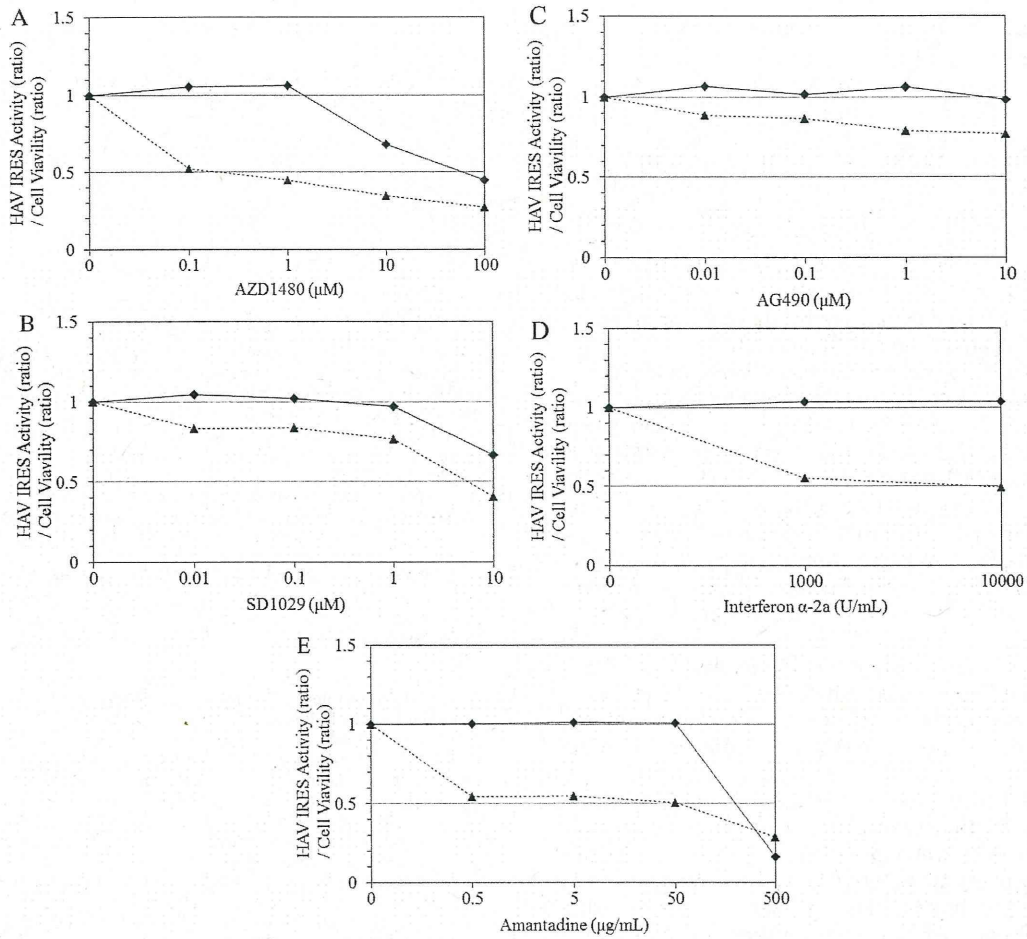

Fig. 1. Effects on cell viability and hepatitis A (HAV) internal ribosomal entry-site (IRES) activity in COS7-HAV-IRES cells. (A) AZD1480, (B) SD1029, (C) AG490, (D) interferon α-2a, (E) amantadine. Cell viability (black diamonds) was evaluated using the MTS assay (Promega). HAV IRES activities (black triangles) were evaluated as previously described [12].

(Fig. 2). At 72 h post-infection, HAV RNA levels in cells treated with1 μM AZD1480 were reduced to 91.1 ± 4.6% (n = 3, p = 0.033) of the untreated control. These results showed that AZD1480 could inhibit HAV replication in the human hepatoma cell line Huh7.

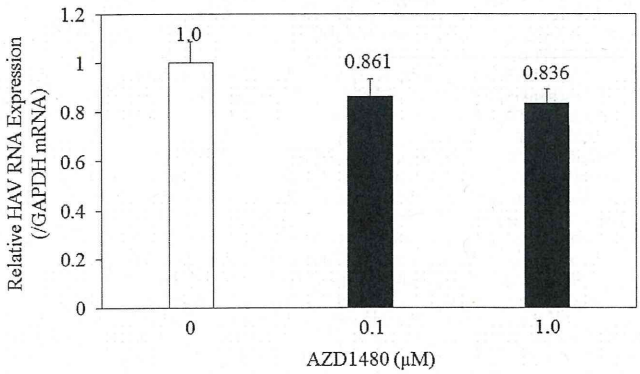

Fig. 2. Inhibition of HAV HA11-1299 genotype IIIA strain replication by AZD1480 in Huh7 cells. Huh7 cells were infected with the HAV HA11-1299 genotype IIIA strain at an MOI of 0.1 24 h after treatment with 0.1 μM or 1 μM AZD1480. At 96 h post-infection, intracellular HAV RNA levels were evaluated by real-time RT-PCR. The data are expressed as means ± standard deviations (SD).

3.4. Effects of AZD1480 on STAT3 and La protein expression in Huh7 cells

To further explore the mechanism behind the above results, we examined the expression of the phosphorylated-(Tyr-705)-STAT3, STAT3 and La proteins in Huh7 cells treated with or without 0.1 μM or 1 μM AZD1480 (Fig. 3). The results showed that AZD1480 inhibited the expression of phosphorylated-(Tyr-705)-STAT3 and

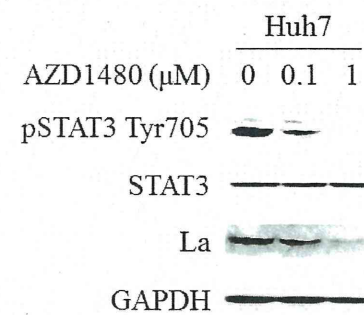

Fig. 3. Effects of AZD1480 on STAT3 and La expression in Huh7 cells. Forty-eight hours after treatment with or without AZD1480, cell lysates were analyzed for phosphorylated-(Tyr-705)-STAT3, STAT3, La and GAPDH expression using specific antibodies.

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La in Huh7 cells, supporting the previous observation that AZD1480 could inhibit the La protein [17].

4. Discussion

HAV IRES-mediated translation and HAV replication are essential steps during HAV infection. We previously demonstrated that HAV IRES-mediated translation is an important target of anti-HAV treatments, resulting in the inhibition of HAV replication [12,14,18–21]. In the present study, we demonstrated that the JAK2 inhibitor AZD1480 could inhibit HAV IRES activity in addition to HAV replication. AZD1480 also inhibited the expression of phosphorylated-(Tyr-705)-STAT3 and La in Huh7 cells.

Our previous study showed that the inhibition of La by JAK inhibitor SD1029 or AG490 led to the efficient inhibition of HAV IRES-mediated translation and HAV replication in the African green monkey kidney cell line GL37 [12]. In the present study, AZD1480 in addition to SD1029 and AG490 led to the efficient inhibition of HAV IRES-mediated translation and HAV replication in Huh7 cells. Nakatake et al. reported that the V617F JAK2 mutation affected p53 response to DNA damage through the upregulation of La antigen and the accumulation of MDM2 in myeloproliferative neoplasma [17]. The authors also showed that AZD1480 inhibited the La protein.

AZD1480 inhibited the expression of phosphorylated-(Tyr-705)-STAT3 as well as the La protein in Huh7 cells. Therefore, the inhibition of the La protein might be one of the mechanisms by which HAV IRES-mediated translation and HAV replication are inhibited [12].

Waris et al. reported the constitutive activation of STAT-3 in a liver biopsy from an HCV-infected patient and suggested a potential role for STAT-3 in HCV RNA replication [22]. Inhibition of the expression of phosphorylated-(Tyr-705)-STAT3 may lead to the inhibition of HAV replication. Because several reports have demonstrated a role for STAT3 in viral replication [23–25], further studies are required to address this issue.

Two methods exist for the use of the HAV vaccine. One is a universal vaccination program, while the other is post-exposure prophylaxis. The national guidelines for hepatitis A control in Australia changed its recommendation to include the use of the hepatitis A vaccine instead of normal human immune globulin for post-exposure prophylaxis [26]. Additionally, anti-HAV drugs that prevent severe HAV infections and promote HAV eradication might contribute to post-exposure prophylaxis.

Among hepatitis A patients, patients with liver disease were hospitalized longer. Moreover, these patients had increased secondary comorbid discharge diagnoses such as liver disease, hypertension, ischemic heart disease, disorders of lipid metabolism and chronic kidney disease [7]. Thus, the availability of an anti-HAV drug would be of clinical importance [11]. In conclusion, AZD1480 significantly inhibited HAV HA11-1299 genotype IIIA strain replication *in vitro*. However, the precise mechanism of the inhibitory effect of AZD1480 was not established. Further studies are required to elucidate the mechanism.

Conflict of interest

Q2 None.

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Impaired induction of *IL28B* and expression of *IFNλ4* associated with non-response to interferon-based therapy in chronic hepatitis C

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ABSTRACT

Background: Interferon (IFN) λ plays an important role in innate immunity to protect against hepatitis C viral (HCV) infection. Single nucleotide polymorphisms (SNPs) near *IL28B* (*IFNλ3*) are strongly associated with treatment response to IFNα therapy in chronic hepatitis C (CHC) patients. Recently, IFNλ4 related to *IL28B*-unfavorable allele was discovered. However, the impact of IFNλs on CHC is unknown. We aimed to investigate the mechanism underlying responsiveness to IFN-based therapy in CHC associated with SNPs near *IL28B*.

Methods: We evaluated the basal mRNA levels and ex-vivo induction of *IFNλ* expression including *IFNλ4* in peripheral blood mononuclear cells (PBMCs) from 50 CHC patients treated with PEG-IFNα/RBV. Furthermore, we investigated the effect of *IFNλ4* on induction of *IL28B* in vitro.

Results: When PBMCs were stimulated with IFNα and poly(I:C), *IL28B* induction was significantly lower in patients with *IL28B*-unfavorable genotype (rs12979860 CT/TT) than those with *IL28B*-favorable genotype (rs12979860 CC; p = 0.049). *IL28B* induction was lower in non-responders than in relapsers (p = 0.04), and it was also lower in non-SVR patients for triple therapy including NS3 protease inhibitors. *IFNλ4* mRNA was detected in 12 of 26 patients with *IL28B*-unfavorable SNP and *IFNλ4* expression was associated with lower *IL28B* induction in patients with *IL28B*-unfavorable genotype (p = 0.04) and non-response to

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IFNα therapy (p = 0.003). Overexpression of *IFNλ4* suppressed *IL28B* induction and promoter activation.

Conclusions: Impaired induction of *IL28B,* related to *IFNλ4* expression in PBMCs of *IL28B*-unfavorable patients, is associated with non-response to IFNα-based therapy for HCV infection.

Keywords: hepatitis C virus, peripheral blood mononuclear cells, pegylated interferon, NS3 protease inhibitor, type III interferon

Abbreviations: HCV, Hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; PEG-, pegylated; RBV, ribavirin; DAA, direct-acting antiviral agents; SNP, single nucleotide polymorphism; IL, interleukin; TLR, Toll like receptor; RLR, RIG-I like receptor; ISG, IFN-stimulated gene; PBMC, peripheral blood mononuclear cells; SVR, sustained virological responder; VR, virological responder; NR, non-responder; poly (I:C), polyinosinic-polycytidylic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BLC, immortalized B lymphocytes; IRF7, interferon regulatory transcription factor 7; ISRE, IFN-stimulated response element; STAT, signal transducers and activator of transcription; BDCA3, blood dendritic cell antigen 3; DC, dendric cell; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; ISDR, IFN sensitivity determining region.

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Introduction

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma [1]. Interferon (IFN)-based therapy has been used to treat chronic hepatitis C (CHC) over the last two decades and the combination therapy with direct-acting antiviral agents (DAAs) improved the treating effect. However, non-responders [2] to previous pegylated interferon α (PEG-IFNα) plus ribavirin (RBV) therapy respond poorly to the triple therapy containing HCV NS3/4A serine protease inhibitors [3, 4]. Moreover, although IFN-free regimen using NS5A inhibitors or NS5B polymerase inhibitors is developed, triple or quadruple therapy including PEG-IFN may still be required to suppress DAA-resistant viruses or difficult-to-treat genotype. Therefore, IFNα responsiveness of host innate immunity remains essential for achieving a good prognosis, and determining the mechanisms responsible for non-response to IFNα is crucial.

In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near *interleukin 28B* (*IL28B*) encoding type III IFN (IFNλ3) were found to be strongly associated with the virological response to PEG-IFNα/RBV therapy in CHC patients [5-8]. IFNλ3 is induced by viral infection through stimulation of Toll-like receptors (TLR) and RIG-I like receptors (RLR) [9-12], and it is also induced by type-I IFN signaling [13]. This interferon stimulates the expression of IFN-stimulated genes (ISGs), including numerous antiviral [13, 14] and immunoregulatory genes [15, 16]. Therefore, IFNλ3 induction may play essential roles in the innate antiviral response [17].

Recently, it was reported that high baseline expression levels of intrahepatic RLR, and lower responsiveness of ISGs to exogenous IFN, were significantly associated with unfavorable *IL28B* SNP and poor treatment outcome in CHC patients [18, 19, 20]. Furthermore, RNA sequencing using primary human hepatocytes revealed that unfavorable allele of dinucleotide polymorphisms near *IL28B* generate *IFNλ4* [21]. The ability of IFNλ4 to

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induce ISGs was reported in human liver tissue samples [22]. Based on these findings, we hypothesized that preactivation of IFN signaling by *IFNλ4* prevents further induction of antiviral genes by exogenous type I IFN, particularly the type I IFN-mediated induction of IFNλs. However, the expression of *IFNλ4* has never been documented in clinical blood samples from CHC patients.

The study aimed to determine the contribution of IFNλ family (IL29 [IFNλ1], IL28A [IFNλ2], IL28B [IFNλ3] and IFNλ4) to the poor response of CHC patients to anti-HCV therapy, and to clarify the mechanisms associated with SNPs near *IL28B*. Since peripheral blood mononuclear cells (PBMCs) are major sources of IFNλ [9, 10], we measured the expression level and investigated the ex vivo induction of *IFNλs* in PBMCs derived from CHC patients receiving PEG-IFNα/RBV therapy. Furthermore, we studied the impact of *IFNλ4* on *IL28B* expression in vitro.

Methods

Patients and Clinical samples. This study included 50 CHC patients with genotype 1b HCV treated with PEG-IFNα-2b/RBV at the Tokyo Medical and Dental University Hospital. Eleven of these patients were re-treated with telaprevir (TVR) or simeprevir (SMV). Exclusion parameters were alcoholic liver injury, autoimmune hepatitis, and decompensated liver cirrhosis. No patient tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody, or had received immunomodulatory therapy before enrollment. The clinical characteristics of the patients immediately before blood collection are shown in Table 1.Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Tokyo Medical and Dental University in accordance with the Declaration of Helsinki.

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Blood samples were collected from each patient during off-therapy periods for gene expression analysis. Human genomic DNA was extracted from whole blood, and SNPs located near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were analyzed using the TaqMan SNP genotyping assay (Applied Biosystems, Carlsbad, CA) [21, 23]. HCV core mutations and IFN-stimulated response element (ISDR) substitutions were determined before the therapy.

Definitions of responsiveness to therapy. The present study used the definition of response to therapy outlined by the AASLD Practice Guideline for Diagnosis, Management, and Treatment of Hepatitis C [2, 3].

Generation of *IL28B* mRNA-specific RT-qPCR systems. For the quantification of *IL28B* mRNA expression, we developed an original real-time quantitative PCR assay that distinguishes *IL28B* from *IL28A*. Gene-specific PCR primers were designed to anneal directly to the cDNA sequences of each gene (Supplementary Table 1).

Real-time detection RT-PCR analysis for *IL28A*, *IL28B*, and *IL29*. Immediately after blood collection, PBMCs were separated by gradient centrifugation with Ficoll-Conray, and incubated in the RPMI 1640 medium (Sigma, St. Louis, MO) with 10% fetal calf serum at 37°C under 5% CO_2. The cells were treated with recombinant IFNα-2b (100 IU/ml) (Schering-Plough, Kenilworth, NJ) for 12 h prior to polyinosinic-polycytidylic acid (poly(I:C)) (Sigma) treatment (10 μg/ml) for 8 h. PBMC RNA was extracted using the RNeasy Mini Kit (Quiagen, Valencia, CA). Total cell RNA (200 ng) was used to generate 10 μl of cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The mRNA expression levels were measured using a ABI 7500 real-time PCR system (Applied Biosystems), and a QuantiTect SYBR Green PCR kit (Quiagen) or TaqMan Universal PCR Master Mix (Applied Biosystems). Expression levels were normalized to the

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expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin. The sequences of the primer sets are provided in Supplementary Table 1.

Analysis of *IFNλ4* mRNA expression. Total cell RNA was pre-treated with DNase-I (Nippon Gene, Tokyo, Japan), followed by RT with SuperScript II, and PCR analysis was performed for 45 cycles using 4 sets of primers (Supplementary Table 1). Primer set #1 could detect 1 copy of IFNλ4 per assay, whereas primer sets #2, 3, and 4 could detect 10 copies of IFNλ4 per assay (Supplementary Fig. 1). The PCR products corresponding to the size of spliced *IFNλ4* mRNA were extracted and the sequences were confirmed. Only the amplicon with ss469415590-ΔG (*) is defined as IFNλ4 (Supplementary Figs. 1, 2).

Generation of the *IL28B* promoter-reporter and stably expressing cell lines. The promoter sequences of human *IL28B* (-1129/+111) were subcloned and the DNA fragment was inserted into the pGL3-basic vector (Invitrogen). The reporter plasmid was transfected into HEK293 cells with pcDNA3.1 (Invitrogen). After cell culture in the presence of the selective antibiotic G418 (Nacalai Tesque, Kyoto, Japan), transfected colonies were isolated to establish a cell line stably expressing the IL28B-Fluc-reporter (HEK293/ IL28B-luc).

Cell culture. HEK293T, Huh7, HepG2, and HeLa cells were maintained in Dulbecco's modified Eagle's Medium (Sigma) supplemented with 10% fetal calf serum (37°C; 5% CO_2). The maintenance medium for the IL28B-promoter-reporter-harboring cell line (HEK293/IL28B-luc) was supplemented with 500 μg/ml of G418 (Nacalai Tesque). Immortalized B lymphocytes (BLC) were generated in-house from human PBMCs by EBV transformation, and maintained in RPMI1640 medium (Sigma) with 10% feral calf serum and 200 ng/ml Cyclosporin-A (Sigma). The HuS/E-2 cells were kindly provided by Dr. Hijikata (Kyoto University, Kyoto, Japan) and cultured as previously described [24].

Expression plasmids and transfections. The expression construct for IFNλ4 (p179) was kindly provided by Dr. Prokunina-Olsson (National Cancer Institute, Bethesda, MD). The

DNA fragments of IRF7 were inserted into the vector pcDNA4/TO/myc-His (Invitrogen). The expression plasmids for p50 and p65 were kindly provided by Dr. Rongtuan Lin (Lady Davis Institute for Medical Research, Baltimore, MD). pcDNA4/TO/myc-His vector (Invitrogen) was used as control for mock transfection.

BLC were transfected with *IFNλ4* plasmids or control plasmids by electroporation using Gene Pulser Xcell Electroporation System (BIO RAD, Hercules, CA). After 24 h, cells were treated with mock, recombinant IFNα-2b (100 IU/ml) (Schering-Plough) for 24h. *IFNλ4* plasmids and *IRF7* plasmids or control plasmids were co-transfected into HEK293T cells with Lipofectamine LTX reagent (Invitrogen) and Opti-MEM medium, according to the manufacturer's instructions. Total RNA was extracted and quantified by real-time qRT-PCR.

Luciferase assays. *IFNλ4* or control plasmids were transfected into HEK293/IL28B-luc cells and the cells were treated with IFNα for 24h next day. HEK293/IL28B-luc cells were co-transfected with IFNλ4 plasmids and IRF7, p50: p65 or control plasmids and incubated for 24h. MTS viability and single luciferase assays were conducted by 1420 Multilabel Counter (ARVO MX, PerkinElmer, Boston, MA) using a CellTiter 96 AQueous One Solution System (Promega, Madison, WI) and a Bright-Glo Luciferase Assay System (Promega), as previously described [25, 26].

Statistical analyses. The data were analyzed using the Welch's *t* test for continuous variables and the chi-square test for categorical data. p values < 0.05 were considered statistically significant.

Results

Genotype of *IL28B* SNP and expression of *IL29*, *IL28A*, and *IL28B* mRNA in PBMC. Three SNPs near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were genotyped. The number of patients with each genotype is shown in Table 1. In agreement with a recent

report from the HapMap Project in Asia [21], the genotype of ss469415590 was completely correlated with that of rs12979860 in this study, while 3 of 50 patients have different genotype between ss469415590 and rs8099917. Baseline mRNA expression levels of *IL29*, *IL28A*, and *IL28B* were not influenced by the rs12979860 genotype (Fig. 1A). However, when PBMCs were stimulated with IFNα and poly(I:C), the induction of *IL28B* expression was significantly lower in patients with the *IL28B*-unfavorable genotype (rs12979860 CT/TT) than in those without (rs12979860 CC) ($p = 0.049$) (Fig. 1B).

Relationship of therapy response with *IL29*, *IL28A,* and *IL28B* mRNA levels in PBMC. We assessed the relationship between the expression level of the *IFNλ*s and the virological response to PEG-IFNα/RBV therapy. At baseline, there was no significant difference in *IFNλ*s expression between the SVR, relapser, and NR patients (data not shown). On the other hand, the induction of *IL28B* expression by IFNα and poly(I:C) decreased with the patients' response to therapy (Fig. 2A). The mRNA levels of NR patients were significantly lower than those for relapsers ($p = 0.04$) as well as VR ($p=0.005$). The induction of IL29 expression of NR patients were lower than those for VR ($p=0.048$). In contrast, the induction of *IL28A* did not reveal any association between mRNA levels and treatment response.

When the *IL28B* induction levels of VR and NR patients were further stratified by genotype, it was significantly lower in NR than in VR patients in both rs12979860 CC and CT/TT subgroups ($p = 0.01$ and 0.02, respectively) (Fig. 2B).

Furthermore, 11 of 32 non-SVR patients were re-treated with NS3 protease inhibitor (TVR or SMV) plus PEG-IFNα/RBV triple therapy; 2 of them were IL28B-favorable and 9 were unfavorable. Even treated with NS3 protease inhibitor, it should be noted that IL28B inductions in non-SVR of the triple therapy were significantly lower than those in SVR of the PEG-IFNα/RBV therapy or triple therapy ($p=0.017$). IL28B inductions in non-SVR were also

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lower than those in SVR of triple therapy (3.5 vs 12.1 fold induction). IL28A inductions in non-SVR of the triple therapy were also significantly lower than those in SVR (p=0.042) (Fig. 2C).

Impact of *IL28B* genotype and induction on *IFNλ4* mRNA expression. We measured the expression level of *IFNλ4* in PBMCs derived from CHC patients. Because we could not detect *IFNλ4* mRNA in PBMCs with RNA sequencing nor the previously reported TaqMan real-time quantitative RT-PCR system [21], we designed a new highly sensitive RT-PCR system using 4 sets of primers. The detection threshold was as low as 1–10 copies/assay (Supplementary Table 1; Supplementary Fig. 1A). This RT-PCR assay allowed us to confirm the full length mRNA sequence of *IFNλ4* in poly(I:C)-treated HepG2, HeLa, HEK293T cells, and BLC from ss469415590-ΔG/ΔG patients by amplicon sequencing (Supplementary Figs. 1B,C).

Using this system, we tested PBMCs from 47 CHC patients for the presence of *IFNλ4* mRNA. Among the 23 patients with *IL28B*-unfavorable rs12979860 [T] and ss469415590 [ΔG]-allele, *IFNλ4* mRNA was detected in 12 patients (7 in non-stimulated PBMCs and 8 in IFN-poly(I:C)-stimulated PBMCs). In marked contrast, *IFNλ4* mRNA was not detected in any of the *IL28B*-favorable patients (Supplementary Fig. 2). There was no significant difference in baseline expression of *IFNλ*s between patients with or without detectable *IFNλ4* expression (Fig. 3A). However, the induction of *IL28B* expression by IFN-poly(I:C) was significantly lower in patients with *IFNλ4* mRNA than those without detectable *IFNλ4* ($p = 0.008$) (Fig. 3B). Even among *IL28B*-unfavorable patients (rs12979860 CT/TT), *IL28B* induction levels were significantly lower in *IFNλ4*-positive patients ($p = 0.04$) (Fig. 3B). Although induction of *IL28A* was lower in *IFNλ4*-positive patients than *IFNλ4*-negative patients ($p = 0.04$), there was no significant relation between *IFNλ4* expression and the induction of *IL28A* and *IL29* among *IL28B*-favorable patients.

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Association between *IFNλ4* expression and clinical response to antiviral therapy. The rate of virological non-response was significantly higher in patients with *IFNλ4* mRNA than in all those without detectable *IFNλ4* ($p = 0.003$; Fig. 3C). Among the *IL28B*-unfavorable patients (rs12979860 CT/TT), the virological non-response rate also tended to be higher in patients expressing *IFNλ4* ($p = 0.08$).

Suppression of *IL28B* induction by *IFNλ4* in vitro. The mechanism behind the lower induction of *IL28B* mRNA in CHC patients expressing *IFNλ4* was investigated by testing whether the expression of *IL28B* is influenced by overexpression of *IFNλ4* in vitro. When IFNλ4 was overexpressed, baseline expression of IL28B was significantly increased in HEK293, BLC (Supplementary Fig. 3). However, as shown in Fig. 4A, IL28B expression was increased by IFNα (1.8 fold induction, $p=0.012$) but that induction was suppressed in the presence of IFNλ4 (1.2 fold induction, $p=0.28$) in BLC. As *IL28B* promoter is known to be activated by the transcription factors such as IRF7 and NFκB [11, 31], we next evaluated IL28B induction by IRF7. *IL28B* mRNA was induced by IRF7 in dose dependent manner and the induction levels were suppressed by *IFNλ4* overexpression significantly (Fig. 4B). *IL28B* promoter activities induced by IFNα, IRF7 and p50:p65 were also inhibited by *IFNλ4* overexpression (Fig. 4C-E).

Discussion

The present study shows that the inducibility of *IL28B* expression is associated with virological responsiveness to IFNα in CHC patients, and it is also related to the *IL28B* genotype. Furthermore, we detected *IFNλ4* mRNA in PBMCs using an original sensitive RT-PCR system. *IFNλ4* suppressed *IL28B* induction and associated with virological non-responses to IFNα-based antiviral therapy.

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Earlier studies reported the lower production of *IL28B* in blood cells of *IL28B*-unfavorable CHC patients [5, 27]. However, the relationship between *IL28B* genotype and expression level remained controversial, probably due to the very low expression level of *IL28B*. In the present study, there was no significant difference in baseline expression level between the *IL28B* genotypes. However, stimulation to PBMCs with IFNα and poly(I:C) raised *IL28B* expression, and this induction was significantly lower in *IL28B*-unfavorable CHC patients. More importantly, the degree of *IL28B* induction was positively correlated to the responsiveness to PEG-IFNα/RBV therapy.

Our findings are consistent with a previous study showing ex vivo induction of *IL28B* by TLR7 agonists [28], and we further confirmed *IL28B* inducibility using IFNα and poly(I:C), which mimic exogenous IFNα administration in HCV patients. Because IFNλ is an essential element of innate anti-HCV responses [16, 29, 30], our data suggest that inadequate induction of IL28B is primarily responsible for virological non-response to IFNα-based therapy.

To elucidate the mechanisms responsible for the genotype-specific inducibility of *IL28B*, we focused on *IFNλ4*. We report, for the first time, the presence of *IFNλ4* mRNA in PBMCs derived from CHC patients with the *IL28B*-unfavorable allele. We could not detect *IFNλ4* mRNA with the previously reported TaqMan real-time RT-PCR system [21]. *IFNλ4* expression was confirmed with a highly sensitive RT-PCR system we designed for this study, which could detect even a single copy of *IFNλ4* mRNA per assay. Although *IFNλ4* mRNA was not detected in 16 of the 23 unstimulated PBMC samples of CHC patients with the *IL28B*-unfavorable genotype, we cannot exclude the presence of *IFNλ4* mRNA under the detection limit of this RT-PCR system in these patients. However, it is important to mention that detectable level of *IFNλ4* expression was associated with NR and more severe impairment of *IL28B* induction. These data suggest that the baseline expression of *IFNλ4* in

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PBMCs is responsible for the non-response to IFNα treatment through suppression of *IL28B* induction.

Our in vitro experiments in cell lines demonstrated that *IL28B* induction by IFNα, IRF7 or NFκB was suppressed by IFNλ4 overexpression. These data are consistent with the relationship between *IFNλ4* and *ISG* induction [20-22]. Our finding of base line *IL28B* induction by *IFNλ4* is also reasonable because *IFNλ* promoters contain IFN-stimulated response element (ISRE) sites [11, 31] that could be activated by IFNλ4 through STAT1 and STAT2 phosphorylation [21]. IFNλ4 may pre-activate IL28B promoter through ISRE activation, and moreover, it may influence NFκB-induced promoter activity by unknown mechanism. Our in vitro data support our observation in the clinical samples, and suggest that the expression of *IFNλ4* in immune cells of *IL28B*-unfavorable CHC patients may weakly induce basal *IL28B* expression, which may be insufficient for HCV eradication [32]. But it may prevent additional induction of *IL28B* by exogenous IFNα treatment through impairment of *IL28B* promoter activity. The molecular mechanism by which *IFNλ4* suppresses *IL28B* mRNA induction and promoter activation should be further investigated, although *IFNλ4* may also have important functions affecting IFN regulation [20, 33, 34].

The lower induction of *IL28B* might be caused by the decrease of the frequency of IFNλs producing cells. However, in the present study, because we measured the expression of *IFNλ*s in all PBMCs, we could not specify the subset of IFNλ4 producer cells. A recent study demonstrated that blood dendritic cell antigen 3 $(BDCA3)^{+}$ dendritic cells (DCs) produce IFNλ3 and expression levels of *IL28B* from BDCA3+ DCs were significantly higher in subjects with *IL28B* major than those with minor type in response to HCV infection [35]. In their experiment, large volumes of blood samples (i.e., 400 ml) were required to sort very small populations of BDCA3+DC (0.054% of all PBMCs), but obtaining such a large amount

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