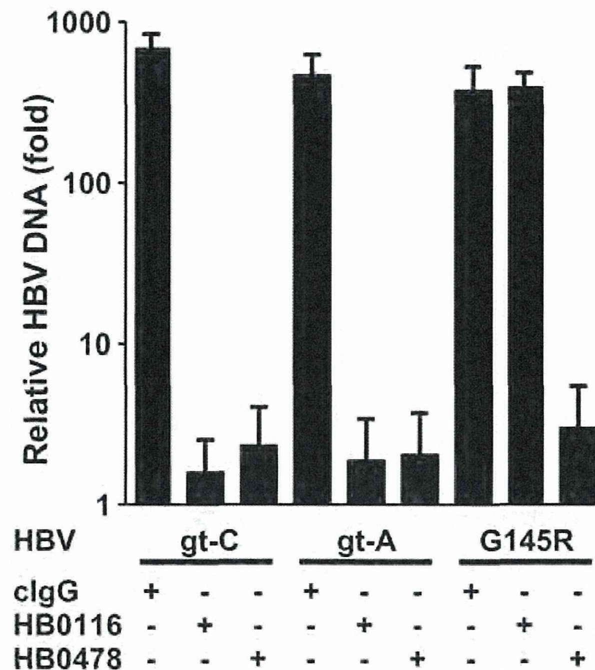

Fig 2. Relative HBV DNA concentrations in the total DNA extracted from HepaRG cells at 7 days after HBV infection. The Y-axis depicts the relative HBV DNA concentrations in the cells, with the concentrations on day 1 set at 1. Mean ± SD of three independent experiments are shown. cIgG, control human monoclonal IgG.

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confirmed by inoculating HBV gt-C at 5 HBV genomes per cell in the presence of 4% PEG 8000. The levels of pregenomic RNA, intracellular HBV DNA, extracellular HBV DNA, and extracellular HBsAg were monitored and it was found that all these viral products gradually increased from 3 to 12 dpi (Fig. 3A). Southern blot analysis of cell lysates revealed the presence of single-stranded HBV DNA as a replication intermediate in the infected PHHs, confirming HBV replication in the cells (Fig. 3B). Furthermore, culture supernatants from HBV-infected donor PHHs were inoculated into newly prepared PHHs. An increase of HBsAg production from the PHHs was observed following exposure of the cells to another culture supernatant containing HBV DNA (Fig. 3C), indicating that the donor PHHs produced infectious HBV virions (also known as Dane particles).

Next, to investigate whether this model can be adapted for the study of neutralizing activities against HBV infection, the effect of HBIG on HBV infection was evaluated in vitro. Fig. 3D shows that HBIG strongly reduced HBV infection but residual infection was detected in the presence of PEG, whereas, in the absence of PEG, the HBV infection was completely blocked by HBIG. These results indicate that, when neutralizing activities against HBV infection were investigated using this PHH system, inoculation without PEG is appropriate for the specificity of the establishment of HBV infection. However, because inoculation without PEG would be less efficient for HBV infection, the efficacy of HBV infection in the absence of PEG was also examined. Various titers of HBV (10, 3, 1, and 0.3 genomes per cell) were inoculated into PHHs and the HBsAg titers in the supernatants were monitored for 22 days (Fig. 3E). Although the HBsAg levels from PHHs infected without PEG were lower than those with

Table 1. In vivo neutralization of HBV infection by monoclonal antibodies (mAbs).

HBV genotype	Group	HB0116 (μg/body)	HB0478 (μg/body)	HBV DNA (copies/mL)
C	Group 1	-	-	9.8×10^3
	(n = 2)	-	-	1.1×10^4
	Group 2	1	-	n.d.
	(n = 3)	1	-	n.d.
		1	-	n.d.
	Group 3	10	-	n.d.
	(n = 3)	10	-	n.d.
		10	-	n.d.
	Group 4	-	1	n.d.
	(n = 3)	-	1	n.d.
		-	1	n.d.
	Group 5	-	10	n.d.
	(n = 3)	-	10	n.d.
		-	10	n.d.
A	Group 6	1	-	n.d.
	(n = 3)	1	-	n.d.
		1	-	n.d.
	Group 7	10	-	n.d.
	(n = 3)	10	-	n.d.
		10	-	n.d.
	Group 8	-	1	n.d.
	(n = 3)	-	1	n.d.
		-	1	n.d.
	Group 9	-	10	n.d.
	(n = 2)	-	10	n.d.
G145R	Group 10	-	-	1.4×10^4
	(n = 2)	-	-	1.0×10^4
	Group 11	10	-	4.4×10^4
	(n = 3)	10	-	1.1×10^4
		10	-	3.3×10^4
	Group 12	-	10	n.d.
	(n = 3)	-	10	n.d.
		-	10	n.d.
	Group 13	1	1	n.d.
	(n = 2)	1	1	n.d.
	Group 14	10	10	n.d.
	(n = 2)	10	10	n.d.
	Group 15	-	1	n.d.
	(n = 2)	-	1	n.d.

n.d.: not detected.

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PEG, the HBsAg levels in the supernatants were well correlated with the initial input of HBV (10 to 0.3 genomes per cells) in the absence of PEG. These results suggest that, albeit with somewhat lower infectivity, inoculation without PEG is available for neutralization assays using the PHH system.

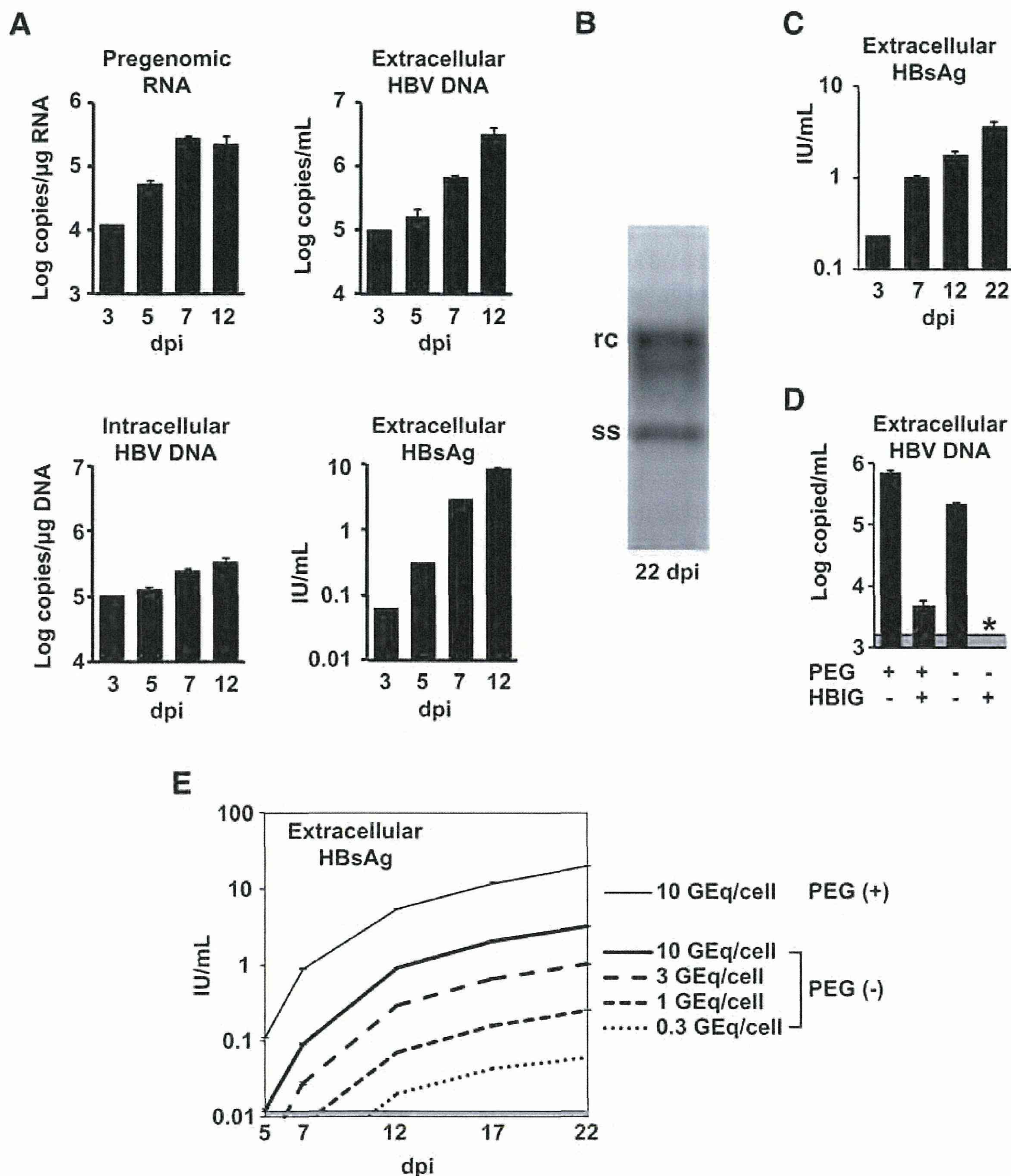

Fig 3. In vitro HBV infection model using PHHs isolated from chimeric mice with human hepatocytes. (A) PHHs were inoculated with HBV gt-C at 5 genomes per cell in the presence of PEG and intracellular pregenomic RNA, intracellular HBV DNA, extracellular HBV DNA and extracellular HBsAg were monitored by real-time quantitative PCR, or by automated ELISA. dpi, days post infection. **(B)** 20 μg of total DNA was extracted from PHHs 22 days after infection with HBV and analyzed by Southern blotting. Single-stranded HBV DNA (ss), a replication intermediate, and relaxed circular HBV DNA (rc) were detected. **(C)** Freshly prepared PHHs were inoculated with the day 52 supernatant from other HBV-infected PHHs. HBsAg secretion was monitored. **(D)** The use of PEG on HBV infection could mask the specificity of neutralization of HBV infection. Residual HBV infection was observed when PHHs were inoculated with a mixture of HBV and HBIG in the presence of PEG. An asterisk indicates a value below detection limit. **(E)** The efficacy of HBV infection without PEG was proportional to the size of the inoculum.

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HB0478 efficiently blocks HBV infection by both gt-C and gt-A

To evaluate the neutralizing activity of HB0478 against HBV infection, various amounts of HB0478 were preincubated with HBV gt-C or gt-A at 10 HBV genomes per cell (6.7×10^5 genomes/well) for 2 hours and exposed to PHHs for 48 hours without PEG (Fig. 4A). Fig. 4B shows the levels of HBV DNA in the supernatants harvested at 22 dpi. HB0478 in the amounts of 550 and 55 mIU completely blocked the infection by both gt-C and gt-A (HBV DNA was never detected in the supernatant). 5.5 mIU of HB0478 also completely inhibited gt-C infection, while it strongly reduced but did not completely inhibit gt-A infection. These results indicate that mAb HB0478 has powerful neutralizing activity against HBV infection and that HB0478 generated by the gt-C type vaccine could protect against HBV infection by both gt-C and gt-A, although less effectively against gt-A.

Discussion

Although the HBV vaccine strain used predominantly worldwide is genotype A2, genotype C strains are prevalent in Japan, where a selective vaccination program for high risk individuals with a gt-C-based vaccine is ongoing. A potential problem is that genotype A2 has been increasing recently as a cause of acute hepatitis B in Japan [10] and little is known about the efficacy of the gt-C-based vaccine against non-C HBV infection. In this report, we demonstrated that two mAbs, HB0478 and HB0116, derived from individuals immunized with the gt-C vaccine (Biimugen) that has been approved in Japan, neutralized HBV infections by both gt-C and gt-A in vitro and in vivo, suggesting that immunization with the gt-C vaccine could prevent infection by non-C HBV strains.

Epidemiological studies have shown that, in countries operating universal childhood vaccination programs using the gt-A2 vaccine, vertical transfer and/or incident infection of non-A2 were prevented efficiently [7]. Some studies have produced data supporting cross-genotype protection by immunization. An analysis of 221 mAbs isolated from volunteer HB vaccinees showed that 97% of them recognized common epitopes shared by all HBV genotypes [5]. The C(K/R)TC motif (amino acids 121–124), located in the N-terminal portion of the first loop of the "a" determinant of HBsAg, is conserved among all HBV genotypes (except for residue 122, K or R determining the serological subtype d or y, respectively) and highly immunogenic [26]. Moreover, a single mouse monoclonal Ab protected chimpanzees from infection by both adr (gt-C) and ayw (genotype D) strains [27].

Along with these findings, our results showed that the mAbs HB0478 and HB0116, generated following immunization with the gt-C type vaccine, neutralized the infectivity of both gt-C and gt-A HBV. In vitro experiments investigating dose dependency using freshly isolated PHHs also demonstrated that HB0478, at doses above 55 mIU, completely protected against both gt-C and gt-A infection, whereas HB0478, at a lower dose, 5.5 mIU, protected against gt-C infection only. It has been reported that analysis of nine HBV DNA positive blood donors in the United States revealed that 5 individuals who had been immunized with an A2-type vaccine were not protected against infections by non-A2 HBV [28]; however, the serum anti-HBs levels of these individuals (3–96 mIU/mL) were relatively low. Interestingly, the infections remained at a subclinical level in these vaccines, who subsequently resolved the HBV infection, suggesting that gt-A2 vaccination could not prevent non-A2 infection but can inhibit the development of clinical manifestations [28]. Therefore, it is possible that HBV specific antibodies, induced by gt-C vaccines, might be able to protect against clinical hepatitis caused by infection with non-C genotypes, even with lower anti-HBs concentrations. Further investigations are needed to determine clinical effectiveness of gt-C vaccine to induce cross-genotype immune responses.

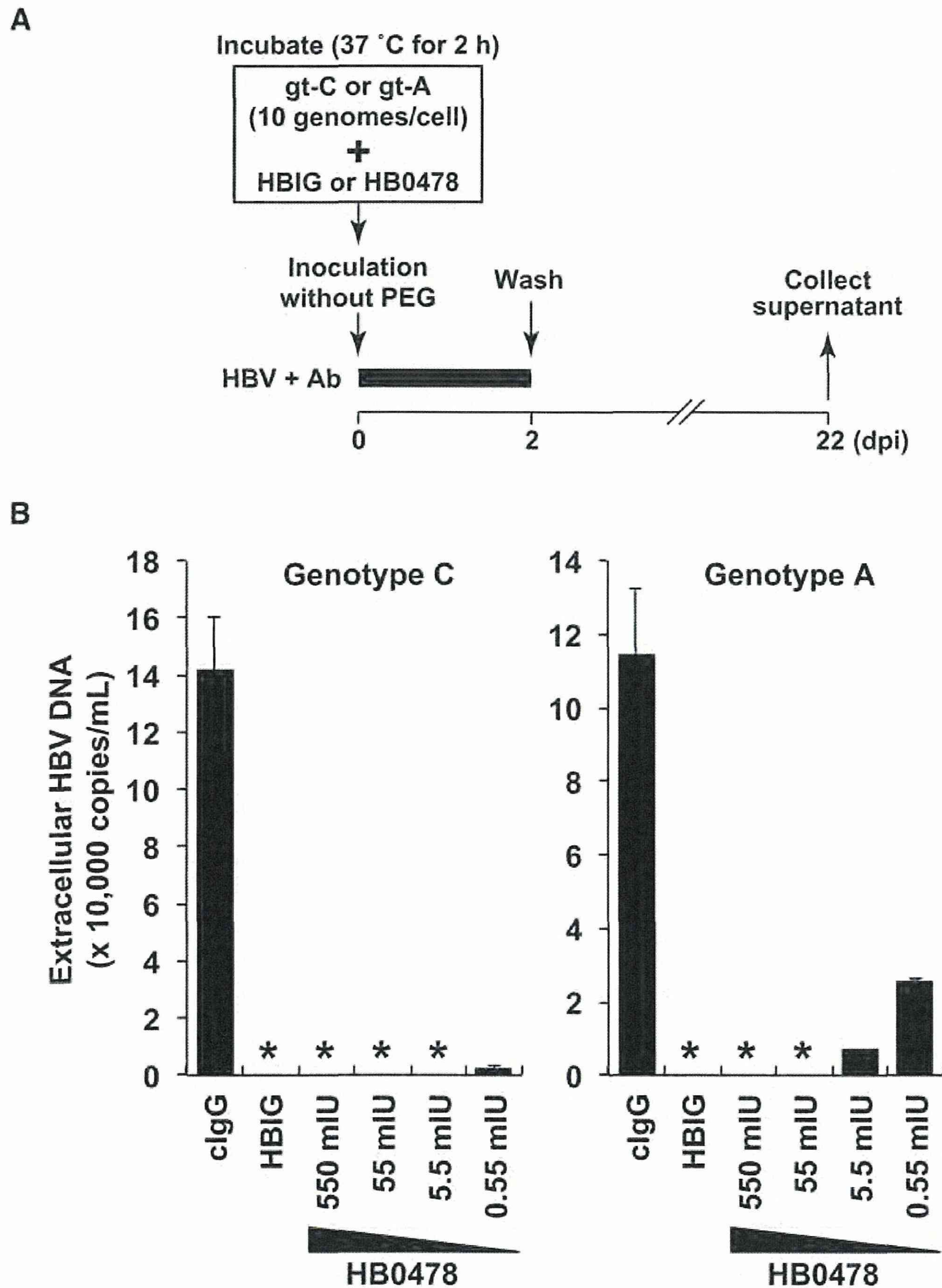

Fig 4. Titration of neutralization of gt-C and gt-A infection by mAb HB0478. HBV gt-C and gt-A were preincubated for 2 hours with 670 ng of control human IgG (cIgG), 100 mIU of HBIG, or 670, 67, 6.7 or 0.67 ng HB0478 (corresponding to 550, 55, 5.5, and 0.55 mIU) and PHHs were inoculated with the products at 10 genomes per cell. The Y-axis depicts the levels of extracellular HBV DNA in the supernatant harvested on 12 days post infection. Asterisks indicate values under the detection limit.

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Meanwhile, virus strains with amino acid substitutions in HBsAg often escape from HB vaccine-induced antibody and HBIG treatment during vertical transmission of HBV [19,20,29]. The substitution reported most frequently is residue 145, glycine to arginine (G145R), located in the second loop of the "a" determinant of HBsAg. This study demonstrated that HB0478 also recognized HBsAg with the G145R substitution and protected against G145R infection in vivo, whereas HB0116 did not bind to the G145R substituted protein or neutralize the mutant. Although how G145R in the second loop affects mAb-binding to the first loop is largely unknown, it is possible that the C(K/R)TC-dependent HB0478 epitope might be more distant from the second loop than that of HB0116, suggesting that HB0478 might not be affected by the conformational change of HBsAg induced by substitution of glycine at residue 145. It is noted that epitopes other than "a" determinant such as those within pre-S2 region [30] could also contributed to the neutralization of escape mutants.

In conclusion, this study raises the possibility that active immunization with a gt-C-based vaccine confers prophylaxis against gt-A, which is spreading in Japan, and against escape mutants such as G145R, when the anti-HBs responses are sufficient. Note that PHHs isolated from chimeric mice with human hepatocytes enabled us to investigate precisely the inhibitory effects of the mAbs, or any antiviral compounds, against HBV infection in vitro.

Author Contributions

Conceived and designed the experiments: SHT EI SM KT TJ YT. Performed the experiments: SHT EI TW SM KM KT TO. Analyzed the data: SHT EI TW SM MI SI TI KM. Contributed reagents/materials/analysis tools: HK AM. Wrote the paper: SHT EI TW YT.

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RESEARCH ARTICLE

Influence of Genes Suppressing Interferon Effects in Peripheral Blood Mononuclear Cells during Triple Antiviral Therapy for Chronic Hepatitis C

Sayuki Iijima[1‡], Kentaro Matsuura[1,2,3‡], Tsunamasa Watanabe[1], Koji Onomoto[4], Takashi Fujita[4], Kyoko Ito[1], Etsuko Iio[1,2], Tomokatsu Miyaki[5], Kei Fujiwara[2], Noboru Shinkai[2], Atsunori Kusakabe[6], Mio Endo[2], Shunsuke Nojiri[2], Takashi Joh[2], Yasuhito Tanaka[1]*

1 Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, **2** Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, **3** Infectious Disease and Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, United States of America, **4** Laboratory of Molecular Genetics, Institute for Virus Research, Kyoto University, Kyoto, Japan, **5** Division of Internal Medicine, Toyokawa City Hospital, Toyokawa, Japan, **6** Division of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya, Japan

‡ These authors contributed equally to this work.
* ytanaka@med.nagoya-cu.ac.jp

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Abstract

The levels of expression of interferon-stimulated genes (ISGs) in liver are associated with response to treatment with pegylated interferon (PEG-IFN) plus ribavirin (RBV). However, associations between the responses of ISGs to IFN-based therapy and treatment efficacy or interleukin-28B (*IL28B*) genotype have not yet been determined. Therefore, we investigated the early responses of ISGs and interferon-lambdas (IFN-λs) in peripheral blood mononuclear cells (PBMCs) during PEG-IFN/RBV plus NS3/4 protease inhibitor (PI) therapy. We prospectively enrolled 50 chronic hepatitis C patients with HCV genotype 1, and collected PBMCs at baseline, 8 and 24 h after the initial administration of PEG-IFN/RBV/PI. Levels of mRNAs for selected ISGs and IFN-λs were evaluated by real-time PCR. All 31 patients with a favorable *IL28B* genotype and 13 of 19 with an unfavorable genotype achieved sustained virological responses (SVR). Levels of mRNA for *A20, SOCS1*, and *SOCS3*, known to suppress antiviral activity by interfering with the IFN signaling pathway, as well as *IRF1* were significantly higher at 8 h in patients with an unfavorable *IL28B* genotype than in those with a favorable one (P = 0.007, 0.026, 0.0004, 0.0006, respectively), especially in the non-SVR group. Particularly, the fold-change of *IRF1* at 8 h relative to baseline was significantly higher in non-SVR than in SVR cases with an unfavorable *IL28B* genotype (P = 0.035). In conclusion, levels of several mRNAs of genes suppressing antiviral activity in PBMCs during PEG-IFN/RBV/PI differed according to *IL28B* genotypes, paralleling treatment efficacy.

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Introduction

Chronic hepatitis C virus (HCV) infection is a significant risk factor for progressive liver fibrosis and hepatocellular carcinoma (HCC). Antiviral treatment improves the natural course in chronic hepatitis C (CHC) [1, 2]. Newly-developed treatments involving direct-acting antivirals (DAAs), including nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in several clinical trials. Thus >70% of patients infected with HCV genotype 1 are reported to achieve sustained virological responses (SVR) [3–5].

Recent genome-wide association studies (GWAS), including our own study on HCV infection [6], have identified a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL28B*) gene encoding type III IFN-λ3 that was strongly associated with the response to PEG-IFN/RBV therapy for chronic HCV genotype 1 infection [6–9]. Furthermore, a recent meta-analysis showed that the *IL28B* genotype was also associated with efficacy of PEG-IFN/RBV plus NS3/4A protease inhibitor (PI) treatment, including telaprevir or boceprevir [10]. However, it is not known how the *IL28B* gene influences the elimination of HCV.

IFNs mediate their potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs). Type I and III IFNs induce the transcription of ISGs by activating the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway through different cell surface receptors [11–14]. Because it has been reported that a high level of expression of intrahepatic ISGs at baseline affects responses to PEG-IFN/RBV therapy [15, 16], several groups have investigated an association between *IL28B* genotype and the expression of intrahepatic ISGs [17, 18]. In addition, intrahepatic expression of genes involved in innate immunity, Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) which are important in signaling pathways for IFN-β induction, were also associated with the *IL28B* genotype and response to PEG-IFN/RBV [19]. Nevertheless, we cannot fully explain treatment outcome by evaluating *IL28B* genotypes and measuring intrahepatic gene expressions at baseline. Changes of intrahepatic gene expressions cannot easily be evaluated due to the risk of complications caused by taking a liver biopsy. For this reason, several groups have assessed the response of ISGs to PEG-IFN/RBV using peripheral blood mononuclear cells (PBMCs) as a surrogate. However, most of these earlier studies found less marked correlations between the expression of ISGs in PBMCs and treatment efficacy or *IL28B* genotype, relative to what was seen in the liver of the same patients [15, 20, 21]. We also analyzed the expression of ISGs, which included previously reported genes [17, 19–21], in PBMCs during PEG-IFN/RBV therapy, indicating that several ISGs that suppressed the antiviral state by interfering with the IFN signaling pathway were associated with the *IL28B* genotype or response to PEG-IFN/RBV therapy. These included *A20*, suppressor of cytokine signaling 1 (*SOCS1*), *SOCS3*. In PEG-IFN/RBV/PI therapy, the expression of ISGs, IFN-λs, and molecules related to the innate immune system is expected to be changed greatly soon after the start of therapy, due to the effects of the PI. Hence, we prospectively collected PBMCs of patients treated with PEG-IFN/RBV/PI, and then evaluated associations between the levels of mRNAs for the selected ISGs or IFN-λs and the *IL28B* genotype or patient's response to treatment.

Patients and Methods

Patients and treatment protocol

We prospectively enrolled a total of 50 CHC individuals infected with HCV genotype 1 who were treated with PEG-IFN/RBV/PI at Nagoya City University Hospital; 32 patients received telaprevir and 18 faldaprevir. All patients had tested positive for HCV RNA for more than 6

months. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other liver diseases such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study.

The regimen of PEG-IFN/RBV/telaprevir therapy was as follows: PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week), RBV (600–1000 mg daily according to body weight), and telaprevir (standard dose of 2250 mg daily given three times a day every 8 hours or reduced dose of 1500 mg daily given twice a day every 12 hours) for 12 weeks, followed by an additional 12 weeks of PEG-IFN/RBV. In several patients, the initial dose of telaprevir was reduced to 1500 mg daily according to age, body weight, gender, or baseline hemoglobin level, at the discretion of the attending physicians. When marked adverse effects such as anorexia, anemia, neutropenia, thrombocytopenia, renal dysfunction or skin rash, developed, the dose of telaprevir was reduced to 1500 mg daily, and that of PEG-IFN or RBV was reduced according to the recommendation on the package inserts or the clinical condition of individual patients. The regimen of PEG-IFN/RBV/faldaprevir was as follows: PEG-IFN-α2a (180 μg subcutaneously once a week), RBV (600–1000 mg daily according to body weight), and faldaprevir (120 or 240 mg once-daily) for 12 or 24 weeks, followed by an additional PEG-IFN/RBV, making a total of 24 or 48 weeks. When marked adverse effects developed, the dose of PEG-IFN or RBV was reduced as mentioned above.

Written informed consent was obtained from each patient and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the ethics review committees of Nagoya City University Hospital.

Definition of virological response to treatment

Treatment outcomes were defined as SVR (undetectable HCV RNA levels 24 weeks after cessation of treatment), transient virological response (TVR; HCV RNA levels became undetectable during treatment but reappeared after the end of treatment), and non-virological response (NVR; HCV RNA levels never became undetectable).

Detection of HCV RNA

Blood samples were obtained before treatment, and at week 1, 2, 4, 8, 12, and every 4 weeks up to treatment completion, and hematologic tests, blood chemistry and HCV RNA assays were performed. Follow-up measurements were obtained at week 4, 12 and 24 weeks after the end of treatment. HCV RNA levels were measured throughout the course of therapy using the COBAS TaqMan HCV test (Roche Diagnostics K.K., Tokyo, Japan). The measurement range of this assay is 1.2–7.8 log IU/mL.

SNP genotyping

Genetic polymorphisms in SNPs of the *IL28B* gene (rs8099917) were determined according to the manufacturer's instructions using TaqMan SNP Genotyping Assays and an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

Measurement of gene expression in PBMCs

Blood samples were collected from the patients at baseline, 8, and 24 hours (h) after the initial administration of PEG-IFN/RBV/PI. PBMCs were isolated from blood by Ficoll gradient centrifugation. Total RNA was extracted from PBMCs using the RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) synthesis was performed using 1.0 μg of total RNA isolated from PBMCs using the High Capacity RNA-to-cDNA kit (Applied Biosystems,