Original article

Combination therapies with daclatasvir and asunaprevir on NS3-D168 mutated HCV in human hepatocyte chimeric mice

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Background: Although the frequency of emergent drug-resistant strains of HCV in patients who failed to respond to simeprevir plus pegylated interferon (PEG-IFN) and ribavirin (RBV) decreased after cessation of the treatment, it is not clear whether or not the NS3-D168 variants affect the outcome of NS5A and NS3 inhibitor combination therapy. In this study, we investigated the relationship between the effect of daclatasvir plus asunaprevir treatment and the frequencies of NS3-D168 variants.

Methods: HCV genotype-1b-infected human hepatocyte chimeric mice with various frequencies of NS3-D168 amino acid substitutions were treated with asunaprevir alone or in combination with daclatasvir for 4 weeks. Frequencies of NS3-D168 substitutions at baseline were analysed by ultra-deep sequencing. Some mice with NS3-D168 substitutions were treated with PEG-IFN or telaprevir for 4 weeks.

Results: Mice with high frequencies of NS3-D168 showed low susceptibility to asunaprevir treatment and failed to respond to daclatasvir plus asunaprevir therapy. In contrast, mice with a low frequency (less than approximately 14%) of NS3-D168 showed a similar susceptibility to wild-type HCV-infected mice and achieved viral eradication with daclatasvir plus asunaprevir therapy. Although treatment with either telaprevir or PEG-IFN resulted in reduction of serum HCV RNA levels, no significant decrease in the frequency of NS3-D168 substitutions was achieved.

Conclusions: Daclatasvir and asunaprevir treatment could eliminate NS3-D168 variant HCV if the frequency was low. It is necessary to confirm that the frequency of NS3-D168 variants has decreased sufficiently before adopting daclatasvir plus asunaprevir therapy in patients with simeprevir plus PEG-IFN/RBV treatment failure.

Introduction

Chronic HCV infection is a leading cause of cirrhosis, liver failure and hepatocellular carcinoma [1]. The goal of chronic HCV infection treatment is eradication of the virus in order to prevent progression to cirrhosis and hepatocellular carcinoma. The development of direct-acting antiviral agents (DAAs) has improved patient outcomes. Simeprevir, an HCV NS3 protease inhibitor (PI), in combination with pegylated interferon (PEG-IFN) and ribavirin (RBV), significantly improved sustained virological response (SVR) rates in treatment-naive patients with chronic HCV genotype-1 infection, and most patients benefited from a shorter 24-week treatment duration [2]. However, some patients with resistance to IFN, including those with an unfavourable *IFNL3* genotype, tend to fail to respond to IFN-based treatment [3]. Mutations conferring resistance to simeprevir were detected in most of the patients who experienced virological failure under simeprevir plus PEG-IFN/RBV treatment, with NS3-D168V as the most frequently detected substitution [2].

The HCV genome is translated as a polyprotein and must be cleaved to produce functional proteins.

The structural proteins are cleaved by host proteases, but the non-structural proteins are cleaved by NS3, a virally encoded protease thought to be important in viral replication [4]. Several PI resistance-associated variants have been reported in NS3, but NS3 PI-resistant HCV variants exhibit reduced fitness, as estimated by viral replicative ability and production of infectious progeny [4,5]. Using deep sequencing analysis, it was reported that the frequency of emerging simeprevir-resistant variants gradually declined and became undetectable over time [6].

Recently, an IFN-free combination therapy including daclatasvir (NS5A replication complex inhibitor [RCI]) plus asunaprevir (NS3 PI) was approved for treatment of genotype-1 in Japan [7]. This combination therapy is well tolerated and can achieve a high rate of SVR. However, the efficacy is affected by the presence of pre-existing drug-resistant variants such as NS3-D168, NS5A-L31 and -Y93. It is not clear whether or not emerging NS3-D168 variants in patients with treatment failure for simeprevir plus PEG-IFN/RBV influence the outcome of daclatasvir plus asunaprevir therapy.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse enables mouse livers to be repopulated with human hepatocytes that can then be infected with HCV [8]. This animal model is useful for evaluating anti-HCV drugs such as PEG-IFN and DAAs [9,10]. Using this animal model, we recently described the successful elimination of HCV genotype-1b by treatment with the combination of BMS-788329, a close analogue of daclatasvir, and the NS3 PI BMS-605339 [11,12]. In this study, using this animal model and ultra-deep sequencing technology, we investigated the relationship between the effect of daclatasvir and asunaprevir combination treatment and the frequencies of drug-resistant NS3-D168 variants.

Methods

Ethics statement

All animal protocols described in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the local committee for animal experiments, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University (protocol number 12-93). Human serum samples were obtained from a patient with chronic hepatitis. The study was approved *a priori* by the ethical committee of Hiroshima University and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The patient provided written informed consent.

Animal treatment

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously [13]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and sacrifice were performed under ether anaesthesia. 8 weeks after hepatocyte transplantation, mice were injected intravenously with 100 µl of genotype-1b HCV-positive human serum samples. Mice serum samples were obtained every 1 or 2 weeks after HCV infection and human serum albumin and HCV RNA levels were measured.

Human serum samples

Human serum containing genotype-1b HCV was obtained from a chronic hepatitis patient who was a non-responder to prior PEG-IFN/RBV treatment. Serum samples were divided into aliquots and stored separately in liquid nitrogen until use.

Treatment of HCV-infected mice with anti-HCV inhibitors

8 weeks after HCV infection, a mouse was administered with 40 mg/kg of simeprevir (Janssen Pharmaceutical KK, Tokyo, Japan) orally for 4 weeks. 4 weeks after treatment, serum from infected mice was inoculated into treatment-naive mice. Mice were administered with 40 mg/kg of asunaprevir (twice a day) with or without 10 mg/kg of daclatasvir (once a day) for 4 weeks. To analyse the susceptibility of NS3-D168-mutated HCV to antiviral drugs, mice were administered with either an intramuscular injection of 30 µg/kg of PEG-IFN-α (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) twice a week or 200 mg/kg of telaprevir (Mitsubishi Tanabe Pharma Co., Osaka, Japan) orally twice per day for 4 weeks.

RNA extraction and amplification

RNA extraction, nested PCR and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [14]. Briefly, RNA was extracted from serum samples and extracted livers using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTra Ace; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV cDNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limit of real-time PCR is 3 $\log_{10}$ copies/ml.

Ultra-deep sequencing

The nucleotide and amino acid sequences of the NS3 and NS5A regions of HCV were determined by deep

sequencing. The primers used to amplify the NS3 region were NS3-3496F (5′-AGA ACC AGG TCG AGG GAG AGG-3′; nucleotides 3496–3516) and NS3-K3736R (5′-AAG TAG AGG TCC GAG CTG CCG-3′; nucleotides 3736–3716) as the former primer pair and NS3-V3717F (5′-GGC AGC TCG GAC CTT TAC TTG GT-3′; nucleotides 3717–3739) and NS3-3950R (5′-AGT TTC CAT AGA CTC AAC GGG-3′; nucleotides 3950–3930) as the latter primer pair. All amplifications were performed as follows: denaturation for 10 s at 98°C, extension for 1 min at 66°C, 3 cycles repeated, denaturation for 10 sec at 98°C, extension for 30 s at 64°C, 4 cycles repeated, denaturation for 10 s at 98°C, extension for 30 s at 62°C, 4 cycles repeated, denaturation for 10 s at 98°C, extension for 1 min at 60°C, 28 cycles repeated, and final extension was performed at 68°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis, and then used for ultra-deep sequencing using the Ion Personal Genome Machine™ (PGM™) Sequencer (Life Technologies, Carlsbad, CA, USA). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA, USA) and the concentration determined using the StepOne Plus RealTime PCR (Life Technologies) and Ion Library Quantitation Kit, according to the instructions provided by the manufacturer. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with Ion OneTouch 200 Template Kit v2 (Life Technologies). Enrichment for templated Ion sphere particles (ISPs) was performed using Ion OneTouch Enrichment System (Life Technologies), according to the instructions provided by the manufacturer. Templated ISPs were loaded onto an Ion 318 chip, and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 Mbase (0.5 M-tag, 200 base read) [15]. The results were analysed with CLC Genomics Workbench software (CLCbio, Aarhus, Denmark) [16]. The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype-1b HCV-J (GenBank Accession number: D90208) [17].

Statistical analyses

All analyses were performed using the R statistical package (R Foundation for Statistical Computing, Vienna, Austria). Non-parametric tests (χ^2 test) were used to detect significant associations. All statistical analysis were two-sided, and $P<0.05$ was considered significant.

Results

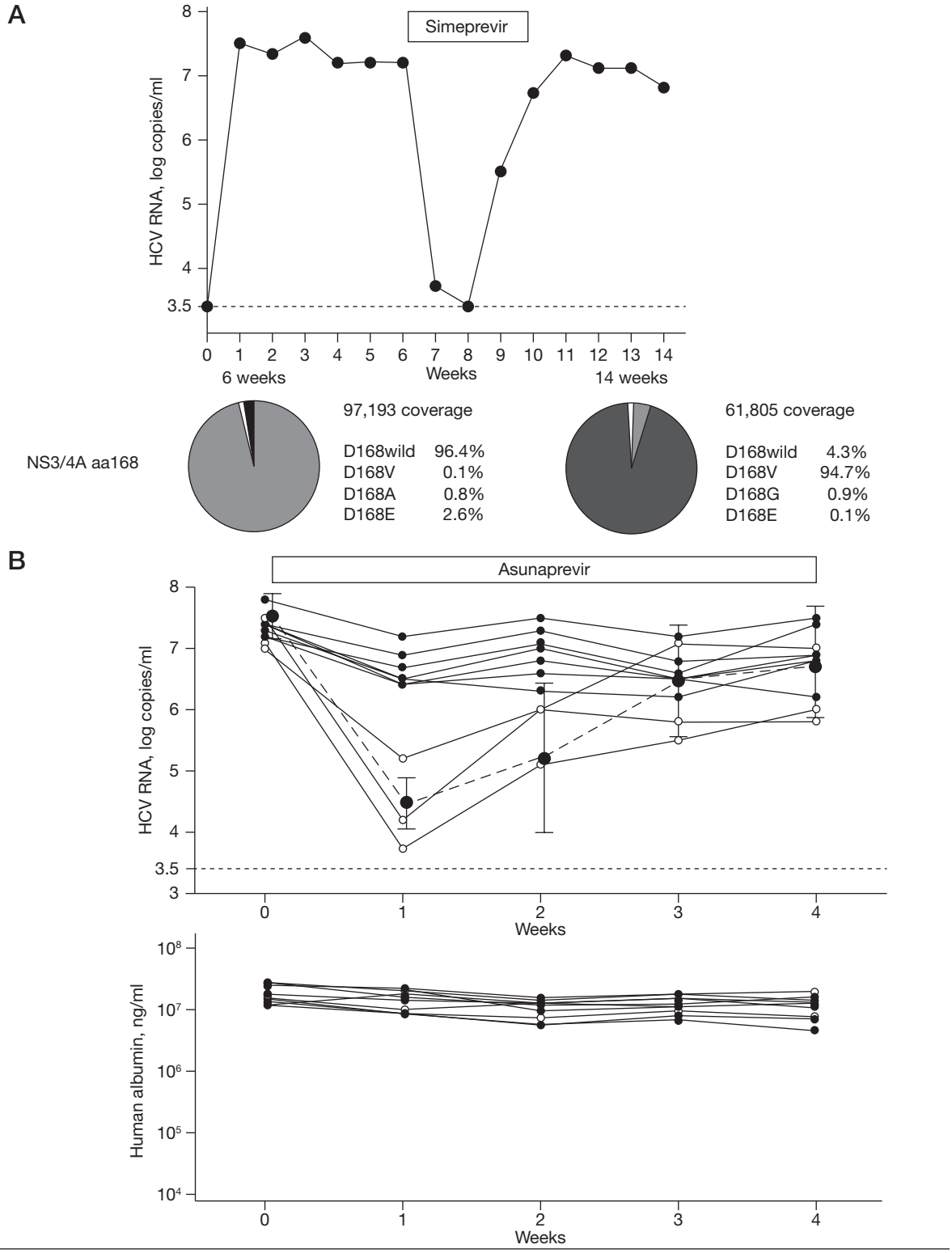
Effects of asunaprevir on drug-resistant HCV in mice

In order to generate NS3-D168 resistance mutants, a human hepatocyte chimeric mouse was infected with HCV, and then 6 weeks after infection, the mouse was treated with simeprevir for 4 weeks (Figure 1A). After 2 weeks of treatment, serum HCV RNA titre transiently decreased below the detectable limit and then rebounded during the treatment. Although ultra-deep sequence analysis showed that the frequency of wild-type of NS3-D168 was 96.4% before simeprevir treatment, NS3-D168 wild-type was replaced by simeprevir-resistant variants NS3-D168V/G/E 4 weeks after cessation of the treatment. Ten mice were injected with serum samples obtained from the simeprevir-treated mouse at 4 weeks after cessation of the treatment. 8 weeks after injection, these 10 mice were treated with 40 mg/kg of NS3 PI asunaprevir for 4 weeks (Figure 1B). In 3 of 10 NS3-D168 variant HCV-infected mice, serum HCV RNA decreased by 1.8, 3.3 and 3.4 log copies/ml after 1 week of treatment, whereas reduction of HCV RNA titres in the remaining 7 mice were minimal after 1 week of treatment (from 7.4 to 6.9 log copies/ml). HCV RNA titres rebounded during treatment in all mice. Ultra-deep sequencing at baseline seemed to reveal high frequencies of NS3-D168 variants in these 7 mice with a minimal HCV RNA reduction under asunaprevir treatment. In three mice in which susceptibility to asunaprevir was similar to that of wild-type HCV-infected mice, the frequencies of D168 variants tended to be low (13.9%, 0.6% and 0.2%, respectively; Figure 1C). All 5 mice with ≥20% of NS3-D168 variants resulted in a slight decrease in the serum HCV RNA titres. In contrast, 3 of 5 mice with <20% of NS3-D168 variants demonstrated more remarkable decrease (P=0.038). These results suggest that low frequencies of NS3-D168 variants are unlikely to have a significant impact on asunaprevir treatment.

Effects of daclatasvir and asunaprevir combination treatment on drug-resistant HCV in mice

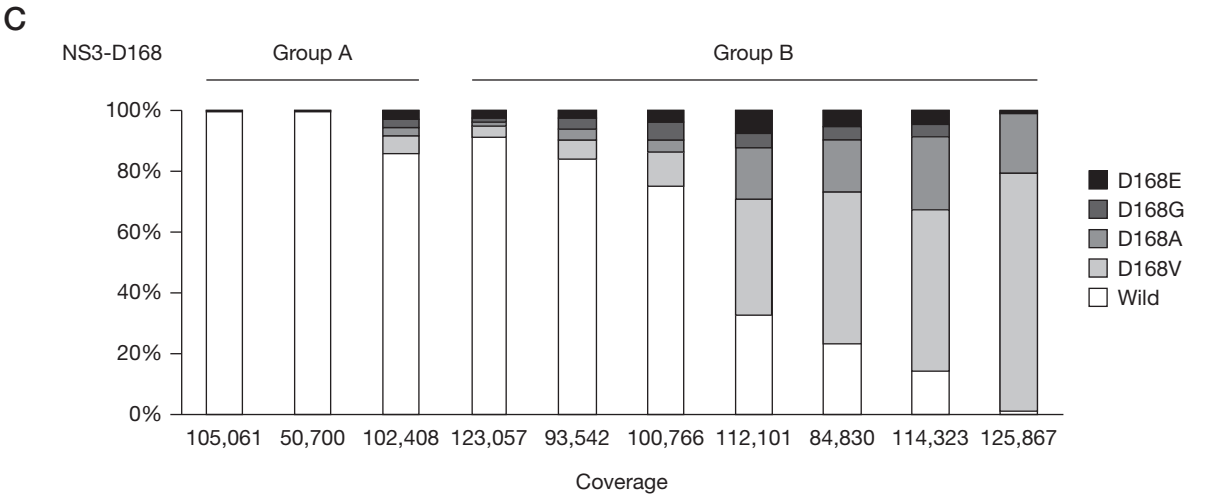
We next analysed the effects of daclatasvir and asunaprevir combination treatment in mice infected with HCV NS3-D168 variants. Four of the mice with HCV NS3-D168 variants were treated with daclatasvir plus asunaprevir for 4 weeks (Figure 2A). Serum HCV RNA titres decreased below the detectable limit and remained negative during the treatment in all mice. In 2 of 4 mice, serum HCV RNA rebounded at 2 and 4 weeks after cessation of the treatment, respectively. In the remaining 2 mice, serum HCV RNA titres remained negative after cessation of treatment, and elimination of the virus was assumed since HCV RNA was undetectable by nested PCR in mouse livers 9 weeks after cessation of the

Figure 1. Effects of asunaprevir on drug-resistant HCV in mice



(A) A human hepatocyte chimeric mouse was infected with HCV and treated with simeprevir for 4 weeks. Mouse serum samples were obtained at the indicated times, and HCV RNA titre was analysed. Deep sequencing at NS3-D168 was performed before simeprevir treatment and 4 weeks after treatment. The horizontal dashed line represents the lower detection limit of HCV RNA. **(B)** Mice were injected with serum samples obtained from either a simeprevir-treated mouse (solid line) or an HCV-infected patient (dashed line, n=6) for 4 weeks. Serum HCV RNA titre (upper panel) and human serum albumin concentration (lower panel) were measured at the indicated time. Data of mice infected with wild type are represented as mean ±SD. Decrease of serum HCV RNA titre was lower in seven mice (closed circles) and similar to wild-type HCV-infected mice in three mice (open circles). **(C)** Frequencies of NS3-D168 variants at baseline in each mouse were analysed by deep sequencing. Mice with similar (Group A) and lower (Group B) susceptibility to asunaprevir compared to wild-type HCV-infected mice are shown. aa, amino acid.

Figure 1. Continued



treatment (Figure 2B). Ultra-deep sequencing revealed that NS3-D168 variants were present at high frequency at baseline in two mice in which HCV RNA rebounded (57.7% and 86.1%, respectively; Figure 2C). In contrast, the frequencies of pre-treatment NS3-D168 variants were low in mice that achieved HCV eradication (3.3% and 13.2%, respectively). Pre-treatment NS5A-L31 and Y93 variants were not detected in any of the mice. These results indicate that daclatasvir and asunaprevir treatment may be effective at eliminating NS3-D168 variants if the initial frequency is low.

The effects of IFN and telaprevir on asunaprevir-resistant NS3-D168 variants

In patients for whom the frequency of NS3-D168 variants is too high for effective treatment with daclatasvir and asunaprevir, HCV eradication might nonetheless be achieved by first reducing the frequency of NS3-168 variants using an alternative approach. Therefore, we investigated whether or not IFN or telaprevir could be used to decrease the frequency of NS3-D168 resistant variants. Eight mice infected with HCV NS3-D168 variants were treated with PEG-IFN or telaprevir for 4 weeks. In PEG-IFN-treated mice, ultra-deep sequencing showed that the frequency of NS3-D168 variant HCV slightly decreased (from 69% at baseline to 31% at the end of treatment) in one mouse and failed to decrease significantly in the other two mice (Figure 3A). In telaprevir-treated mice, ultra-deep sequencing showed a slight decrease in the frequency of NS3-D168 variants in one mouse, but no significant decrease in the frequency of NS3-D168 was observed in the remaining four mice (Figure 3B). These results suggest that neither IFN nor telaprevir treatment was effective in reducing the frequency of NS3-D168 variants.

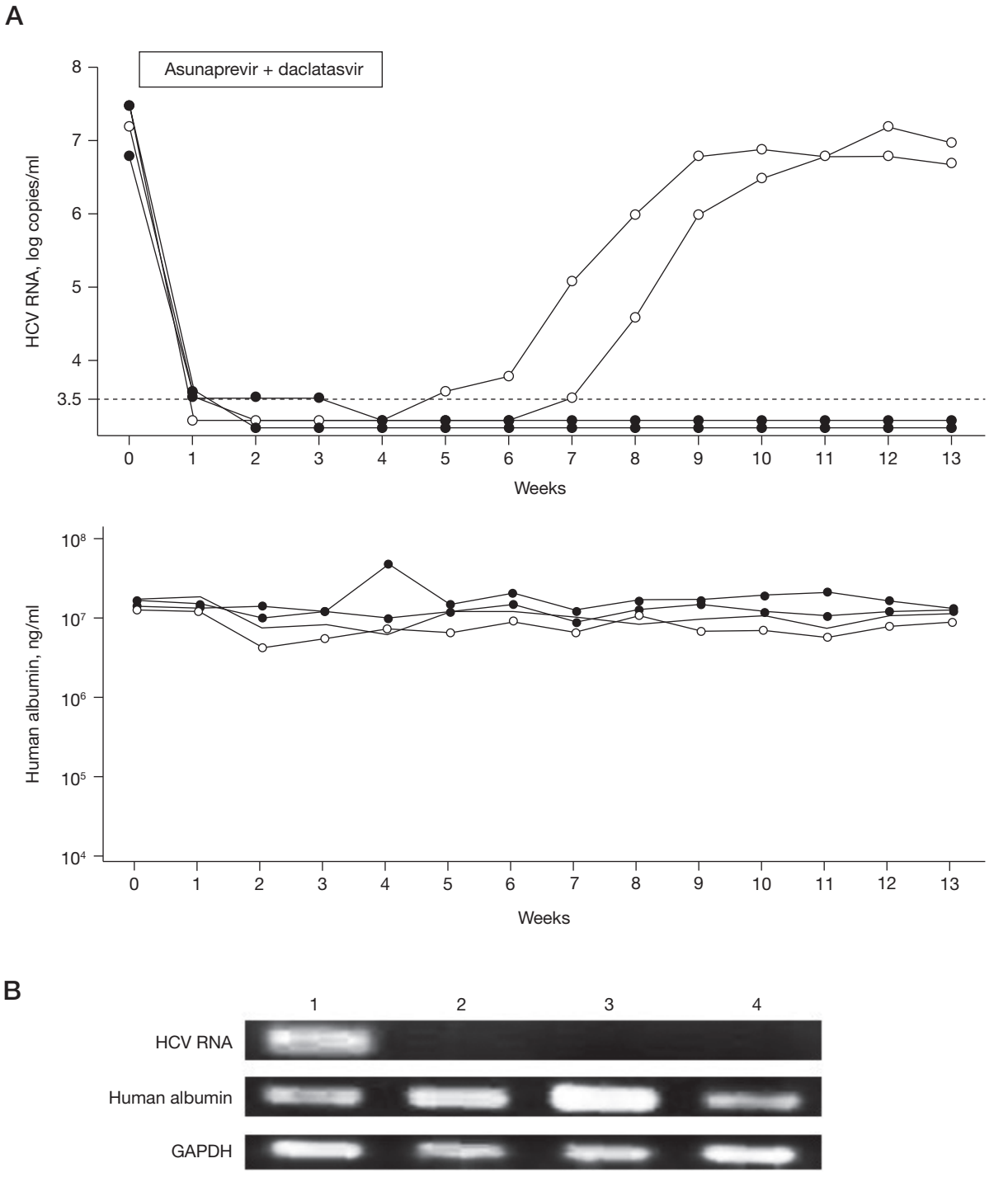
Discussion

Although the frequencies of HCV NS3-D168 resistance variants emerging in patients treated with simeprevir plus PEG-IFN/RBV treatment failure has been found to gradually decrease after cessation of the treatment, it is not clear whether or not NS3-D168 variants affect the outcome of therapy with daclatasvir and asunaprevir. In this study, we investigated the relationship between the frequencies of PI-resistant NS3-D168 variants and the effect of daclatasvir and asunaprevir combination treatment.

First, we investigated the efficacy of asunaprevir monotherapy in mice with NS3-D168V substitutions at various baseline frequencies. The antiviral effect of asunaprevir was reduced in mice with high baseline frequencies of NS3-D168 variants (Figure 1B and 1C). In contrast to the effect on mice with high frequencies of NS3-D168 variants, the effect of asunaprevir in mice with low frequencies of NS3-D168 variants was similar to that of mice with wild-type HCV.

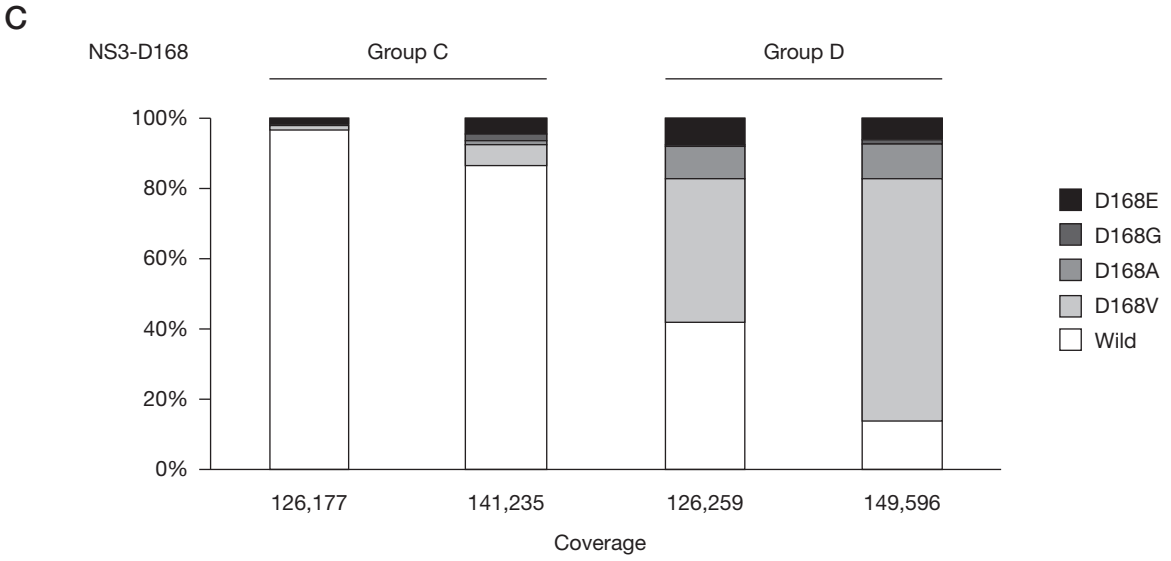
Second, we investigated the effect of asunaprevir and daclatasvir combination therapy on mice with various baseline frequencies of NS3-D168 variants. In all mice, neither NS5A-L31 nor -Y93 substitutions were detected prior to treatment. Serum HCV became negative in all mice, but HCV RNA rebounded in mice with high initial NS3-D168 variant frequencies (Figure 2A and 2C). In contrast to mice with high frequencies of NS3-D168 variants, HCV was eradicated in mice with low frequencies of NS3-D168 variants. Therefore, it is necessary to confirm that the frequency of NS3-D168 variants has decreased sufficiently before adopting asunaprevir and daclatasvir combination therapy in patients who failed to respond to simeprevir plus PEG-IFN/RBV treatment.

Figure 2. Effects of daclatasvir and asunaprevir combination treatment on drug resistant HCV in mice



(A) Four mice infected with NS3-D168 mutated HCV were treated with daclatasvir and asunaprevir for 4 weeks. Serum HCV RNA titre was measured at the indicated time points. Serum HCV RNA rebounded after cessation of the treatment in two mice (open circles) or remained negative at 9 weeks after cessation of the treatment in two mice (closed circles). **(B)** Nested PCR of HCV RNA, human serum albumin and GAPDH in mouse livers. Livers from two mice negative for HCV RNA 9 weeks after cessation of the treatment were obtained (lanes 2 and 3). Mouse livers with HCV infection (lane 1) or without (lane 4) were also analysed. **(C)** Frequencies of NS3-D168 variants in each mouse were analysed by deep sequencing. Mice negative for HCV RNA 9 weeks after cessation of the treatment (Group C) and mice with HCV RNA rebound (Group D) are shown.

Figure 2. Continued



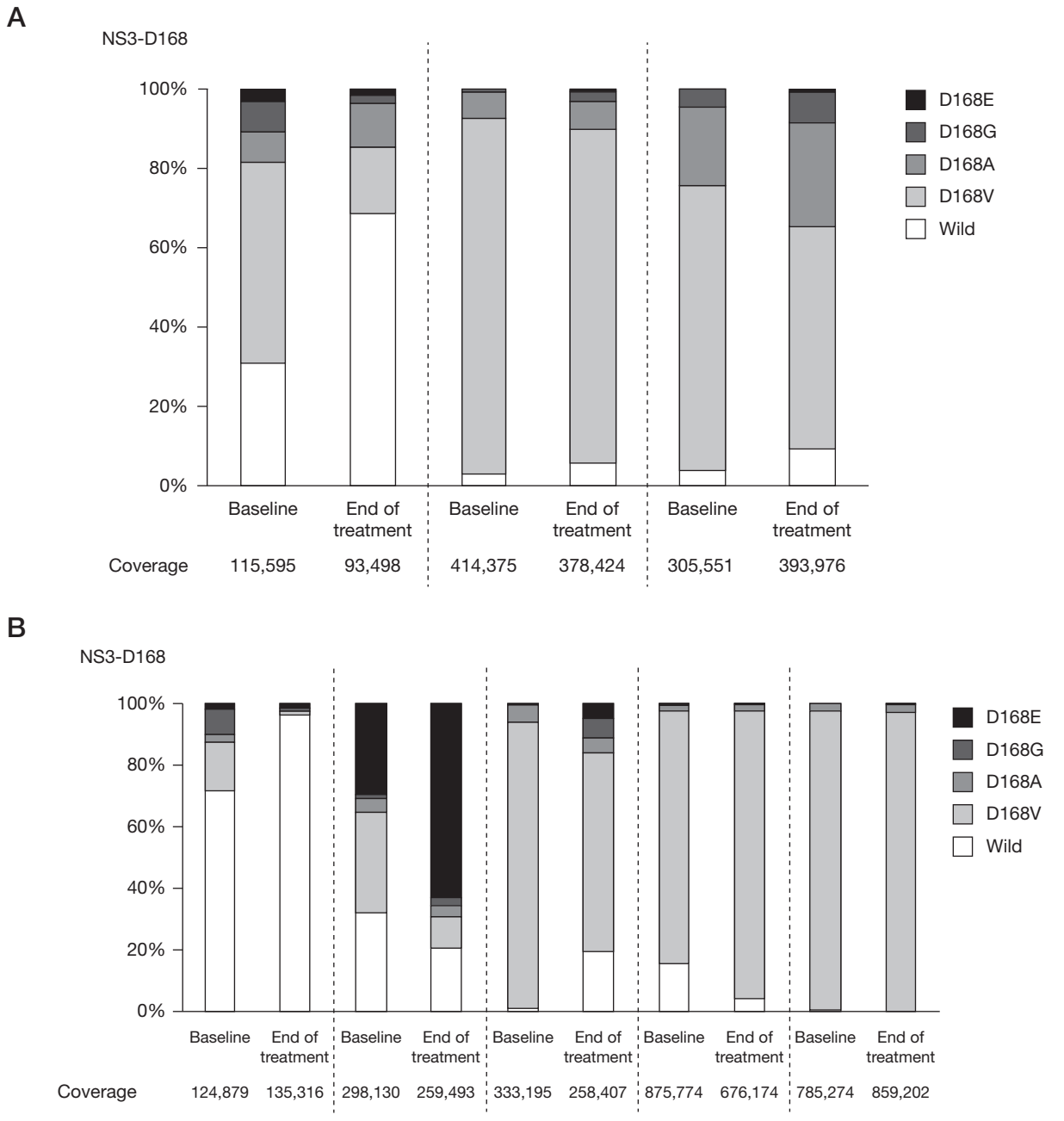
Pre-existing NS5A inhibitor-resistant variants affect the antiviral properties of daclatasvir plus asunaprevir treatment; thus high SVR rates are expected in patients without drug-resistant variants as determined by direct sequence analysis [18]. In this study, chimeric mice with less than approximately 14% of NS3-D168V variants at baseline are likely to show a similar response to that of NS3-D168 wild-type and are expected to be able to achieve viral eradication by treatment with daclatasvir and asunaprevir combination therapy. However, in clinical practice, it is unknown whether patients with less than 14% of NS3-D168V variants actually achieve viral eradication by daclatasvir and asunaprevir therapy. Our study has some limitations due to the use of human hepatocyte transplanted mice. Because we treated chimeric mice with antivirals for only 4 weeks, patterns of emergence of drug resistant variants in mice are likely to differ from that of humans treated for more than 4 weeks with respect to genetic distance. Sato *et al.* [19] reported that in the analysis of viral quasispecies composition in non-SVR patients, the genetic distance of HCV over time changed during treatment. In fact, we have observed a patient who experienced viral breakthrough during daclatasvir plus asunaprevir combination therapy who had previously failed to respond to simeprevir plus PEG-IFN/RBV treatment but for whom the frequency of NS3-D168 variants had returned completely to wild type and who had wild-type NS5A-L31 and Y93 (data not shown). Further analyses will be required.

NS3 PI-resistant HCV variants have reduced fitness, as estimated by viral replicative ability, and have a high susceptibility to IFN [4,14]. Telaprevir, a first wave PI, has a different resistance profile than that of the second wave PI simeprevir [20]. If telaprevir or a similar drug is able to accelerate the reduction of NS3-D168 variants in patients who experienced simeprevir plus PEG-IFN/RBV treatment failure, these patients could receive daclatasvir plus asunaprevir treatment more promptly. Therefore, using a mouse model, we analysed whether or not telaprevir or IFN could reduce the frequencies of NS3-D168 variants. However, no significant reduction in the frequencies of NS3-D168 variants was observed using either telaprevir or IFN (Figure 3). A more effective method is needed to decrease the frequency of NS3-D168 variants in patients with simeprevir plus PEG-IFN/RBV treatment failure prior to retreatment.

Deep sequence analysis has recently been applied to analyse viral mutations, but the method is complex and expensive to perform and impractical for widespread clinical use. We recently developed a rapid NS5A-Y93H strain detection system based on the Invader assay [21]. Our assay system achieved a better lower detection limit for NS5A-Y93H, estimated to be 1% to 2%, compared to direct sequencing, although it is not as sensitive as deep sequencing. It is expected that a similar system to detect NS3-D168 variants could be established for clinical use.

In conclusion, virus eradication was achieved by daclatasvir and asunaprevir combination treatment in genotype-1b HCV-infected chimeric mice with low baseline frequencies of NS3-D168 variants. It is necessary to confirm that NS3-D168 variants are suppressed sufficiently before adoption of asunaprevir and daclatasvir combination therapy in patients with simeprevir plus PEG-IFN/RBV treatment failure.

Figure 3. The effects of antivirals on NS3-D168 variant HCV-infected mice



NS3-D168 variant HCV-infected mice were treated with either **(A)** pegylated interferon-α or **(B)** telaprevir for 4 weeks. Frequencies of NS3-D168 variants at baseline and at the end of treatment were analysed by deep sequencing in each mouse.

Acknowledgements

The authors thank Rie Akiyama and Yoko Matsumoto (Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan) for their expert technical help, Bristol–Myers Squibb Research and Development for providing daclatasvir and asunaprevir, and Mitsubishi Tanabe Pharma Co. for providing telaprevir. This study was supported in

part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor, Health and Welfare (grant number 26380101).

Disclosure statement

KC received honoraria from MSD KK, Bristol–Myers Squibb and research funding from AbbVie, Dainippon Sumitomo Pharma, and The Institute of Physical and Chemical Research (RIKEN). MI received honoraria and research funding from Bristol–Myers Squibb. MT received research funding from Bristol–Myers Squibb. The other authors declare no conflicts of interest.

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Accepted 27 October 2015; published online 12 November 2015