Figure 1 Flow diagram of patient recruitment. HCV, hepatitis C virus; IFN, interferon; LDLT, living-donor liver transplantation; LT, liver transplantation; RBV, ribavirin; SVR, sustained viral response.

patients develop cirrhosis within 5 years after LT, and approximately 50% within 10 years.[13,16,17] LT recipients with recurrent HCV are treated with a combination of PEGIFN/RBV for 48 weeks. However, eradication with IFN therapy after LT is hampered by the use of immunosuppressive agents, anemia, frequent side-effects, and the need to discontinue or reduce therapy. The outcome of PEGIFN/RBV antiviral therapy after LT is poor, with the SVR rate ranging from 10% to 30% for HCV-1-infected patients.[18-24]

However, Fukuhara *et al.*[8] reported that in patients with recurrent HCV infection after LT, combination analyses of SNP of *IL28B* in both the donor and recipient tissues and mutations in HCV–RNA allow the prediction of SVR to PEGIFN/RBV therapy.

We reported previously the effectiveness of the treatment of recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy).[25] Others also reported SVR rates of 34% and 50% under the same treatment, respectively.[26,27]

In the present study, we analyzed the viral response to long-term PEGIFN/RBV therapy in patients according to the major and minor genotypes of the polymorphic *IL28B* gene.

Methods

Patients. Sixty-five patients underwent living-donor LT (LDLT) for HCV-related end-stage liver disease between 2000 and January 2011. Among them, 22 patients died before the start of therapy, one was naturally negative for HCV–RNA before LT, one did not become positive for HCV–RNA after LDLT, and four obtained SVR by IFN therapy before LT, thus leaving 37 patients treated with IFN therapy at our institution. Of these, two patients are currently continuing antiviral therapy. A total of 35 patients were enrolled in this retrospective study.

There were 28 patients with HCV-1, and seven with HCV-2. The data of eight of the 28 patients with HCV-1 were excluded from

the analysis due to the use of standard IFN/RBV in four patients, and cessation due to side-effects in four patients. Thus, the study included 20 patients with HCV-1 (Fig. 1).

Protocol of antiviral therapy. Patients received PEGIFN-α-2b subcutaneously once weekly combined with RBV (200 mg/day). The dose of the latter was increased to 800 mg/day in a stepwise manner, according to individual tolerance within the first 12 weeks of therapy. The combination PEGIFN/RBV therapy was continued for more than 48 weeks after the disappearance of serum HCV–RNA. At the end of the active treatment, patients were followed for another 24 weeks without treatment. In patients who remained positive for HCV–RNA in spite of treatment for more than 48 weeks, PEGIFN was switched to PEGIFN-α-2a, and treatment was continued as described earlier.

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the local ethics committees of all participating centers. Written, informed consent was obtained from all participating patients.

Assessment of therapy efficacy. HCV–RNA levels were measured using one of several reverse transcription–polymerase chain reaction (RT–PCR)-based methods (*Taq*Man RT–PCR test) at weeks 4, 8, and 12, and thereafter every 4 weeks of treatment, and at 24 weeks after the cessation of therapy.

SNP genotyping and quality control. Because the two reported significant *IL28B* SNP (rs8099917 and rs12979860) are in strong linkage disequilibrium, we examined only rs8099917 in this study. Some samples obtained from patients with HCV-1 were determined using the Illumina HumanHap610-Quad Genotyping BeadChip (San Diego, CA, USA), whereas the remaining samples were genotyped using the Invader assay (Third Wave Technologies, Madison, WI, USA), as described previously.[28,29]

Table 1 Characteristics of 20 patients with recurrent hepatitis C genotype 1 after living-donor liver transplantation

Age (years)[†]	58 (44–70)
Sex (male/female)	15/5
Body mass index (kg/m²)[†]	24.3 (18.8–42.2)
Viral load at therapy (LogIU/mL)[†]	6.6 (4.9–7.8)
Time from transplantation to therapy (months)[†]	4 (1–41)
No. mutations in the ISDR (0–1/2–5)	12/8
HCV core70 region (mutant/wild)	12/8
HCV core 91 region (mutant/wild)	10/10
Donors' *IL28B* genotype TT/TG + GG	15/5
Recipients' *IL28B* genotype TT/TG + GG	14/6
Combination of donors' and recipients' *IL28B* genotype (TT : TT/TT : TG + GG/TG + GG : TT/TG + GG : TG + GG)	11/4/3/2
Immunosuppression (tacrolimus/cyclosporine)	16/4
Adherence to PEGIFN ≥ 70/< 70 (%)[†]	11/9
Adherence to RBV ≥ 50/< 50 (%)[†]	8/12

[†]Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

Analysis of the nucleotide sequences of the core and non-structural 5A regions.

The amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region and mutation at the IFN sensitivity-determining region were analyzed in the non-structural 5A region of HCV by the direct sequencing method, as described previously by our group.[25,30,31] Samples after LT were used.

Statistical analysis.

Non-parametric tests (χ^2-test and Fisher's exact probability tests) were used to compare the characteristics of the groups. Univariate logistic regression analysis was used to determine those factors that significantly contributed to early viral dynamics. The odds ratios and 95% confidence intervals were also calculated. All *P*-values < 0.05 using two-tailed tests were considered significant. Variables that achieved statistical significance (*P* < 0.05) or marginal significance (*P* < 0.10) in the univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Statistical analyses were performed using PASW 18 statistical software (SPSS, Chicago, IL, USA).

Results

Patient characteristics.

Table 1 shows the baseline characteristics of the 20 patients with recurrent hepatitis C after LT who completed PEGIFN/RBV treatment. The median age of the patients (15 males and 5 females) was 58 years, and the median body mass index was 24.3. The median latency between transplantation and the initiation of antiviral therapy was 4 months. The median pretreatment serum HCV–RNA viral load was 6.6 LogIU/mL. The *IL28B* genotype (rs8099917) of the donors was TT in 15 patients, and TG + GG in five patients, whereas that of the recipients was TT in 14, and TG + GG in six. Immunosuppressive therapy included tacrolimus in 16, and cyclosporine in four.

Efficacy and tolerance of IFN therapy and side-effects.

Figure 1 shows the effects of IFN therapy according to genotype. The SVR rate was 54.2% (19/35) for all patients. Among the patients infected with HCV-1, one of eight patients who were treated with mono-IFN/RBV or ceased treatment had SVR. Twelve of 20 patients with HCV-1 who completed IFN therapy achieved SVR. Thus, the SVR rate was 46.4% (13/28) for those with HCV-1, and 85.7% (6/7) with HCV-2. In patients with HCV-1, four ceased IFN therapy due to adverse effects. These included general fatigue in one, rejection in two, and cerebral hemorrhage in one patient.

Relationship between IL28B and viral response in patients infected with HCV genotype 1.

Data or eight of 28 patients with HCV-1 were excluded from the analysis due to standard-IFN plus RBV in four patients, and the cessation of IFN therapy due to adverse effects in four patients. Thus, the data of 20 patients with HCV-1 were available for the analysis of *IL28B*.

In the donors, the SVR rate of the TT group (73.3% [*n* = 11/15]) was higher than that of the TG + GG group (20% [*n* = 1/5], *P* = 0.053, Fig. 2a). In the recipients, the SVR rate of the TT group (64.2% [*n* = 9/14]) was similar to that of the TG + GG group (50% [*n* = 3/6]) (Fig. 2b). The SVR rate of the TT : TT group (donors' *IL28B* : recipients' *IL28B*) was 81.8% (*n* = 9/11), which was higher than the SVR rate of the TT : TG + GG group (50% [*n* = 2/4], Fig. 2c). The SVR rate of the TG + GG : any group (donors' *IL28B* : recipients' *IL28B* of either TT or TG + GG) was 20% (*n* = 1/5), which was lowest among the three groups. There was significant difference between the SVR of the TT : TT group and TG + GG : any group (*P* = 0.036). We also analyzed the viral response (VR) rate according to the combination of donors' and recipients' *IL28B*. The VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. The VR rate of the TT : TT group was 63.6% (*n* = 7/11), which was higher than the VR rate of the TG + GG : any group (0% [*n* = 0/5]) at 24 weeks. The VR rate of the TT : TT group was 100% (*n* = 11/11), which was higher than the VR rate of the TG + GG : any group (20% [*n* = 1/5]) at the end of treatment. The SVR rate of the TT : TT group was 100% (*n* = 11/11), which was higher than the SVR rate of the TG + GG : any group (20%, *n* = 1/5) at 24 weeks at the end of treatment (Fig. 3).

Analysis of factors associated with SVR in HCV-1 patients with recurrent hepatitis C.

The univariate analysis identified three parameters that correlated with SVR either significantly or marginally: the combination of donors' and recipients' *IL28B* (TT : TT *P* = 0.037), donors' *IL28B* (TT genotype; *P* = 0.053), and adherence to RBV therapy (≥ 50; *P* = 0.076, Table 2). The combination of donors' and recipients' *IL28B* (TT : TT genotype) and adherence to RBV (> 50; *P* = 0.076) were entered into the multiple logistic regression analysis to identify significant independent predictive factors. The multivariate analysis identified the combination of donors' and recipients' *IL28B* (TT : TT) as the only significant and independent factor that influenced the SVR: (odds ratio: 15.0, 95% CI: 1.2–185.1, *P* = 0.035).
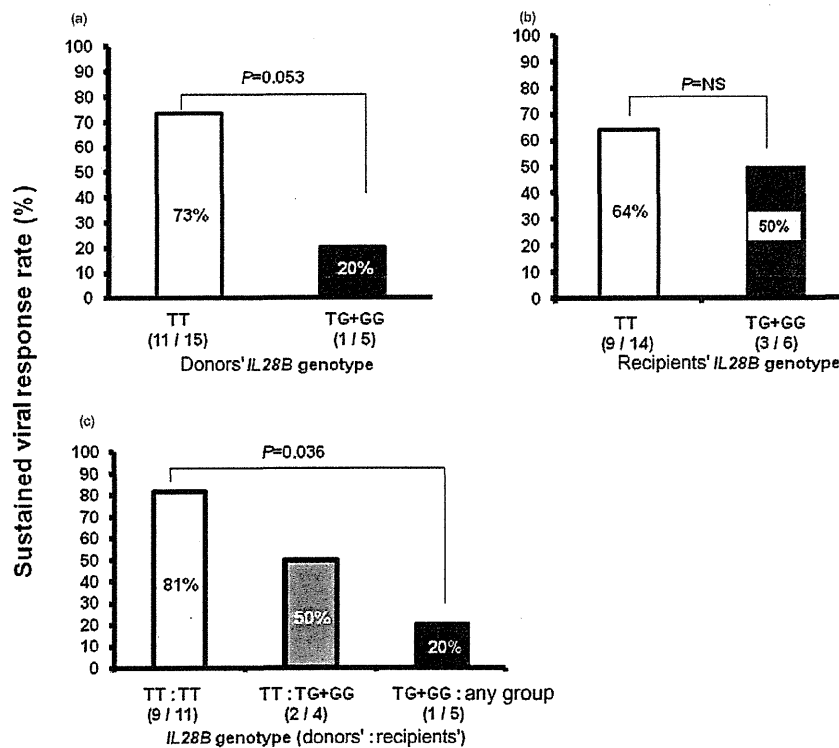
Figure 2 Sustained viral response rates according to (a) donors' interleukin-28B (*IL28B*), (b) recipients' *IL28B*, and (c) donors' and recipients' *IL28B* in patients infected with hepatitis C virus genotype 1. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). NS, not significant.
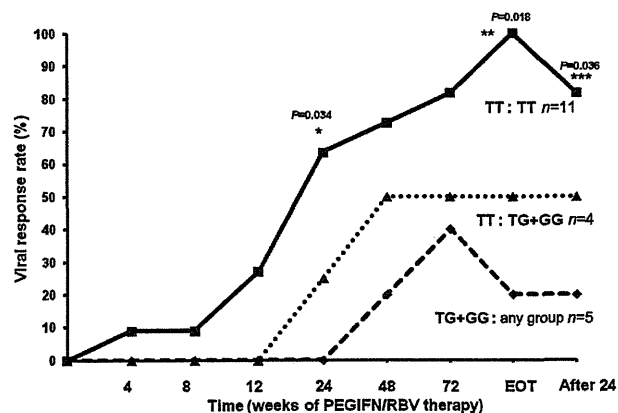

Figure 3 Viral response rates according to donors' and recipients' interleukin-28B (*IL28B*) genotyping. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). *Viral rate (VR) of the TT : TT group was 63.6% (*n* = 7/11), which was higher than the VR rate of the TG + GG : any group (0%, *n* = 0/5) at 24 weeks. **VR rate of the TT : TT group was 100% (*n* = 11/11), which was higher than the VR rate of the TG + GG : any group (20%, *n* = 1/5) at the end of treatment (EOT). ***Sustained VR (SVR) rate of the TT : TT group was 100% (*n* = 11/11), which was higher than the SVR rate of the TG + GG : any group (20%, *n* = 1/5) at 24 weeks at the EOT. PEGIFN, pegylated interferon; RBV, ribavirin.

Discussion

The SVR rate has improved since the introduction of PEGIFN/ RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with a history of HCV-1 infection is 30–50%.[21–24,26,27] These results are much better than those reported in the 1990s and early 2000s; however, more than half of recipients still suffer from recurrent chronic hepatitis C.

Although many studies have determined the predictive factors of the viral response for PEGIFN/RBV among patients with chronic hepatitis C, recent molecular biological analyses and genome-wide analyses of the human genome have identified genetic variations of *IL28B* and amino-acid substitution of HCV core 70 as the most significant predictive factors for IFN response.[3–5,32,33] *IL28B* encodes a cytokine distantly related to type I IFN and the IL-10 family. It has been reported that the expression level of the *IL28* gene in peripheral blood mononuclear cells is significantly lower in individuals with minor alleles than in individuals with major alleles.[5]

Several studies have determined the predictive factors for the viral response to PEGIFN/RBV in patients with recurrent post-LT hepatitis C viral infection, and recent molecular and genome wide analyses of the human genome have demonstrated that genetic variation of *IL28B* is the most significant predictive factor of the response to IFN.[8,34–37] In the present study, we examined whether the same factors can also predict the response to PEGIFN/RBV in LT recipients. Several groups have reported that recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent hepatitis C after LT.[8,36,37] Furthermore, others

Table 2 Univariate analysis of factors associated with sustained viralresponse (SVR) during interferon therapy in genotype 1 patients with recurrent hepatitis C

	SVR ($n = 12$)	Non-SVR ($n = 8$)	*P*-value
Age (years)[†]	60 (44–69)	57 (47–65)	0.48
Sex (male/female)	10/2	5/3	0.3
Body mass index (kg/m²)[†]	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.4
Viral load at therapy (LogIU/mL)[†]	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.52
Time from transplantation to therapy (months)[†]	4 (1–41)	3 (1–6)	1.7
No. mutations in the ISDR (0–1/2–5)	7/5	5/3	1.0
HCV core70 region (mutant/wild)	7/5	5/3	1.0
HCV core 91 region (mutant/wild)	7/5	3/5	0.6
Donors' *IL28B* genotype TT/TG + GG	11/1	4/4	0.053
Recipients' *IL28B* genotype TT/TG + GG	9/3	5/3	0.6
Donors' and recipients' *IL28B* genotype TT : TT/others	9/3	2/6	0.037
Immunosuppression (tacrolimus/cyclosporine)	9/3	7/1	1.0
Adherence to PEGIFN ≥ 70/< 70 (%)[†]	8/4	3/5	0.3
Adherence to RBV ≥ 50/< 50 (%)[†]	7/5	1/7	0.076

[†]Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT,[34] and that recipients' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C.[35,36]

The results of the present study indicate that both donors' and recipients' *IL28B* influence the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C. Both recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in recurrent hepatitis C after LT; however it is not clear whether the recipients' or donors' *IL28B* influenced the SVR to PEGIFN/RBV.

However, the donors' *IL28B* might have influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C more than the recipients' *IL28B*. This conclusion is based on the following results: although the SVR rate of the TT group (64.2%) was similar to that of the TG + GG group (50%), according to the recipients' *IL28B*, the SVR rate of the TT group (73.3%) was higher than that of the TG + GG group (20%), according to the donors' *IL28B*. Furthermore, the VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. That is, the time to VR of the TG + GG : any group was the latest among the three groups. Lange *et al.* reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT.[34] In this regard, Hiraga *et al.*[38] reported that IFN-stimulated gene expression levels in mice livers measured at 2 weeks after IFN treatment were significantly higher in mice transplanted with donor human hepatocytes (*IL28B*; TT) than from donor (*IL28B*; TG + GG) mice. Furthermore, previous studies reported that the expression level of IFN- λ-3, coded for the *IL28B* gene, was higher in hepatocytes than hematopoietic cells.[39]

However, we demonstrated the feasibility of treatment of LT recipients with PEGIFN/RBV until HCV–RNA reached undetectable levels, followed by the continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy). In fact, the SVR rate (50%) of the recipients' *IL28B* TG + GG group was higher than that

reported by others[8] (SVR rate: 11%). Furthermore, the SVR rate (81%) of the combination of donors' and recipients' *IL28B* (TT : TT) group was higher than that reported by Fukuhara *et al.*[8] (SVR rate: 56%). However, the SVR rate of the donors' *IL28B* TG + GG group (SVR rate: 20%) was similar to that reported by Fukuhara *et al.*[8] (SVR rate: 9%). We believe that the treatment of LT recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by the continuation of treatment for at least 48 weeks, is not useful for donors with *IL28B* TG + GG.

In Japan, LDLT is more common than orthotopic LT. In finding a suitable donor, it is better to select a donor with TT of the *IL28B* gene than a TG or GG donor. In conclusion, our results demonstrated the suitability of donors with the TT *IL28B* genotype, and that long-term PEGIFN/RBV therapy seems useful for recipients of LDLT who develop recurrent hepatitis C after transplantation.

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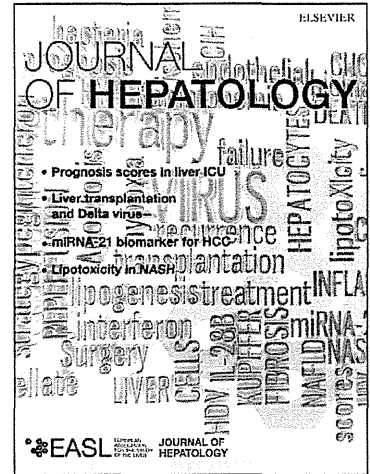
Accepted Manuscript

Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir

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Abbreviations: DAA, direct-acting antiviral; HCV, hepatitis C virus; SVR, sustained virologic response; GT, genotype; alfa/RBV, peginterferon alfa and ribavirin; DCV, daclatasvir; ASV, asunaprevir; LLOQ, lower limit of quantitation; PCR, polymerase chain reaction; FU, follow-up; RAV, resistance-associated variant; BL, baseline; VBT, viral breakthrough; SD, standard deviation.

2

Abstract

Background and Aims: Daclatasvir and asunaprevir are NS5A- and NS3 protease-targeted antivirals currently under development for treatment of chronic hepatitis C virus infection. Clinical data on baseline and on-treatment correlates of drug resistance and response to these agents are currently limited.

Methods: Hepatitis C virus genotype 1b Japanese patients (prior null-responders to peginterferon-alfa/ribavirin [n=21] or peginterferon-alfa/ribavirin ineligible or intolerant [n=22]) were administered daclatasvir/asunaprevir for 24 weeks during a phase 2a open-label study. Genotypic and phenotypic analyses of NS3 and NS5A substitutions were performed at baseline, after virologic failure, and post-treatment through follow-up Week 36.

Results: There were three viral breakthroughs and four relapsers. Baseline NS3 polymorphisms (T54S, Q80L, V170M) at amino acid positions previously associated with low-level resistance (<9-fold) to select NS3 protease inhibitors were detected in four null-responders and three ineligibles but were not associated with virologic failure. Baseline NS5A polymorphisms (L28M, L31M, Y93H) associated with daclatasvir resistance (<25-fold) were detected in five null-responders and six ineligibles. All three viral breakthroughs and 2/4 relapsers carried a baseline NS5A-Y93H polymorphism. NS3 and NS5A resistance-associated variants were detected together (NS3-D168A/V,NS5A-L31M/V-Y93H) after virologic failure. Generally, daclatasvir-resistant substitutions persisted through 48 weeks

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post-treatment whereas asunaprevir-resistant substitutions were no longer detectable. Overall, 5/10 patients with baseline NS5A-Y93H experienced virologic failure while 5/10 achieved a sustained virologic response.

Conclusions: The potential association of a pre-existing NS5A-Y93H polymorphism with virologic failure on daclatasvir/asunaprevir combination treatment will be examined in larger studies. The persistence of treatment-emergent daclatasvir- and asunaprevir-resistant substitutions will require assessment in longer-term follow-up studies.

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Introduction

The introduction of direct-acting antivirals (DAA) targeting hepatitis C virus (HCV) NS3 protease activity has substantially increased sustained virologic response (SVR) in chronic HCV genotype 1 (GT1) infection. In combination with peginterferon-alfa and ribavirin (alfa/RBV), treatment with the recently approved protease inhibitors boceprevir or telaprevir results in SVR rates of around 70–75% in treatment-naïve patients [1, 2]. Despite these improvements, SVR rates vary by genotype and remain suboptimal in some patients, such as null-responders to alfa/RBV [3], and patients for whom alfa/RBV is poorly tolerated or medically contraindicated. Furthermore, alfa/RBV is associated with frequent side effects [3], and the addition of these DAAs results in elevated rates of anemia and additional events such as dysgeusia (boceprevir), or rash, pruritis, and nausea (telaprevir) [4, 5].

Daclatasvir (DCV) and asunaprevir (ASV) are currently undergoing clinical development for HCV infection. DCV (BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad HCV genotypic coverage [6] that has demonstrated antiviral efficacy and good tolerability in combination with alfa/RBV [7]. ASV (BMS-650032) is a selective inhibitor of NS3 protease with antiviral activity *in vitro* against GT1 and GT4 [8]; it has also been shown to be efficacious and generally well tolerated in combination with alfa/RBV [9]. Clinical interest is increasingly focusing on exploring DAA-only regimens without alfa/RBV, whose potential benefits might include better tolerability and compliance, and a reduced duration of therapy. One recent alfa/RBV-sparing study of DCV plus ASV (AI447017) has examined the efficacy and safety of this combination for 24 weeks in a small cohort of ten GT1b null-responders, in which an SVR rate of 90% was

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observed [10]. The study was then expanded to include an additional cohort of null-responders and a group of patients ineligible to receive, or intolerant of, alfa/RBV [11].

As with other antiviral agents, the efficacy of DCV and ASV can be compromised by the development of drug resistance. *In vitro* data suggest that DCV and ASV should provide additive or synergistic activity that enhances the genetic barrier to resistance [8]. Here we characterize virologic escape observed on DCV plus ASV treatment in the expanded AI447017 study [11], its associations with baseline characteristics including *IL28B* genotype and HCV polymorphisms, and an assessment of on- and off-treatment genotypic changes in NS5A and NS3 protease and their phenotypic consequences.

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Patients and methods

Study design and patients

This was an open-label, Phase 2a study (AI447017; clinicaltrials.gov identifier NCT01051414) evaluating the antiviral activity and safety of DCV plus ASV in 43 patients with HCV GT1 infection. Patients comprised (a) 21 alfa/RBV null-responders (<2 $\log_{10}$ decline in plasma HCV-RNA after 12 weeks); and (b) 22 patients who discontinued previous alfa/RBV within 12 weeks for intolerance or were considered medically poor candidates for alfa/RBV for reasons such as advanced age, complications of depression, anemia, myelosuppression, diabetes, or cardiovascular or renal dysfunction. Patients enrolled in four cohorts; two each of null-responders and ineligible/intolerant patients. The initial sentinel cohort of null-responders has been described previously [10]. All enrolled patients were infected with GT1b.

Patients received DCV 60mg once-daily with ASV 200mg twice-daily for 24 weeks, with a further 48 weeks' post-treatment follow-up. ASV dosing in the expanded study was reduced from the 600mg twice-daily administration used in the sentinel cohort following reports of hepatic enzyme elevations at this dose in another clinical study [12].

The full study design, including inclusion/exclusion criteria, and safety/efficacy endpoints, is described elsewhere [11]. Briefly, eligible patients were men and women aged 20–75 years with HCV genotype 1 infection ≥6 months and HCV RNA ≥10^5 IU/mL. Patients were excluded if they had evidence of liver cirrhosis within 24 months of screening; a history of

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hepatocellular carcinoma, other chronic liver disease, variceal bleeding, hepatic

encephalopathy, or ascites requiring diuretics or paracentesis; coinfection with hepatitis B

virus or HIV; or other clinically significant medical conditions.

Laboratory assessments

Plasma samples for resistance testing were collected at baseline and study Weeks 1, 2, 4, 6,

8, 10, 12, 16, 20, and 24 and post-treatment weeks 4, 8, 12, 24, 36, and 48. HCV-RNA was

determined at a central laboratory using the Roche COBAS® TaqMan® HCV Auto assay,

(Roche Diagnostics KK, Tokyo, Japan) with a lower limit of quantitation (LLOQ) of 15 IU/mL.

HCV genotype and subtype, and *IL28B* genotype (rs12979860 single-nucleotide

polymorphism) were determined by polymerase chain reaction (PCR) amplification and

sequencing.

Genotypic and phenotypic analysis of clinical samples

Testing was performed on all baseline samples and on samples indicative of slow virologic

response at Week 1 or virologic failure with HCV-RNA levels ≥1000 IU/mL. Virologic failure,

for the purpose of the study, was defined as an HCV-RNA level (a) ≥LLOQ at Week 4 (futility

rule), (b) >1 $\log_{10}$ IU/mL above nadir or ≥LLOQ after confirmed undetectable (virologic

breakthrough), or (c) ≥LLOQ at any follow-up visit after being undetectable at end of

treatment (relapse).

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Population sequencing of PCR amplicons was performed using methods described elsewhere [13-15]. For clonal analysis, amplicons were cloned into the TOPO vector and transformed into TOP10 *Escherichia coli* using a commercially available kit (TOPO® TA-cloning® kit, Invitrogen, Carlsbad, CA) according to manufacturer's instructions, with ≥20 individual colonies expanded and sequenced for each analysis.

Phenotypic analyses of resistance-associated substitutions were performed by employing *in vitro* HCV replicon systems according to previously published methodologies [15-17].

Results

Viral response to DCV and ASV

Overall, plasma HCV-RNA was undetectable in 77% (33/43) of patients at 24 weeks post-treatment. SVR was higher among the null-responders than in the alfa/RBV ineligible population; all viral breakthroughs (n=3) and relapses (n=4) occurred in the ineligible/intolerant subpopulation. Three patients discontinued the study without subsequent SVR or virologic failure (Tables 1 and 2) [11].

Null-responders

Virologic response

Rapid and similar decreases in plasma HCV-RNA levels were observed among patients who initiated treatment with ASV 600mg (Fig. 1A) or ASV 200mg (Fig. 1B). Mean reduction in HCV-RNA at Week 1 was comparable for both groups (−4.4 versus −4.3 $\log_{10}$ IU/mL,

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respectively). Of the patients still receiving treatment (P-6 discontinued at Day 16 due to an

AE), all but one patient (P-13) had HCV-RNA <15 IU/mL at Week 4 and 52% had

undetectable HCV-RNA at this time.

Baseline analysis

Baseline *IL28B* genotype and naturally occurring polymorphisms associated with ASV or DCV

resistance (resistance-associated variants [RAVs]) are shown in Table 1. As anticipated for

this prior null-responder population, the majority (18/21) were non-CC *IL28B*. The NS5A

polymorphism Y93H (24-fold DCV resistance [13]) was observed in three patients. Other

polymorphisms conferring minimal (2- to 3-fold) DCV resistance were detected in two

patients (NS5A-L28M-R30Q and NS5A-L31M). Polymorphisms associated with minimal to

low-level resistance to select NS3 protease inhibitors (one patient, NS3-T54S-Q80L; one

patient, NS3-Q80L-V170I/M; two patients, NS3-Q80L) [4, 5, 18] were also observed.

Baseline polymorphisms and *IL28B* genotype did not appear to influence either the Week 1

response or SVR rate (Fig. 2A). Five patients had RNA levels ≥1000 IU/mL after 1 week, of

whom one (P-21) had significantly slower initial HCV-RNA declines when compared with

mean reductions (standard deviation [SD]) in HCV-RNA for null-responders on the study (−

3.4 versus -4.35 ± 0.49 $\log_{10}$ IU/mL). This patient had a CC *IL28B* genotype and an NS5A

polymorphism (Q54L; no fold-change in DCV resistance). The other four patients had

polymorphisms that have been associated with DCV and NS3 protease inhibitor low-level

resistance [13, 19]—specifically NS5A-Q54H/Q-Q62Q/E-Y93H/Y with NS3-T54S-Q80L (P-1,

no fold-change to DCV/ASV), NS3-Q80L-V170I/M (P-2, no fold-change to ASV), NS5A-R30Q

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with NS3-S122G (P-20, no fold-change to either DCV/ASV), or NS5A-Q54H (P-13, no fold-change to DCV). P-13 was the only patient with HCV-RNA <15 IU/mL (target detectable) at Week 6 and was, therefore, considered a treatment failure. Treatment-emergent resistance at Week 1 in the five patients could not be determined because of PCR failure. A comparison of initial virologic response versus dose and polymorphisms associated with resistance revealed no differences. Among null-responders who received ASV 600mg, mean HCV-RNA declines at Week 1 for those with versus without RAVs were -4.6 versus -4.3 $\log_{10}$ IU/mL, which were similar to the Week 1 declines among those who received ASV 200mg (-4.5 $\log_{10}$ IU/mL with RAVs (one patient) versus -4.3 $\log_{10}$).

Baseline HCV-RNA levels did not impact response to treatment; patients with high baseline viral load still experienced rapid and robust responses to therapy (Fig. 1; Table 1).

Ineligible/intolerant patients

Virologic response

Virologic response at Week 4 was greater in alfa/RBV ineligible patients than null-responders. Undetectable HCV-RNA at Week 4 was observed in 86% of the ineligible group versus 52% of null-responders. However, by Week 12, undetectable HCV-RNA was similar in both groups. Early HCV-RNA declines appeared unaffected by *IL28B* genotype, the presence of baseline polymorphisms associated with resistance, or virologic outcome (Fig. 3). Adherence to therapy, assessed through pill counts, was found to be high in six of the seven patients experiencing virologic failure. However, DCV/ASV exposures were high in the one non-compliant patient (P-31) who subsequently experienced relapse.

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Baseline analysis

Baseline *IL28B* genotype, polymorphisms associated with resistance, and virologic outcome are shown in Table 2 and Fig. 2B. Three patients presented with DCV resistance at baseline: one (P-25) with an NS5A-L31M-Y93H combination (7,105-fold DCV resistance [13]), and two with an NS5A-Q54Y-Y93H (58-fold resistance). All three subsequently experienced viral breakthrough at Week 10 or 16.

Other patients had baseline polymorphisms conferring minimal or low-level resistance to DCV and/or protease inhibitors; NS5A-Y93H (n=4), NS5A-L28M-R30L (n=1), NS3-T54S (n=1), and NS3-Q80L (n=5). Variable responses were observed among these patients (Fig. 2B); the majority responded, but two patients with baseline NS5A-Y93H experienced post-treatment relapse. One patient (P-24) with baseline NS5A-L28M-R30L-Q54H-A92T and NS3-Q80L-S122G had a slower response to treatment at Week 1 when compared with mean HCV-RNA reductions (SD) for ineligible/intolerant patients on the study (-3.4 versus -4.74 [0.58] $\log_{10}$ IU/mL) but subsequently achieved SVR with only 16 weeks' treatment. Neither NS3-Q80L-S122G nor NS5A-L28M-R30L-Q54H-A92T conferred resistance to ASV or DCV, respectively.

Baseline viral load did not appear to affect response; mean HCV-RNA levels (SD) were 6.4 (0.7) $\log_{10}$ IU/mL among patients achieving SVR compared with 6.8 (0.3) $\log_{10}$ IU/mL among patients experiencing virologic failure. However, four of six patients with the *IL28B* CT allele subsequently failed treatment (three breakthroughs, one relapse) versus only three of 16 patients with *IL28B* CC (all relapsed).

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Genotypic analysis of patients with viral breakthrough

Treatment-emergent RAVs were assessed through post-treatment Week 48 in the three

patients with virologic breakthrough (Table 3).

Patient P-25: This patient was *IL28B* CT genotype with baseline HCV-RNA 6.8 $\log_{10}$ IU/mL and

a linked baseline NS5A-L31M-Y93H/Y polymorphism. Despite undetectable HCV-RNA by

Week 4 (Fig. 4A), viral breakthrough occurred at Week 16, associated with high-level

resistance to both DCV (NS5A-L31M-P58A-Y93H; 65,000-fold) and ASV (D168A; ~120-fold in

GT1b). Other minor variants detected at baseline by clonal analysis (NS5A-Q62R, -A92T)

were not present at breakthrough. NS5A variants present at end of therapy persisted

through follow-up Week 48, and although P58A had largely changed to P58G (73% of 33

clones, Fig. 5A) by Week 36, a similar ratio of P58G to A was detected at follow-up Week 48.

By contrast, NS3-D168A had mostly been replaced by wild-type at Week 48 (83% of 64

clones).

Patient P-29: This patient was *IL28B* CT genotype, with baseline HCV-RNA 6.7 $\log_{10}$ IU/mL

and a pre-existing linked NS5A-Q54Y-Y93H/Y (Fig. 5B) and NS3-Q80L. Undetectable HCV-

RNA by Week 3 was followed by viral breakthrough at Week 16 (Fig. 4A) associated with

NS5A-L31M-Q54Y-Y93H (6,467-fold DCV resistance) and NS3-Q80L-D168V (~280-fold ASV

resistance). These RAVs remained stable through 48 weeks post-treatment.

Patient P-43: This patient was *IL28B* CT genotype with baseline HCV-RNA 7.0 $\log_{10}$ IU/mL,

and a pre-existing NS5A-Q54Y-Y93H variant (Fig. 5C). HCV-RNA was undetectable at Week 2

and breakthrough occurred at Week 10 (Fig. 4A), associated with a linked NS5A-L31M-Q54Y-

Y93H variant (Fig. 5C; 6,467-fold DCV resistance) and a NS3-D168V variant (~270-fold ASV

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resistance). Again, NS5A variants remained stable through Week 48 post-treatment while NS3-D168V was replaced by wild-type (100% of 60 clones).

For the three patients experiencing viral breakthrough, DCV and ASV trough exposures were less than drug levels required to achieve a 90% effective concentration (EC_{90}) value against emergent RAVs (Table 3).

Genotypic analysis of patients experiencing post-treatment relapse

Four ineligible patients with undetectable HCV-RNA at end of treatment experienced relapse (Fig. 4B). Resistance polymorphisms through Week 48 off-treatment are shown in Table 3. Baseline polymorphisms associated with resistance were not detected in two patients (P-32 and P-36), but both displayed post-relapse resistance by follow-up Weeks 8 and 4, respectively. Patient P-32 relapsed with NS5A-L31M-P58L-Y93H (8,300-fold DCV resistance) and NS3-D168V (270-fold ASV resistance). Patient P-36 relapsed with a NS5A-L31V/M-Y93H genotype (L31V-Y93H: 14,789-fold DCV resistance versus L31M-Y93H: 7,105-fold) [13] and NS3-D168V. The remaining two patients had detectable NS5A-Y93H at baseline (24-fold DCV resistance) and additional substitutions at NS5A-L31 and NS3-D168 were detected after relapse. Patient P-31 displayed NS5A-L31M-Y93H (7,105-fold DCV resistance) [13] and NS3-D168A (~120-fold ASV resistance); patient P-37 relapsed with the same NS5A-L31V/M-Y93H and NS3-D168V, as described for patient P-36.

Baseline HCV-RNA and *IL28B* genotype did not appear to influence relapse; three of four relapse patients were *IL28B* CC genotype and baseline HCV-RNA was not appreciably higher

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