Table 5. Prevalence of anti-HCV drug resistant mutations among the treatment-naïve patients.

Residue and Position	Drugs	Number of patients with mutated clones (%)	Frequency of the mutated clones (%)*
Resistant mutation to NS3/4A protease inhibitor			
T54S/A	Telaprevir Boceprevir	20/27 (74.1%)	0.49 (0.21–86.9)
V55A	Boceprevir	16/27 (59.3%)	0.4 (0.23–1.53)
Q80R/K	TMC435350	16/27 (59.3%)	0.36 (0.24–1.37)
V36A/M	Telaprevir Boceprevir	12/27 (44.4%)	0.47 (0.20–0.88)
V170A/T	Boceprevir	11/27 (40.7%)	0.52 (0.20–1.03)
A156T/V	Telaprevir	7/27 (25.9%)	0.35 (0.20–0.80)
R155K/T/Q	Telaprevir Boceprevir ITMN191/R7227 MK-7009 TMC435350 BI-201335	5/27 (18.5%)	0.42 (0.22–0.62)
A156S	Telaprevir Boceprevir	3/27 (11.15)	0.35 (0.24–0.83)
D168A/V/T/H	ITMN191/R7227 MK-7009 TMC435350 BI-201335	0/27 (0%)	
Resistant mutation to NS5B polymerase inhibitor			
V499A	BI-207127	20/27 (74.1%)	0.59 (0.20–99.1)
M423T/I/V	Filibuvir	12/27 (44.4%)	0.41 (0.21–1.48)
P495S/L/A/T	BI-207127	9/27 (33.3%)	0.37 (0.21–0.87)
P496A/S	BI-207127	1/27 (3.7%)	0.32
S282T	R7128	0/27 (0%)	

* Values are median (range).

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strated that IFN treatment resulted in no selective decrease of the viral clones comprising the previously defined mutational changes that were associated with a response to anti-viral therapy. Moreover, immediate virologic responders showed no common baseline nucleotide alterations that are efficiently eliminated in response to the administration of peg-IFNα2b plus RBV. Thus, our data suggest that an HCV sequence variation itself at a specific single nucleotide position does not directly reflect the virologic features regarding the sensitivity to IFN therapy in each viral clone, at least at the early stage of IFN administration. In contrast, several studies have provided evidence of the pre-existence of viral strains with an inherent resistance to IFN in patients who subsequently experienced a viral breakthrough or relapse [24,28]. Thus, there is room for further investigation to identify IFN-resistant clones by comparing the viral clones at baseline with those at the point of relapse using ultra-deep sequencing technology.

Notably, a distinct pattern of dynamic changes of HCV quasispecies was present between immediate responders and non-responders. Immediate responders showed a significant decrease of genetic complexity spanning all the viral genetic regions, resulting in a more homogeneous viral population after 1 week of peg-IFNα2b plus RBV administration. In contrast, non-responders showed no significant change in the genetic complexity in any of the HCV genomic regions. Our findings are consistent with the previous study showing that the early changes in HCV quasispecies determined by E1/E2 sequences provided prognostic information as early as the first 2 weeks after starting IFN therapy [28]. Moreover, the findings that there is no difference in the level of genetic complexity between early responders and non-responders at baseline and that almost none of the pre-existed HCV clones were eliminated in non-responder cases might suggest that the absence of sensitivity to IFN treatment in non-responders is due to host factors. Consistent with this hypothesis, recent studies revealed that host genetic variations at the IL28B gene are associated with a virologic response to peg-IFNα plus RBV combination therapy [29-32]. Alternatively, it is possible that a particular HCV protein of certain HCV mutants contributed to the strong inhibition of IFN-mediated anti-viral response in the liver of non-responders. Although dynamic changes in HVR1 sequences revealed that the minor viral clones were promptly eliminated in immediate virologic responders, the originally-inhabited major viral clones persisted 1 week after peg-IFNα2b plus RBV administration. Thus, further analyses are required to clarify how viral heterogeneity might be associated with the response to anti-viral therapy.

DAAs are promising drugs that could be more effective than peg-IFNα plus RBV therapy [33]. These DAAs include HCV NS3/4A protease and NS5B RNA-dependent RNA polymerase inhibitors, both of which have currently advanced to phase 1-3 trials. Increasing evidence, however, has clearly revealed that monotherapy with DAAs poses a high risk for the selection of resistant variants because of the high genetic heterogeneity of HCV [20]. Several studies reported the low prevalence of DAAs resistant mutants as the dominant clones in treatment-naïve cases [21,34-36]. For example, Kuntzen et al showed that drug-resistant mutations were detectable by conventional sequencing at individual frequencies between 0.3% and 2.8% in a treatment-naïve genotype 1 HCV-infected population [21]. In sharp contrast, ultra-deep sequencing identified that DAAs-resistant variants are common among treatment-naïve patients. Indeed, ultra-deep sequencing showed that 26 of 27 (96%) treatment-naive Japanese patients enrolled in this study possessed at least two clones resistant to DAAs, while 70.2% of the mutants presented as a very minor population (less than 1%) in each individual. It remains unclear whether these minor drug-resistant mutations have clinical significance, because the DAAs are not yet approved here in Japan. Recent *in vitro* findings, however, showed that minor but preexisting resistant mutants in HCV replicon cells were selected and expanded after DAAs therapy [37]. Lu et al revealed

that M414T mutants preexisting at a frequency of 0.22% and 0.18% in the treatment-naïve replicon population rapidly increased upon treatment with DAAs in a dose-dependent manner, reaching frequencies of 25% and 60% after 4 days of treatment. These findings suggest that those preexisting minor mutants might cause resistance against DAAs through the selection of dominant mutations. Thus, the significance of low-abundance variants in treatment-naïve patients requires further exploration.

The present study raises two limitations of ultra-deep parallel sequencing technology in the analyses of viral quasispecies. First, because the massive parallel ultra-deep sequencing platform is based on multitudinous short reads, it is difficult to separately evaluate the association between nucleotide sites mapped to different viral genome regions in a single viral clone. Indeed, it is difficult to clarify the potential mutational linkage between different viral genomic regions because of the short read length of the shotgun sequencing approach. Second, it is difficult to accurately analyze highly polymorphic regions such as the HVR by ultra-deep sequencing, because mutation findings strongly depend on mapping to the reference genome sequences. Thus, utilization of both conventional and ultra-deep sequencing technology might be necessary to fully clarify the significance and clinical relevance of the prominent HCV genomic heterogeneity.

In summary, using ultra-deep sequencing technology, we clearly demonstrated the extremely large genetic complexity in the genotype1b HCV derived from chronically infected patients. Although there was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at baseline, immediate virologic responders, but not non-responders, showed a rapid reduction in the viral sequence variability at an early phase of peg-IFNα2b plus RBV administration. We also showed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, indicating a putative risk for the expansion of resistant clones to DAAs. Further studies with a large number of patients are needed to fully elucidate the significance of viral heterogeneity in the clinical outcome of patients receiving anti-viral therapy.

Materials and Methods

Patients

The participants comprised 27 Japanese adult chronic hepatitis patients with genotype 1b HCV infection and the mean baseline level of serum HCV RNA determined by TaqMan RT-PCR (Applied Biosystems, Foster City, CA) was 6.9 log IU/ml. All patients received conventional peg-IFNα2b plus RBV combination therapy (Schering-Plough, Kenilworth, NJ) at Kyoto University and affiliated hospitals from February 2007 to December 2008. Indications for IFN-based combination therapy included high serum values of alanine aminotransferase and positivity for serum HCV RNA. Patients were treated with peg-IFNα2b (1.5 μg/kg) once per week, combined with daily oral RBV for 48 weeks [38]. The RBV dose was 600 mg/day in patients weighing less than 60 kg, 800 mg/day in those weighing at least 60 kg but less than 80 kg, and 1000 mg/day in those weighing 80 kg or more.

In this study, immediate virologic responders were defined as patients whose serum HCV RNA levels declined by more than 2 log IU/mL after 1 week of treatment with peg-IFNα2b plus RBV, while non-responders were defined as those whose serum HCV RNA levels declined less than 2 log IU/mL after peg-IFNα2b plus RBV administration. Of the original 27 patients, the serum before and 1 week after initiating treatment with peg-IFNα2b plus RBV of 16 cases was available for further analyses, and 8 of these cases were defined as immediate virologic responders and 8 cases were defined as non-responders. Among these non-responder cases, the serum HCV RNA levels in 6 of 8 (75.0%) patients changed by less than 1 log IU/mL after 1 week of treatment. The decline in HCV RNA levels in the remaining 2 cases was slightly over 1 log IU/mL (1.2 and 1.4 log IU/mL).

The ethics committee at Kyoto University approved the studies, and written informed consent for participation in this study was obtained from all patients.

Direct population Sanger sequencing

To define the representative reference sequences of full-length HCV in each clinical specimen, all samples were first subjected to direct population Sanger sequencing using Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) [39]. Serum samples were obtained before the start and at 1 week after initiation of peg-IFNα2b and RBV combination therapy. Total RNA was extracted from 140 μL of serum using a QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and reverse-transcribed in a volume of 20 μL with the One step RNA PCR Kit AMV (Takara Bio, Ohtsu, Japan).

HCV genomes were amplified using Phusion High-Fidelity DNA polymerase (FINZYMES, Espoo, Finland). Oligonucleotide primers were designed to amplify the first-half (~5,000 bps) and the latter-half (~4,500 bps) of the genotype 1b HCV genome sequences (Table S3).

PCR products purified by the QIAquick Gel Extraction kit (Qiagen) were assayed for direct sequencing [40]. Nucleotide sequences of PCR products were determined using an ABI Prism Big Dye Terminator Ready Reaction Kit (Applied Biosystems). The serum of a healthy volunteer was used as a negative control.

Massive-parallel ultra-deep sequencing

Paired-end sequencing with multiplexed tags was carried out using the Illumina Genome Analyzer II. End-repair of DNA fragments, addition of adenine to the 3′ ends of DNA fragments, adaptor ligation, and PCR amplification by Illumina-paired end PCR primers were performed as described previously [41].

Briefly, the viral genome sequences were amplified with high-fidelity PCR and sheared by nebulization using 32 psi N2 for 8 min and the sheared fragments were purified and concentrated using QIAquick PCR purification Kit (Qiagen). The overhangs resulting from fragmentation were then converted into blunt ends using T4 DNA polymerase and Klenow enzymes, followed by the addition of terminal 3′ adenine-residues. Next, one of the adaptors containing six unique base pair (bp) tags, such as "ATCACG" and "CGATGT" (Multiplexing Sample Preparation Oligonucleotide Kit, Illumina), was ligated to each fragment using DNA ligase. Adaptor-ligated DNAs in the range of 200 to 350 bp were then size-selected by agarose gel electrophoresis. These libraries were amplified independently using a minimal PCR amplification step of 18 cycles with Phusion High-Fidelity DNA polymerase and then purified using a QIAquick PCR purification Kit for a downstream assay. Cluster generation and sequencing was performed for 64 cycles on the Illumina Genome Analyzer II following the manufacturer's instructions. Obtained images were analyzed and base-called using GA pipeline software version 1.4 with default settings provided by Illumina.

Genome Analyzer sequence data analysis

Using the high performance alignment software "NextGene" (SoftGenetics, State College, PA), the 64 base tags obtained from the Genome Analyzer II reads were aligned to the reference HCV RNA sequences of ~9200 bp that were determined by direct population Sanger sequencing in each clinical specimen. Entire reads were removed from the analysis when the median quality value score was below 20 and when containing more than 3 uncalled nucleotides. The low quality bases were trimmed from reads when more than 3 consecutive bases fell below a quality value score of 16. Based on the above criteria, reads with 90% or more bases matching a particular position of the reference sequence were aligned. Each position of the viral genome was assigned a coverage depth, representing the number of times the nucleotide position was sequenced.

Statistical analysis

Results are expressed as mean or median values and range (minimum and maximum). Pretreatment values were compared using the Mann–Whitney U-test. Categorical variables were analyzed by Fisher's exact test. P values of less than 0.05 were considered statistically significant. The viral quasispecies nature was evaluated by analyzing the genetic complexity based on the number of different sequences present in the population. Genetic complexity was determined by Shannon entropy values calculated as follows:

$$Sn = -\frac{\sum_{i=1}^{n} fi(\ln fi)}{N}$$

where n is the number of different species identified, fi is the observed frequency of the particular variant in the quasispecies, and N is the total number of clones analyzed [23,42]. Statistical comparisons of complexity between two groups were made using the Wilcoxon rank sum test or the Mann–Whitney U-test.

Supporting Information

Figure S1 Relationship between serum HCV RNA levels and the number of resistant mutant. No correlation was observed between serum HCV RNA levels (log IU/ml) and the number of resistant mutations against direct-acting antivirals in 27 cases in this study.
(TIF)

Table S1 Aligned reads, nucleotides, and mean coverage of each reference sequence in all patients.
(DOC)

Table S2 Mean genetic complexity in each viral genomic region of the 8 immediate virologic responders and 8 non-responders at pre-treatment and 1 week after IFN therapy.
(DOC)

Table S3 The oligonucleotide primers for PCR amplifying the whole HCV sequences.
(DOC)

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Author Contributions

Conceived and designed the experiments: AN HM. Performed the experiments: AN HM. Analyzed the data: AN HM NN TF FS KS TC YU. Contributed reagents/materials/analysis tools: AN HM YO YY TI TT. Wrote the paper: AN HM KT TC.

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Hepatology Research

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Case Report

Two patients treated with pegylated interferon/ribavirin/telaprevir triple therapy for recurrent hepatitis C after living donor liver transplantation

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It is difficult to use protease inhibitors in patients with recurrent hepatitis C virus (HCV) infection after liver transplantation (LT) due to interaction with immunosuppressive drugs. We report our experience with two patients treated with telaprevir (TVR) combined with pegylated interferon/ribavirin (PEG IFN/RBV) for recurrent HCV genotype 1 infection after LT. The first was a 63-year-old man with HCV-related liver cirrhosis, who failed to respond to IFN-β plus RBV after LT. Treatment was switched to PEG IFN-α-2b plus RBV and TVR was started. The donor had TT genotype of interleukin (IL)-28 single nucleotide polymorphisms (SNP) (rs8099917). The recipient had TT genotype of IL-28 SNP (rs8099917). Completion of 12-week triple therapy was followed by PEG IFN-α-2b plus RBV for 36 weeks. Finally, he had sustained viral response. The second was a 70-year-old woman with HCV-related liver cirrhosis and hepatocellular carcinoma. She failed to respond to PEG IFN-α-2b plus RBV after LT, and was subsequently switched to PEG IFN-α-2b/RBV/TVR. Genotype analysis showed TG genotype of IL-28 SNP for the donor, and TT genotype of IL-28 SNP for the recipient. Serum HCV RNA titer decreased below the detection limit at 5 weeks. However, triple therapy was withdrawn at 11 weeks due to general fatigue, which resulted in HCV RNA rebound 4 weeks later. Both patients were treated with cyclosporin, starting with a small dose to avoid interactions with TVR. TVR is a potentially suitable agent for LT recipients who do not respond to PEG IFN-α-2b plus RBV after LT.

Key words: hepatitis C virus, liver transplantation, telaprevir

INTRODUCTION

THE HEPATITIS C virus (HCV) has infected 170 million people worldwide, which progresses in some patients to liver cirrhosis and/or hepatocellular carcinoma (HCC).[1] The current treatment for patients infected with HCV genotype 1 is the combination of pegylated interferon-α and ribavirin (PEG IFN/RBV) for 48 weeks.[2] However, this treatment produces sustained viral response (SVR) in only approximately 50% of patients with genotype 1 HCV infection. In 2011, the first direct-acting antiviral agent (DAA) for the treatment of HCV genotype 1, telaprevir (TVR), was approved and treatment with this agent improved SVR to approximately 70–80% of patients with genotype 1 HCV infection.[3,4]

Recurrence of HCV infection after liver transplantation (LT) is one of the major causes of morbidity and allograft loss after LT.[5,6] Because the outcome of post-LT therapy with the classic antiviral agents PEG IFN/RBV are at most moderate with respect to SVR, LT patients constitute one of the classic difficult-to-treat groups.[7–9] The newly introduced triple therapy of protease inhibitors (PEG IFN/RBV/TVR) offers promising perspectives

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for the management of LT patients, although TVR is not yet approved for use in LT patients.

Although there is urgent need for effective treatment of HCV recurrence after LT, significant concern has been expressed about the safety and efficacy of HCV protease inhibitors in this setting because of the side-effect profile and the potential for drug–drug interactions with immunosuppressive agents.[10] Both cyclosporin and tacrolimus are substrates of cytochrome P450 3A and P-glycoprotein. Thus, co-administration of TVR, a potent cytochrome P450 3A4 substrate and inhibitor with the potential to saturate or inhibit intestinal P-glycoprotein, substantially increases the blood levels of cyclosporin and tacrolimus.[11] Consequently, the blood concentration of tacrolimus increased 78-fold, and that of cyclosporin increased fourfold by interaction with TVR.[11] In their recent pilot study, Werner *et al.*[10] described the response to 12-week treatment with TVR plus tacrolimus, cyclosporin or sirolimus in nine patients. Pungpapong *et al.*[12] also reported the preliminary data of 35 patients treated with TVR plus cyclosporin and those of another group of 25 patients treated with boceprevir. Here, we report our preliminary data on protease inhibitors used in combination with PEG IFN/RBV for the treatment of recurrent HCV genotype 1 infection after LT.

CASE REPORT

Case 1

THIS PATIENT WAS a 63-year-old man with HCV-related liver cirrhosis. Living donor LT (LDLT) was performed after obtaining informed consent at May 2009. In August 2009, the patient was started on IFN-β (600 μg) plus RBV (200 mg) due to depression. Because serum HCV RNA titer never fell below the detection limit (1.2 log IU/mL) over the 48-month treatment period, tacrolimus was switched to cyclosporin. In April 2012, treatment was changed to PEG IFN-α-2b (100 μg) plus RBV (200 mg, due to anemia) and TVR (1500 mg) because of depression. At the start of triple therapy, the platelet count was 24.6×10^4/μL, alanine aminotransferase (ALT) was 45 IU/L, genotype was 1b and HCV RNA was 6.8 log IU/mL. Further analysis showed six amino acid (a.a.) substitutions in interferon sensitivity-determining region (ISDR), and mutant- and wild-type amino acids at a.a.70 and a.a.91 in the core region, respectively. The donor had TT genotype of IL-28 single nucleotide polymorphisms (SNP) (rs8099917) and TT/TT genotype of λ4 (ss469415590). The recipient had TT genotype of interleukin (IL)-28 SNP (rs8099917) and TT/TT genotype of λ4 (ss469415590) (Table 1, Fig. 1). Cyclosporin was started at 10 mg/day after triple

Table 1 Laboratory data of patient 1 at start of triple therapy after LT

CBC		LDH	219 IU/L	Tumor marker	
WBC	4630/μL	ALP	357 IU/L	AFP	4.8 ng/mL
RBC	4.01×10^6/μL	γ-GT	20 IU/L		
Hb	12.4 g/dL	TP	7.3 g/dL	HCV virus markers	
Ht	37.8%	Alb	4.0 g/dL	HCV RNA	6.8 KIU/mL
Plt	24.6×10^4/μL	TC	164 mg/dL	Genotype	1b
		TTT	12 U		
Blood coagulation test		ZTT	15 U		
PT	120%	BUN	24.6 mg/dl	a.a. substitution in ISDR	6
		Cr	1.07 mg/dl	a.a.70 in the core region	Mutant
Blood chemistry		CRP	0.10 mg/dl	a.a.91 in the core region	Wild
T-Bil	0.5 mg/dL	NH_3	32 μg/mL	*IL-28B* donor	TT genotype
AST	30 IU/L			*IL-28B* recipient	TT genotype
ALT	45 IU/L			ss469415590 donor	TT/TT genotype
FBS	98 mg/dL			ss469415590 recipient	TT/TT genotype
HbA1c	5.5%			AUC of telaprevir	103 μgh/mL

γ-GT, γ-glutamyltransferase; a.a. substitution in ISDR, amino acid substitutions in the interferon sensitivity-determining region; AFP, α-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under curve; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; CRP, C-reactive protein; FBS, fasting blood sugar; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ht, hematocrit; LDH, lactate dehydrogenase; LT, liver transplantation; RBC, red blood cells; Plt, platelets; PT, prothrombin time; T-Bil, total bilirubin; TC, total cholesterol; TP, total protein; TTT, thymol turbidity test; WBC, white blood cells; ZTT, zinc sulfate turbidity test.

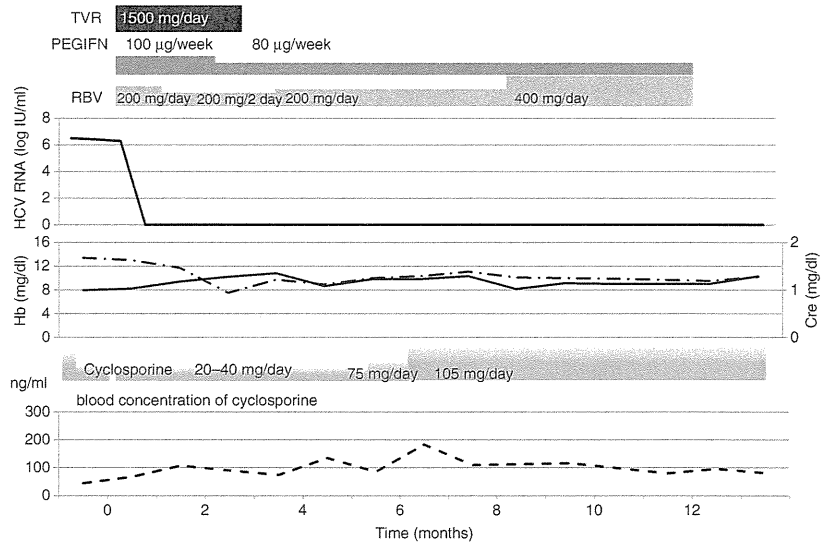

Figure 1 Clinical course of patient 1. Cre, creatinine; Hb, hemoglobin; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir. ——, Cre; — ·, Hb.

therapy, but subsequently increased (based on measurement of its level in the peripheral blood during follow up) to 105 mg/day. The area under the curve (AUC) of TVR was 103 μgh/mL. Serum HCV RNA titer fell below the detection limit (1.2 log IU/mL) at 2 weeks after triple therapy. After 12-week triple therapy, PEG IFN-α-2b and RBV were continued for 36 weeks until April 2013. Finally, he achieved SVR.

Case 2

The patient was a 70-year-old woman with HCV-related liver cirrhosis and HCC. LDLT was performed in May 2006 after obtaining informed consent. Postoperatively, the patient was treated with PEG IFN-α-2b (80 μg) plus RBV (200 mg, due to anemia), which commenced in August 2006. Because serum HCV RNA titer never decreased below the detection limit (1.2 log IU/mL) in the subsequent 48 months, tacrolimus was changed to cyclosporin, and PEG IFN-α-2b plus RBV was changed to the combination of PEG IFN-α-2b (100 μg), RBV (200 mg, due to anemia) and TVR (1500 mg). At the start of triple therapy, platelet count was $19.8 \times 10^4/\mu L$, ALT was 15 IU/L, genotype was 1b, and HCV RNA was 6.2 log IU/mL. Further analysis showed no a.a. substitutions in the ISDR, but mutant- and wild-type a.a. at a.a.70 and a.a.91 in the core region, respectively were detected. The donor had TG genotype of IL-28 SNP (rs8099917) and TT/ΔG genotype of λ4 (ss469415590), while the recipient had TT genotype of IL-28 SNP (rs8099917) and TT/TT genotype of λ4 (ss469415590) (Table 2, Fig. 2). Cyclosporin was started at 10 mg/day, and based on measurement of its concentration in peripheral blood, the dose was increased gradually to 40 mg/day. Subsequent analysis showed a rise in serum creatinine and uric acid, but parameters improved following transfusion. Skin rashes of grade 2 appeared during the triple therapy, which was successfully treated with steroid cream. On the other hand, serum HCV RNA titer decreased below the detection limit (1.2 log IU/mL) at 5 weeks. However, triple therapy was stopped at 11 weeks due to general fatigue. HCV RNA rebounded 4 weeks later.

DISCUSSION

THE SVR RATE has improved since the introduction of PEG IFN/RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with history of HCV genotype 1 infection is 30–50%.[13–18] 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.

It is often difficult to use protease inhibitors for HCV recipients after LT due to potential interaction with immunosuppressive drugs. We reported here our experience with two patients treated with protease inhibitors combined with PEG IFN/RBV for the treatment of recurrent post-LT hepatitis caused by genotype 1 HCV.

A recent study that examined the effect of TVR on the pharmacokinetics of cyclosporin and tacrolimus

Table 2 Laboratory data of Patient 2 at start of triple therapy after LT

CBC		LDH	241 IU/L	Tumor marker	
WBC	7530/μL	ALP	294 IU/L	AFP	5.6 ng/mL
RBC	4.23×10^6/μL	γ-GT	17 IU/L		
Hb	13.3 g/dL	TP	6.4 g/dL	HCV virus markers	
Ht	39.7%	Alb	3.5 g/dL	HCV RNA	6.2 log IU/mL
Plt	17.8×10^4/μL	TC	219 mg/dL	genotype	1b
		TTT	7 U		
Blood coagulation test		ZTT	12 U		
PT	121%	BUN	12.6 mg/dL	a.a. substitution in ISDR	0
		Cr	0.50 mg/dL	a.a.70 in the core region	Mutant
Blood chemistry		CRP	0.11 mg/dL	a.a.91 in the core region	Wild
T-Bil	0.7 mg/dL	FBS	106 mg/dL	*IL-28B* donor	TG genotype
AST	20 IU/L	HbA1c	6.9%	*IL-28B* recipient	TT genotype
ALT	15 IU/L	NH_3	57 μg/mL	ss469415590 door	TT/⊿G genotype
				ss469415590 recipient	TT/TT genotype

γ-GT, γ-glutamyltransferase; a.a. substitution in ISDR, amino acid substitutions in the interferon sensitivity-determining region; AFP, α-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under curve; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; CRP, C-reactive protein; FBS, fasting blood sugar; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ht, hematocrit; LDH, lactate dehydrogenase; LT, liver transplantation; RBC, red blood cells; Plt, platelets; PT, prothrombin time; T-Bil, total bilirubin; TC, total cholesterol; TP, total protein; TTT, thymol turbidity test; WBC, white blood cells; ZTT, zinc sulfate turbidity test.

reported a 78-fold increase in tacrolimus blood concentration and fourfold rise in cyclosporin blood concentration through interaction with TVR.[11] For this reason, we changed tacrolimus to cyclosporin before triple therapy. We also started cyclosporin using a small dose and checked the blood concentration of cyclosporin on a daily basis. Based on these measures, cyclosporin blood concentration remained at approximately 100 ng/mL. Considered collectively, it is important to change the dose of immunosuppressive drugs and frequently monitor cyclosporin blood concentrations.

It is noteworthy that the blood concentration of TVR also increased by interaction with cyclosporin. The AUC of TVR in patient 1 was 103 μgh/mL, while the AUC of TVR of 10 chronic hepatitis C patients treated with PEG IFN/RBV was 52 μgh/mL in our hospital (data not shown). These findings highlight the need for awareness of the potential side-effects of TVR. In fact, various side-

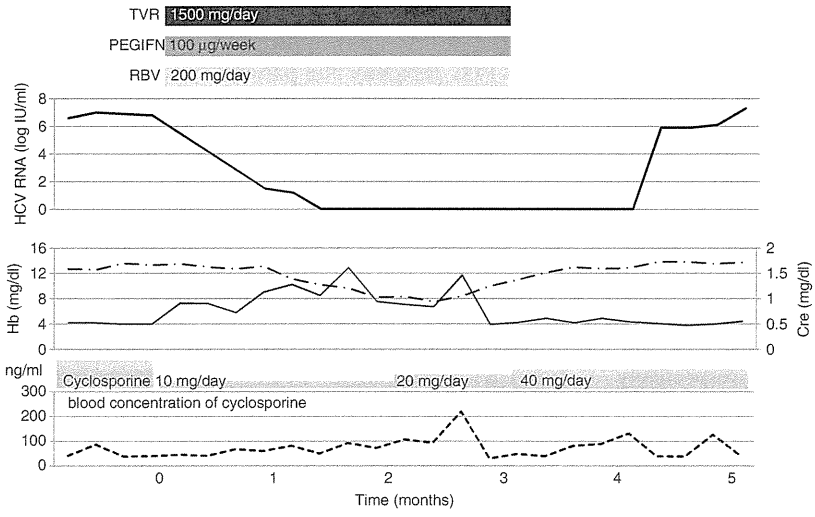

Figure 2 Clinical course of patient 2. Cre, creatinine; Hb, hemoglobin; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir. ——, Cre; — ·, Hb.

effects were reported by patient 2, including anemia, renal dysfunction and skin rashes. Consequently, the triple therapy was discontinued at 11 weeks in this patient.

What are the indications for triple therapy? While there are no standardized rules for the initiation of this mode of treatment, we believe that triply therapy should be used under the following conditions: (i) laboratory tests should show normal hemoglobin and serum creatinine levels to avoid potential side-effects of TVR; and (ii) recipients who develop HCV RNA relapse while receiving PEG IFN/RBV dual therapy after LT. In naïve cases, we recommend PEG IFN/RBV therapy. There are some reports of triple therapy for recipients after LT.[19–21] However, there is no evidence in safety of triple therapy for recipients. Furthermore, Coilly *et al.* recommends PEG IFN/RBV dual therapy for naïve cases in review.[22]

Third, both the donor and recipient must have good SNP (IL28B or λ4). On the other hand, we recommend withholding triple therapy for patients who fail to respond to PEG IFN/RBV and those who have minor SNP (IL28B or λ4) of donor and recipient. In this regard, several groups have reported that *IL28B* of both recipients and donors influenced the SVR to PEG IFN/RBV in patients with recurrent hepatitis C after LT.[23–26]T.[19–22]

Another important question regarding treatment of recurrent post-LT HCV infection is the duration of IFN therapy. The answer to this question is difficult and currently there are no data on the ideal duration of triple therapy. However, we recommend long-term PEG IFN/RBV therapy following triple therapy from 12 to 36 weeks, with a total duration of treatment of 48 weeks. This is based on our previous finding that the majority of patients with genotype 1b in whom HCV RNA reached undetectable levels were able to achieve SVR (87.5%; 7/8).[23] Eradication of HCV by triple therapy should increase the SVR rate. In fact, Pungpapong *et al.* used 12-week triple therapy followed by 36-week PEG IFN/RBV therapy and reported an SVR rate associated with this regimen of 100% (7/7) for genotype 1b recipients.[12]

On the other hand, for such hard-to-treat patients after LT, DAA will become a standard therapy in the future. Because SVR rate and safety of DAA therapy is more higher than triple therapy.[27–29] However, there is a problem of mutation of HCV against DAA therapy.[30,31] In these instances, it may be necessary to recommence triple therapy. The experience of the present study provides a good reference for such an occurrence (e.g. dose of TVR and dose of immunosuppressive agents).

In conclusion, we reported our experience with two patients who developed recurrent HCV genotype 1 infection after LT and were treated with protease inhibitors combined with PEG IFN/RBV. The results point to possible achievement of SVR by triple therapy; however, more studies are needed to evaluate the clinical benefits and side-effects of triple therapy for recurrent post-LT HCV infection.

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Attenuation of Portal Hypertension by Continuous Portal Infusion of PGE1 and Immunologic Impact in Adult-to-Adult Living-Donor Liver Transplantation

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Background. Small-for-size syndrome remains the greatest limiting factor of expanding segmental liver transplantation from living donors. Portal hyperperfusion is considered to substantially contribute to small-for-size syndrome. We investigated the impact of continuous portal infusion of prostaglandin E1 (PGE1) on small-for-size grafts (SFSGs) in adult-to-adult living-donor liver transplantation (LDLT).

Methods. From July 2003 to December 2009, LDLT was performed in 122 patients. We introduced continuous portal infusion of PGE1 to five SFSG patients (PG group) from November 2007 to December 2009 and retrospectively compared them with a historical control group of eight relevant SFSG patients without PGE1 infusion (non-PG group) from July 2003 to October 2007 to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Splenectomy cases were excluded from analysis.

Results. The PG group demonstrated significantly lower postoperative portal pressure than the non-PG group. Moreover, the PG group demonstrated significantly improved liver function in the early posttransplantation period and significantly better recovery from hyperammonemia at 1 week after transplantation and from hyperbilirubinemia in the late posttransplantation period. Overall survival was significantly better in the PG group than in the non-PG group. Three patients in the non-PG group died of rejection-related reasons. Interestingly, immunomonitoring assay revealed that antidonor immune responses were significantly accelerated in the non-PG group compared with the PG group after LDLT. In contrast, the PG group showed well-suppressed antidonor immune responses.

Conclusion. Continuous portal infusion of PGE1 for SFSG attenuated portal hypertension, improved graft function, and suppressed antidonor immune responses, resulting in better survival.

Keywords: Living-donor liver transplantation, Small-for-size graft, Portal hypertension, Alloimmune response, Prostaglandin E1.

(*Transplantation* 2013;95: 1521–1527)

Segmental liver transplantation based on cadaveric splitting or living-donor liver transplantation (LDLT) has been developed for treating patients with end-stage liver disease. It is also a means of overcoming organ shortage and wait-list mortality. However, small-for-size syndrome (SFSS) remains the greatest limiting factor for the expansion of segmental liver transplantation from either cadaveric or living donors (*1, 2*). If the volume of the engrafted liver is considerably less than the standard liver weight in patients with end-stage liver disease who are undergoing partial liver transplantation, excessive portal venous inflow might cause early portal hypertension (*3, 4*) and increased morbidity and mortality due to SFSS (*5*). Previous data have suggested that, in recipients of adult-to-adult LDLT, one of the most challenging tasks is to match a good size graft. Emphasis has more recently been placed not only on the evaluation of the ratio between donor and recipient liver volume but also on the degree of portal hypertension and the stage of liver disease in the recipient, consistent with the result in a pig model (*6–8*). Therefore, the importance of portal pressure during LDLT is now recognized.

We have demonstrated that continuous portal infusion of prostaglandin E1 (PGE1) considerably improved the congestion

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of the residual liver after extended hepatectomy in a rat model (*9*). Based on this result, we applied a continuous portal infusion of PGE1 for small-for-size grafts (SFSGs) in LDLT in the clinical setting.

We here investigated the clinical significance of controlling portal pressure by continuous portal infusion of PGE1 after surgery in LDLT with SFSGs, focusing on portal decompression, postoperative liver function, survival, and the antidonor immune status of the recipient retrospectively.

RESULTS

Patients' Demographic and Clinical Characteristics

Thirteen patients receiving SFSGs were retrospectively analyzed in this study. The patients' demographic and clinical characteristics are shown in Table 1. Of these patients, five received a continuous portal injection of PGE1 after transplantation (PG group) from November 2007 to December 2009 (era 2), whereas eight were historical controls from July 2003 to October 2007 (era 1) without PGE1 infusion (non-PG group). There was no significant difference in age or underlying disease between the two groups. Preoperative examination of the hepatic reserve showed similar Child-Pugh scores (PG group, 10.0±0.71; non-PG group, 9.00±0.83). Patients' model for end-stage liver disease scores, which were used as recipient severity indices, was similar between groups (mean [range], 16.8 [8–30] and 15.1 [9–28], respectively). Portal vein pressure (PVP) at laparotomy was also similar between the two groups (25.2 [17–34] and 20.3 [17–24] mm Hg, respectively). Concerning the graft, one patient in each group showed minimal fatty metamorphosis (<0.1%) on histology and there was no significant difference in graft-to-recipient body weight ratio (GRWR) between the two groups (0.680 [0.63–0.71] and 0.655 [0.51–0.72], respectively).

Furthermore, factors related to surgical invasiveness in those two groups, such as hemorrhage level, operation time, and graft ischemia duration, were similar. No donor had donor-specific antigens, and there was no difference in the number of human leukocyte antigen (HLA) mismatch (Table 1). Three donor candidates in each group underwent liver biopsy. Among them, one in each group showed minimal fatty metamorphosis (<0.1%) on histology. Of note, three of five patients in the PGE1 group and three of eight patients in the non-PGE1 group received right-lobe grafts. All patients receiving right lobes in both groups had grafts with middle hepatic vein (MHV) tributaries more than 5 mm in diameter, and all draining tributaries were reconstructed with the

TABLE 1. Patients' demographic and clinical characteristics

Variables	PG group (n=5)	Non-PG group (n=8)	*P*
Recipient factors			
Age, years	56.4±3.4	57.9±4.4	0.510[a]
Gender, male/female	5/0	3/5	0.075[b]
Child-Pugh score	10.0±1.6	9.0±1.9	0.325[a]
MELD score	16.8±8.2	15.1±5.8	0.702[a]
PVP, mm Hg, at laparotomy	25.2±6.1	20.9±3.0	0.199[a]
Disease background			
Viral hepatitis (B/C)	1/2	1/5	>0.999[b]
Alcoholic	1	1	>0.999[b]
Acute hepatic failure	1	0	0.385[b]
Cholestatic disease	0	1	>0.999[b]
Donor factors			
Age, years	26.2±3.3	33.3±10.5	0.113[a]
Gender, male/female	0/5	5/3	0.075[b]
Graft factors			
Graft type, right/left	3/2	3/5	0.592[b]
GRWR, %	0.68±0.03	0.66±0.09	0.510[a]
Reconstruction of hepatic vein	3	3	0.592[b]
HLA class I mismatch	1.20±0.49	1.63±0.23	0.453[a]
HLA class II mismatch	0.60±0.24	1.00±0.00	—
DSA	0	0	—
Surgical factors			
Operation time, min	781.0±153.6	755.9±106.0	0.758[a]
Bleeding, mL	5322.0±2295.3	5751.4±6371.2	0.866[a]
Total ischemia time, min	117.0±35.5	118.9±31.4	0.925[a]

[a] Unpaired *t* test with Welch's correction.
[b] Fisher's exact test.
DSA, donor-specific antibody; HLA, human leukocyte antigen; GRWR, graft-to-recipient body weight ratio; MELD, model for end-stage liver disease; PVP, portal vein pressure.

recipients' native MHV trunk as reported previously (*10*). There was no thrombosis in those reconstructed tributaries after surgery. One patient of each group had grafts with inferior right hepatic vein, which were reconstructed using direct anastomosis to inferior vena cava in each case.

Continuous PGE1 Infusion Attenuated Portal Hypertension After Reperfusion in SFSGs

After laparotomy, we inserted a catheter from the mesenteric vein to the distal side of the portal vein and measured the PVP during the operation. All patients exhibited portal hypertension during laparotomy. In the PG group, after reflow of the portal and hepatic veins was confirmed, we started PGE1 infusion into the portal vein through a catheter. Continuous infusion of PGE1 resulted in a significant reduction of PVP at the time of abdominal closure in the PG group compared with the non-PG group ($P<0.005$; Fig. 1A). The mean PVP at the time of abdominal closure was 15.4±1.17 mm Hg in the PG group and 20.5±1.47 mm Hg in the non-PG group (Fig. 1A). Furthermore, the PVP ratio at the end of the operation, compared with that at laparotomy, showed effective portal decompression in the PG group and non-PG group, respectively (0.62±0.04 vs. 0.99±0.06; $P<0.001$; Fig. 1B). Importantly, none of the patients in the PG group developed hypoperfusion after PGE1 portal infusion.

Clinical Course of Graft Liver Function

Graft liver function markers, including serum transaminases, arterial ketone body ratio (AKBR), ammonia, and total bilirubin, after surgery were compared between the PG group and the non-PG group.

Elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly attenuated in the PG group compared with the non-PG group on days 1 and 2 (Fig. 2). Similarly, the AKBR, which reflects the hepatic mitochondrial redox state and is considered an accurate index of the functional reserve of the graft liver after transplantation, was significantly higher in the PG group. However, these values became comparable between the two groups after day 3. Strikingly, significantly better recovery from hyperammonemia was seen in the PG group for 1 week after surgery. The serum total bilirubin level was comparable between the two groups by day 28 after LDLT. Nonetheless, hyperbilirubinemia was significantly improved in the PG group after day 28 but remained prolonged in the non-PG group. These results indicate that continuous infusion of PGE1 significantly improved the liver function after LDLT with SFSGs.

Complications and Prognosis

In the PG group, no complications associated with the portal vein catheter were observed after surgery (e.g., post-removal bleeding, catheter infection, or portal thrombosis). One patient in the non-PG group and none in the PG group developed SFSS. Postoperative death occurred in 5 patients of the non-PG group and in none in the PG group. In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In contrast, in the PG group, the 1-and 2-year survival rates were both 100%, a difference that was statistically significant ($P<0.05$; Fig. 3). The main causes of death in the non-PG group were graft dysfunction, rejection, and subsequent infection as well as bacterial sepsis after biliary stenosis. No patients in the PG group had a

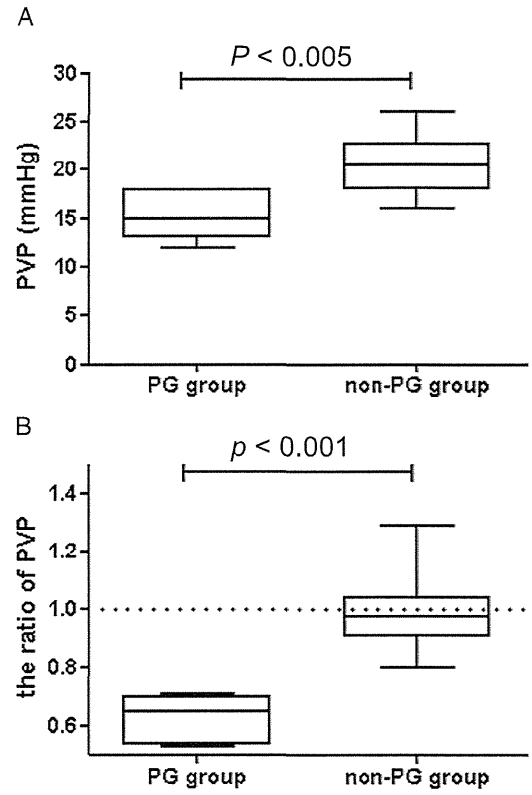

FIGURE 1. PVP value at the end of the operation (A) and ratio of PVP at the end of the operation to that at laparotomy (B) in the PG group and the non-PG group. An unpaired *t* test with Welch's correction was used to compare PVP and the ratio of PVP between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. *$P<0.05$; ***$P<0.001$. PVP, portal vein pressure.

rejection episode. Rejection was diagnosed by liver biopsy and histologic findings showed features of SFSG and/or portal hypertension with rejection (see **Figure S1, SDC**, http://links.lww.com/TP/A807). The 2-year survival of SFSG patients (non-PG group) in era 1 (July 2003 to October 2007) was significantly worse than that of the non-SFSG patients in the same period (37.5% vs. 77.8%; $P<0.05$), whereas the 2-year survival of SFSG patients (PG group) in era 2 (November 2007 to December 2009) was not statistically different from that of the non-SFSG patients in the same period (100% vs. 77.1%). Of note, the 2-year survival of non-SFSG patients was similar between eras 1 and 2 (Fig. 4).

Estimation of Immunosuppressive Status After Surgery by Using the Carboxyfluorescein Diacetate Succinimidyl Ester-Mixed Lymphocyte Reaction Assay

Because the main cause of death in 3 patients in the non-PG group was related to rejection, we retrospectively analyzed the immunosuppressive postoperative status of both groups. All patients and their donors consented to be

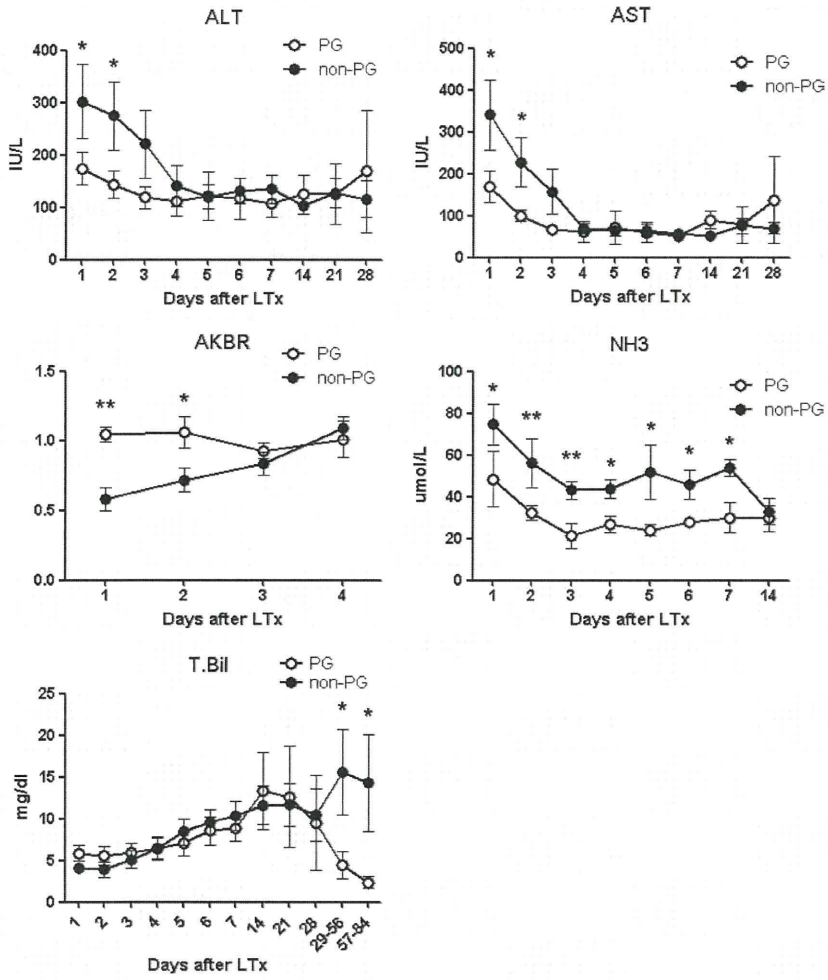

FIGURE 2. Liver function tests (ALT, AST, AKBR, NH_3, and T.Bil) of patients with (PG group; open circle) or without PGE1 portal infusion (non-PG group; closed circle) after LDLT. Data are mean±SEM for individual groups. An unpaired *t* test with Welch's correction was used to compare each of the indicated parameters between the PG group and the non-PG group. **P*<0.05; ****P*<0.01. AKBR, arterial ketone body ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDLT, living-donor liver transplantation; LTx, liver transplantation; NH_3, ammonia; T.Bil, total bilirubin.

subjected to a mixed lymphocyte reaction (MLR) assay with the carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique. In all five patients of the PG group, suppressed $CD8^+$ T-cell proliferation, which is defined as a stimulation index (SI)<2, was observed in the antidonor MLR assay (i.e., a hyporesponse to donor; mean SI, 1.10±0.13; Fig. 4A). The mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells, which are activated cytotoxic T cells, was 9.24±5.93 (Fig. 4B). In contrast, in five of the eight patients in the non-PG group, accelerated $CD8^+$ T-cell proliferation was observed in the antidonor MLR assay (i.e., a hyperresponse to donor; mean SI, 2.85±0.50; Fig. 4A). Furthermore, the mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells was 63.82±8.63 (Fig. 4B). These differences between the two groups were significant. Of note, three patients in the non-PG group who showed high antidonor response (i.e., SI of $CD8^+$ T cells>3) required steroid pulse treatment and died of graft dysfunction or infection after rejection. Two patients who showed a relatively high antidonor response (i.e., SI of $CD8^+$ T cells>2) required an increase in immunosuppressant doses. These results indicated that patients with SFSGs show accelerated antidonor immune responses and that continuous portal infusion of PGE1 suppressed this type of antidonor immune response.

DISCUSSION

Various approaches to controlling excessive portal flow and pressure have been proposed, such as dual grafting to increase graft volume (*11, 12*). Although this concept is simple, it requires two healthy living donors and involves increased risk to donors. Another approach is portal decompression with a portosystemic shunt (*13, 14*) or splenic artery manipulation, including splenectomy, embolization, and ligation (*15–17*). This method is more favored in terms of availability and donor risk. Nonetheless, there is

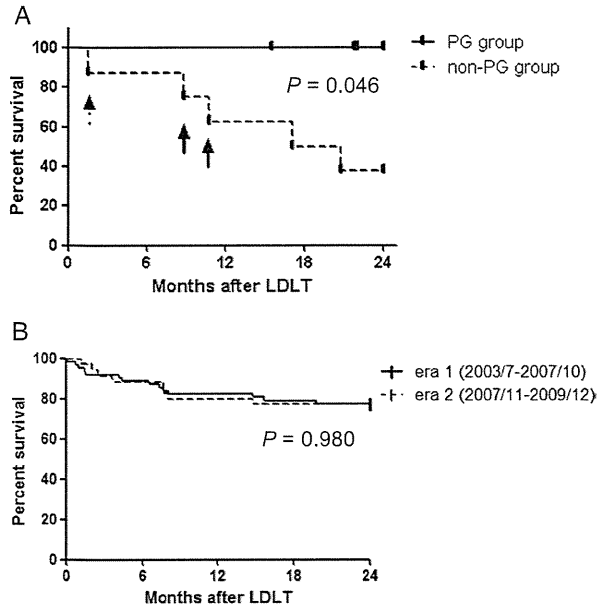

FIGURE 3. A, Kaplan–Meier patient survival curves of patients with (PG group; n=5; solid line) or without PGE1 portal infusion after LDLT (non-PG group; n=8; dotted line). In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In the PG group, the 1- and 2-year survival rates were both 100%, a difference that was statistically significant. *P<0.05. Dashed arrow represents a patient's death due to SFSS and rejection followed by infection, and solid arrows represent patients' death due to rejection-related reasons. B, Kaplan–Meier patient survival curves of non-SFSG patients in era 1 (from July 2003 to October 2007; n=62; solid line) or era 2 (from November 2007 to December 2009; n=35; dotted line). In the era 1 and era 2 groups, the 2-year survival rate was 77.4% and 77.1%, respectively, with no statistical difference (P=0.980). ABO-incompatible cases and splenectomy cases were excluded from analysis. LDLT, living-donor liver transplantation; PGE1, prostaglandin E1; SFSG, small-for-size graft; SFSS, small-for-size syndrome.

a considerable risk of infection in splenectomy or splenic artery ligation (*18*). Moreover, significantly higher mortality was observed in patients who had splenectomy mainly due to septic complications in liver transplantation (*19, 20*). In fact, we experienced one SFSG case in which the patient died of sudden sepsis without any primary focus 4 years after transplantation with splenectomy. Therefore, another method to control portal pressure and preserve the spleen is likely more preferable.

We have reported that portal administration of PGE1, a vasodilator of vessels containing smooth muscle (*21, 22*), prevented congestion of residual liver tissues in a rat extended hepatectomy model. In this study, we tried various vasodilators; however, residual liver congestion after hepatectomy was improved only by continuous portal infusion of PGE1. We also tried systemic continuous venous infusion of PGE1 at the same dose, but this was not effective. This suggests the therapeutic potential of portal PGE1 injection to prevent portal hypertension after LDLT with SFSGs.

We translated this method to adult LDLT with SFSGs, and portal infusion of PGE1 successfully reduced PVP, resulting in improved liver graft function in both early and late posttransplantation periods. This result was unexpected because the portal infusion of PGE1 was given for only the first week yet improved the long-term survival of recipients.

We used a CFSE-MLR assay to objectively evaluate the antidonor responses of the recipients (*23, 24*). The lack of $CD8^+$ and $CD25^+$ T-cell proliferation in antidonor MLR reflects the suppression of the antidonor response. In this immunologic investigation, all patients given the continuous portal infusion of PGE1 showed a well-suppressed response of the antidonor $CD8^+$ T cells (Fig. 4). In contrast, surprisingly, patients without the PGE1 treatment showed

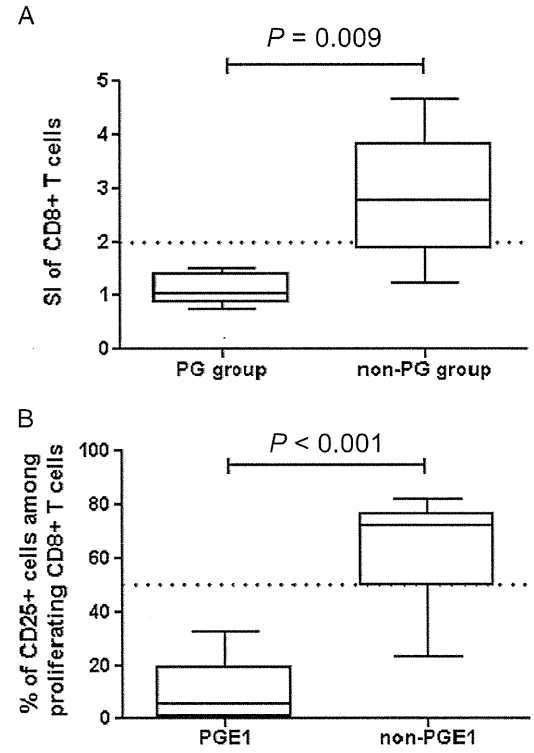

FIGURE 4. SIs of $CD8^+$ T-cell subsets in the antidonor MLR assay of patients in the PG group (n=5) and the non-PG group (n=8) on the third to fourth weeks after transplantation (A) and percentage of $CD25^+$ cells among proliferating $CD8^+$ T cells in patients of the PG group and the non-PG group (B). $CD8^+$ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the $CD4^+$ and $CD8^+$ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of the allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic (self to donor) combination by that of the self-control. An unpaired t test with Welch's correction was used to compare the SI and percentage of $CD25^+$ cells between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. **P<0.01; ***P<0.001. MLR, mixed lymphocyte reaction; SI, stimulation index.

an accelerated response of antidonor $CD8^+$ T cells despite the use of the same immunosuppressive protocol. Therefore, SFSG likely accelerated the antidonor response, enhanced rejection, and might result in a worse survival rate, although SFSS is multifactorial in nature. This finding is consistent with the results in an animal model (*25*) and our preliminary data using mouse model (data not shown). To our knowledge, this is the first clinical report to state that SFSG may accelerate antidonor immune responses in LDLT.

We previously reported that liver sinusoidal endothelial cells (LSECs) of grafts induce allospecific immunotolerance by suppressing reactive T cells through Fas ligand and/or PD-L1 signaling (*26–28*). Based on this concept, one possible mechanism is that the sinusoidal structure and LSECs could be damaged by portal hypertension and lose their tolerogenicity, resulting in accelerated antidonor immunoresponse and rejection. This interpretation is consistent with that of previous reports stating that portal hypertension disrupts sinusoids and LSECs in the liver (*8*). Another possibility is antirejection and/or the anti-inflammatory effect of PGE1 itself. It has been reported that PGE1 could prevent ischemia-reperfusion injury by inducing heat shock protein (*29*) or by inhibition of neutrophil adherence (*30*). It has been also reported that administration of PGE1 could prevent and suppress the rejection process in heart transplantation (*31*) and in renal transplantation (*32*). Furthermore, it has been reported that PGE1 protects human LSECs from apoptosis, which is consistent with our findings (*33*).

The main limitation of our study is its retrospective nature. Another limitation is the relatively small number of patients in each subgroup analyzed, although the background characteristics of each group and the survival rates of non-SFSG patients were similar. A randomized study is ideal; however, performing a prospective randomized study for this approach is difficult because of the high mortality of SFSG patients without PGE1 perfusion, as shown in Results.

In conclusion, continuous infusion of PGE1 is suggested to be useful in improving SFSG function and survival after LDLT. Improved understanding of underlying mechanisms may have important implications for clinical managements such as antirejection therapy or preventing ischemia-reperfusion injury in liver transplantation with SFSGs.

MATERIALS AND METHODS

Patients

From July 2003 to December 2009, LDLT was performed on 122 adult patients with end-stage liver disease. We introduced continuous portal infusion of PGE1 to five patients with SFSGs that exhibited a GRWR less than 0.72% from November 2007 to December 2009 (era 2). These patients (PG group) were the subjects of this retrospective case–control study. We compared them with a historical group of eight relevant patients who received SFSG without PGE1 infusion (non-PG group) from July 2003 to October 2007 (era 1) to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Because we introduced the portal infusion of PGE1 in November 2007, all patients with SFSGs in era 2 received PGE1 infusion. One SFSG case with splenectomy in era 1 was excluded from this study. The study protocol was approved by the ethics committee of Hiroshima University, and all patients provided informed consent before surgery. None of the patients receiving PGE1 portal infusion showed clinical evidence of insertion site infection or bleeding after catheter removal throughout the follow-up period.

Operation, PVP Measurement, and Continuous Portal Infusion of PGE1

The graft-harvesting technique, recipient surgery, perioperative recipient management, and immunosuppression regimens were conducted as described previously with minor modifications (*34, 35*). In brief, the right lobe without the MHV or the left lobe with the MHV was harvested from the donor as follows. Before parenchymal transection, the right or left lobe was mobilized and the short hepatic veins were transected. For the right lobe, during parenchymal transection, the major right tributaries of the MHV were clamped using a vascular clip and then transected. After hepatectomy, ex vivo perfusion of the graft was performed through the portal vein. The initial perfusate was saline solution (500 mL); then, the University of Wisconsin solution (1000 mL) was used as the perfusate.

To measure the PVP in the recipient during the operation, an 18G catheter was inserted from the mesenteric vein to the portal vein after laparotomy (*36*). The implantation was performed after total hepatectomy. The graft vein was anastomosed to the equivalent vein of the recipient in an end-to-end fashion. Thereafter, the graft was reperfused before microsurgical reconstruction of the hepatic artery (end-to-end anastomosis of the graft hepatic artery to the recipient hepatic artery). The bile duct of the graft liver was anastomosed in an end-to-end fashion to the recipient's common hepatic bile duct. In the non-PG group, the portal catheter was removed at the time of abdominal closure. In the PG group, PGE1 was administered through a portal catheter at the graft portal reflow. PGE1 was continuously administered for 1 week (0.01 g kg^{-1} min^{-1}) and then the catheter was noninvasively removed. AST, ALT, serum bilirubin, serum ammonia, and AKBR levels were measured as liver function indices. The initial immunosuppressive regimen consisted of tacrolimus and steroids. Doppler ultrasonography and computed tomographic scans were routinely performed daily and biweekly, respectively.

Immunosuppression was initiated with a protocol based on tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan) and methylprednisolone. Methylprednisolone was withdrawn gradually according to the protocol. The dose of tacrolimus was controlled according to blood concentration and adjusted daily. The target trough level was set at 15 ng/mL for 2 weeks and 10 ng/mL for another 2 weeks. Continuous venous infusion of heparin for therapeutic heparinization was routinely done to prevent thrombosis, which was monitored using coagulation tests. Rejection was diagnosed and proven by biopsy histologically and MLR assay. Patients were followed for 2 years after LDLT, and survival was defined as the period between LDLT and death.

Immune Monitoring by In Vitro CFSE-MLR Assay

CFSE-MLR was routinely performed to evaluate the recipient's antidonor immune response 2 to 4 weeks after surgery.

For CFSE-MLR, peripheral blood mononuclear cells prepared from the blood of the recipients (autologous control), donors, and healthy volunteers with the same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy. Those obtained from the recipients for use as the responder cells were labeled with 5 mM CFSE (Molecular Probes, Eugene, OR), as described previously (*24*). The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates in a total volume of 2 mL culture medium at 37°C under 5% CO_2 for 5 days. After culture for MLR, $CD4^+$ and $CD8^+$ T-cell proliferation, CD25 expression of proliferating T-cell subsets and SI were quantified by flow cytometry as described previously (*37, 38*) and described in the SDC Materials and Methods in detail (see http://links.lww.com/TP/A807).

Statistical Analysis

Statistical analysis and comparisons were performed using PRISM version 4.0 (GraphPad, San Diego, CA). Data are expressed as mean±SEM. An unpaired *t* test with Welch's correction was used to compare groups. *P* values <0.05 were considered statistically significant.

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HEPATOLOGY

Interleukin-28B single nucleotide polymorphism of donors and recipients can predict viral response to pegylated interferon/ribavirin therapy in patients with recurrent hepatitis C after living donor liver transplantation

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Key words
core, hepatitis C virus, interferon sensitivity-determining region, interleukin-28B, liver transplantation.

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Abstract

Background and Aim: Interleukin-28B (*IL28B*) single nucleotide polymorphism (SNP) influences viral response (VR) to interferon (IFN) therapy in patients with hepatitis C. We studied the relationship between VR and the *IL28B* polymorphism (rs8099917) in patients on long-term pegylated IFN plus ribavirin (PEGIFN/RBV) therapy for recurrent hepatitis C after living-donor liver transplantation (LDLT).

Methods: Thirty-five patients with recurrent hepatitis C after LDLT were treated with PEGIFN/RBV. We evaluated the effect of *IL28B* SNP on the outcome in 20 patients infected with hepatitis C virus genotype 1 who completed IFN therapy.

Results: The sustained VR (SVR) rate was 54% (19/35) for all patients; 46% (13/28) for genotype 1. The SVR rate of donors' TT group (major genotype) was higher than that of donors' TG + GG group (minor genotype) (73% *vs* 20%), while that of recipients' TT group was similar to that of recipients' TG + GG group (64% *vs* 50%). With regard to the combined effect of donors' and recipients' *IL28B* SNP, the SVR rates of TT : TT (donors' : recipients'), TT : TG + GG, TG + GG : any group were 81%, 50%, and 20%, respectively. The VR rate of TT : TT, TT : TG + GG and TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, 20%, and those at the end of treatment were 100%, 50%, 20%, respectively. The multivariate analysis identified *IL28B* of donors : recipients (TT : TT) as the only independent determinant of SVR (odds ratio 15.0, $P = 0.035$).

Conclusion: Measurement of donors' and recipients' *IL28B* SNP can predict the response to PEGIFN/RBV therapy, and the donors' *IL28B* SNP might be a more significant predictor than that of the recipients.

Introduction

Hepatitis C virus (HCV) has infected 170 million people worldwide, and such infection sometimes progresses to liver cirrhosis and/or hepatocellular carcinoma.[1] The current treatment for patients infected with HCV genotype 1 (HCV-1) is the combination of pegylated interferon-α and ribavirin (PEGIFN/RBV) for 48 weeks.[2] However, this treatment results in sustained viral response (SVR) in only approximately 50% of patients with HCV-1 infection.

In a recent genome-wide association study, a single nucleotide polymorphism (SNP) upstream of the interleukin (IL)-28B (*IL28B*) gene on chromosome 19, coding for IFN-λ-3, was found to be strongly associated with SVR rate in treatment-adherent HCV-1 patients.[3–8] The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas a T nucleotide was found to be associated with a fair response to treatment (major allele) in Japanese patients.

HCV-related end-stage liver disease is currently the leading indication for liver transplantation (LT). However, the outcome of LT for patients with HCV-related liver disease has been less satisfactory than those with HCV-negative liver disease.[9–15] HCV recurrence is universal after LT with accelerated progression of liver fibrosis. Approximately 20–25% of HCV-positive

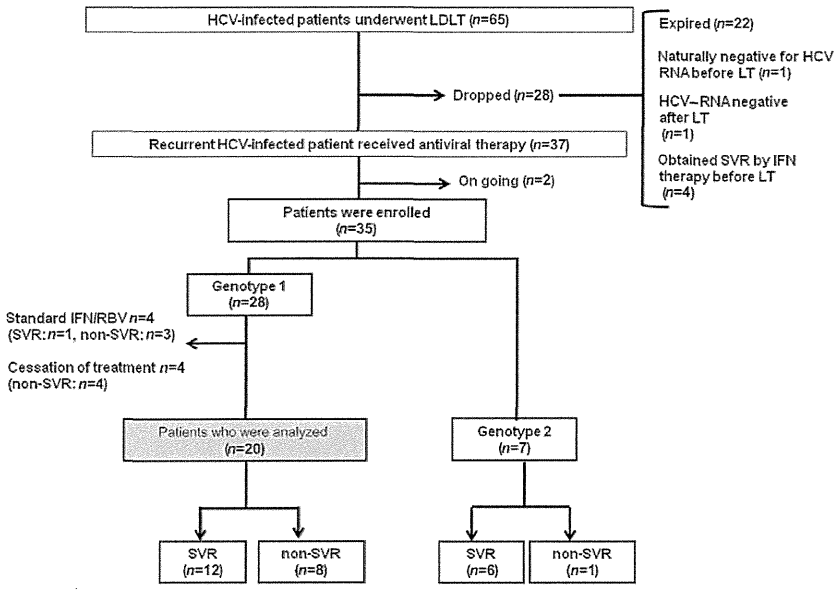

Figure 1 Flow diagram of patient recruitment. HCV, hepatitis C virus; IFN, interferon; LDLT, living-donor liver transplantation; LT, liver transplantation; RBV, ribavirin; SVR, sustained viral response.

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, $P = 0.035$).