Fig. 3. Inuzuka et al.

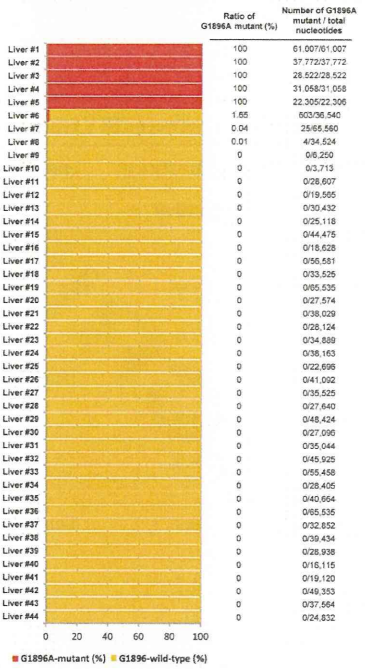

Table 1. Clinical characteristics of patients with reactivation from occult HBV and HBsAg carrier status BEFORE viral exacerbation

Case	Age/ Sex	Anti-HBs	Primary disease	Treatment	Use of steroids	HSCT	Period between HBV reactivation and start of treatment (months)	Period between HBV reactivation and end of treatment (months)
Reactivation from occult HBV carrier status								
#1	48M	+	ML	Fludarabine	+	+	57.7	39.8
#2	25M	−	AML	IDA+AraC	+	+	27.0	19.2
#3	59M	Unknown	Colon cancer	S-1	−	−	3.6	During treatment
#4	61M	Unknown	ML	R-CHASE	+	+	13.8	9.5
#5	64M	−	MM	MP→CAD	+	+	13.6	6.4
#6	72M	−	ML	MTX+AraC →Rituximab	+	−	10.9	During treatment
#7	78M	Unknown	ML	R-CVP	+	−	34.7	34.2
#8	66M	Unknown	MM	MP	+	−	49.1	6.6
#9	61F	−	ML	R-FND	+	−	1.0	During treatment
#10	66M	Unknown	Psoriasis	Cyclosporine	−	−	37.8	During treatment
#11	79F	Unknown	ML	R-CHOP	+	−	3.7	During treatment
#12	81F	−	ML	R-CVP	+	−	11.2	7.6
#13	84F	Unknown	ML	R-CHOP	+	−	17.4	During treatment
#14	87F	+	MM	MP	+	−	23.1	During treatment
							median: 15.6	median: 9.5
Reactivation from HBsAg carrier status								
#15	32F	−	Sjögren synd.	PSL	+	−	15.1	During treatment
#16	63F	−	Raynaud's dis.	PSL	+	−	20.4	During treatment
#17	42F	−	Aortitis synd.	PSL	+	−	122.2	During treatment
#18	59M	−	Lung cancer	Chemotherapy[a]	+	−	17.9	During treatment
#19	54M	−	RA	MTX+PSL	+	−	11.5	During treatment
#20	72M	−	RA	Bucillamine	−	−	6.7	During treatment
							median: 16.5	

[a]carboplatin, paclitaxel → docetaxel → gemcitabine, vinorelbine → cisplatin, irinotecan

AML, acute myeloid leukemia; AraC, cytarabine; dis, disease; CAD, cyclophosphamide, doxorubicin, dexamethasone; F, female; HBsAg, hepatitis B surface antigen; HSCT, hematopoietic stem cell transplantation; IDA, idarubicin; M, male; ML, malignant lymphoma; MM, multiple myeloma; MP, melphalan, prednisolone; MTX, methotrexate; PSL, prednisolone; RA, rheumatoid arthritis; R-CHASE, rituximab, cyclophosphamide, cytosine arabinoside, etoposide, dexamethasone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine,

prednisolone; R-CVP, rituximab, cyclophosphamide, doxorubicin, prednisolone; synd, syndrome; R-FND, rituximab, fludarabine, mitoxantrone, dexamethasone.

Table 2. Clinical courses of patients with reactivation from occult HBV and HBsAg carrier status AFTER viral exacerbation

Case	At diagnosis of HBV reactivation				ETV treatment*	Period to HBsAg disappearance** (months)
	HBV Genotype	HBeAg/ anti-HBe	HBV DNA level (log copies/mL)	ALT[a] level (IU/mL)		
Reactivation from occult HBV carrier status						
#1	C	+/−	8.2	1,915	+	13.3
#2	C	+/−	6.2	24	+	2.8
#3	C	+/−	6.4	2,019	+	0.6
#4	C	+/−	8.3	720	+	3.1
#5	C	+/−	5.4	681	n.t.	−
#6	C	+/−	8.4	15	+	−
#7	B	+/−	7.7	1,983	+	2.9
#8	B	+/−	6.2	97	+	−
#9	C	−/+	5.0	18	+	1.7
#10	C	−/+	6.6	2,028	+	0.9
#11	C	−/+	5.4	38	+	13.5
#12	B	−/+	9.0	503	+	10.5
#13	B	−/+	6.5	623	+	−
#14	B	−/+	8.5	705	+	−
			median: 6.6	median: 652		median: 2.9
Reactivation from HBsAg carrier status						
#15	C	+/−	8.8	499	+	−
#16	C	+/−	7.1	1,740	+	−
#17	C	−/+	7.8	628	+	−
#18	C	−/+	5.5	1,674	+	−
#19	B	−/+	5.8	619	+	−
#20	C	−/+	8.8	813	+	0.4
			median: 7.5	median: 716		

ALT, alanine aminotransferase; anti-HBe, antibodies to hepatitis B e antigen; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; n.t., not treated

* All patients except case #5 were treated with ETV immediately after diagnosis of HBV reactivation to suppress viral activity.

** Period (months) between ETV administration and HBsAg disappearance

[a] normal range 10-42 IU/L.

Table 3. Mean mutation rate of the reactivated HBV clones in patients with reactivation from occult HBV and HBsAg carrier status

	Occult HBV carrier status (n=14)	HBsAg carrier status (n=6)
Average aligned reads	605,890	630,253
Average aligned nucleotides	52,814,651	52,812,297
Average coverage	16,712	16,632
Mutation rate* (%)	0.015	0.114

Mutation rate * (%): the ratio of total different nucleotides from the representative HBV reference sequences.

Table 4. Overview of nucleotide 1896, 1762 and 1764 sequencing data with the deep sequencing analyses

Case	G1896A		A1762T		G1764A	
	Base counts	(%)	Base counts	(%)	Base counts	(%)
Reactivation from occult HBV carrier status						
#1	1/10,833	(0.0)	0/6,391	(0.0)	1/6,491	(0.0)
#2	1/10,200	(0.0)	0/9,213	(0.0)	3/9,216	(0.0)
#3	8/27,694	(0.0)	1/16,506	(0.0)	4/16,851	(0.0)
#4	4/13,008	(0.0)	2/12,007	(0.0)	0/11,857	(0.0)
#5	0/6,860	(0.0)	0/6,175	(0.0)	0/6,307	(0.0)
#6	273/31,622	(0.9)	8/29,996	(0.0)	4/30,400	(0.0)
#7	22/12,561	(0.2)	0/3,405	(0.0)	1/3,492	(0.0)
#8	1/11,500	(0.0)	0/4,964	(0.0)	1/5,089	(0.0)
#9	12,897/12,904	(100)	11,676/11,677	(100)	11,653/11,659	(100)
#10	11,432/11,444	(100)	1/6,153	(0.0)	2/6,217	(0.0)
#11	9,533/9,539	(99.9)	7,669/7,671	(100)	7,681/7,685	(99.9)
#12	10,944/10,945	(100)	2/10,874	(0.0)	1/11,325	(0.0)
#13*	9,358/9,411	(99.4)	2/10,900	(0.0)	0/11,298	(0.0)
#14*	11,174/11,179	(100)	0/6,579	(0.0)	2/6,773	(0.0)
Reactivation from HBsAg carrier status						
#15	734/12,544	(5.9)	7,593/7,596	(100)	7,556/7,570	(99.8)
#16	2/7,469	(0.0)	0/6,481	(0.0)	2/6,618	(0.0)
#17	12,251/12,701	(96.5)	5,110/5,241	(97.5)	5,180/5,239	(98.9)
#18	9,649/9,660	(99.9)	0/10,026	(0.0)	0/10,069	(0.0)
#19	18,402/18,413	(99.9)	1/15,677	(0.0)	3/16,045	(0.0)
#20*	11,158/11,160	(100)	0/6,671	(0.0)	3/6,929	(0.0)

* Patients who developed fatal acute liver failure.

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Dynamics of Defective Hepatitis C Virus Clones in Reinfected Liver Grafts in Liver Transplant Recipients: Ultradeep Sequencing Analysis

Shigeru Ohtsuru,[a,b] Yoshihide Ueda,[a] Hiroyuki Marusawa,[a] Tadashi Inuzuka,[a] Norihiro Nishijima,[a] Akihiro Nasu,[a] Kazuharu Shimizu,[c] Kaoru Koike,[b] Shinji Uemoto,[d] Tsutomu Chiba[a]

Department of Gastroenterology and Hepatology,[a] Department of Primary Care and Emergency Medicine,[b] and Department of Surgery,[d] Graduate School of Medicine, and Department of Nanobio Drug Discovery, Graduate School of Pharmaceutical Sciences,[c] Kyoto University, Shogoin, Sakyo-ku, Kyoto, Japan

Hepatitis C virus (HCV) reinfects liver allografts in transplant recipients by replicating immediately after transplantation, causing a rapid increase in blood serum HCV RNA levels. We evaluated dynamic changes in the viral genetic complexity after HCV reinfection of the graft liver; we also identified the characteristics of replicating HCV clones using a massively parallel ultradeep sequencing technique to determine the full-genome HCV sequences in the liver and serum specimens of five transplant recipients with genotype 1b HCV infection before and after liver transplantation. The recipients showed extremely high genetic heterogeneity before transplantation, and the HCV population makeup was not significantly different between the liver and blood serum specimens of the individuals. Viral quasispecies complexity in serum was significantly lower after liver transplantation than before it, suggesting that certain HCV clones selectively proliferated after transplantation. Defective HCV clones lacking the structural region of the HCV genome did not increase in number, and full-genome HCV clones selectively increased in number immediately after liver transplantation. A re-increase in the same defective clone existing before transplantation was detected 22 months after transplantation in one patient. Ultradeep sequencing technology revealed that the genetic heterogeneity of HCV was reduced after liver transplantation. Dynamic changes in defective HCV clones after liver transplantation indicate that these clones have important roles in the HCV life cycle.

The hepatitis C virus (HCV) has an approximately 9.6-kb plus-strand RNA genome that encodes the viral core, envelope glycoprotein 1 (E1), E2, and p7 structural proteins and the NS2, NS3, NS4A, NS4B, NS5A, and NS5B nonstructural proteins (1). A characteristic of HCV infection is its remarkable genetic diversity with a high degree of genetic heterogeneity in each patient, which is referred to as a quasispecies. In heterogeneous HCV clones, a dominant viral population might evolve as a result of its viral replicative fitness and concurrent immune selection pressures that drive clonal selection.

In HCV-positive liver transplant recipients, HCV reinfection of the liver allograft occurs at the time of transplantation, and replication of HCV begins immediately after transplantation. Blood serum HCV RNA levels then rapidly increase to levels that are 10- to 20-fold higher than pretransplant levels. It is thus hypothesized that specific HCV clones that have growth advantages increase after liver transplantation. Although several studies have attempted to clarify the change in genetic heterogeneity following liver transplantation, the abundant diversity and complexity of HCV have been obstacles to a detailed evaluation of viral genetic heterogeneity. The recent introduction of ultradeep sequencing technology, which is capable of producing millions of DNA sequence reads in a single run, however, is rapidly changing the landscape of genome research (2, 3).

In this study, we performed ultradeep sequencing analyses to unveil the levels of viral quasispecies of genotype 1b HCV in the liver and the serum specimens from 5 patients who underwent living donor liver transplantation (LDLT) and clarified the changes in viral genetic complexity after reinfection of HCV in the graft liver. In the analyses, we found that the population of defective HCV clones that lack structural regions of the HCV genome changed after liver transplantation. We then clarified the dynamics and characteristics of the defective HCV clones.

MATERIALS AND METHODS

Patients. The participants comprised 5 Japanese adult patients with end-stage liver disease with genotype 1b HCV infection who underwent LDLT at Kyoto University Hospital between May 2006 and September 2008. Serum samples were obtained before and 1 month after liver transplantation. In addition, a blood serum sample from a patient in the chronic hepatitis phase 22 months after liver transplantation was obtained and analyzed. Liver tissue samples were obtained from 4 patients (patients 1–4) at the time of transplantation, frozen immediately, and stored at −80°C until use.

Tacrolimus with a steroid or mycophenolate mofetil was administered to induce immunosuppression in the patients. A patient who received an ABO blood type-incompatible transplant was treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone (4).

The ethics committee at Kyoto University approved the studies (protocol no. E1211), and written informed consent for participation in this study was obtained from all patients.

Virologic assays. The HCV genotype was determined using a PCR-based genotyping system developed by Ohno et al. (5) to amplify the core region using genotype-specific PCR primers for the determination of the HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. The blood serum HCV RNA load was evaluated before LDLT 1 month post-LDLT and then every 3 months after LDLT using PCR and an Amplicor HCV assay (Cobas

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Address correspondence to Yoshihide Ueda, yueda@kuhp.kyoto-u.ac.jp.
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Amplicor HCV monitor; Roche Molecular Systems, Pleasanton, CA) until April 2008 or a real-time PCR-based quantitation method for HCV (Cobas AmpliPrep/Cobas TaqMan HCV test; Roche Molecular Systems) starting May 2008.

Direct population Sanger sequencing. To define the representative reference sequences of full-length HCV in each clinical specimen, serum samples collected before liver transplantation were first subjected to direct population Sanger sequencing using the Applied Biosystems 3500 genetic analyzer (Applied Biosystems, Foster City, CA) (6). Total RNA was extracted from 140 μl of serum using a QIAamp viral RNA minikit (Qiagen, Valencia, CA) and reverse transcribed in a volume of 20 μl with the OneStep RNA PCR kit AMV (TaKaRa Bio, Ohtsu, Japan). The HCV genomes were amplified using Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). Oligonucleotide primers were designed to amplify the first half (~5,000 bp) and latter half (~4,500 bp) of the genotype 1b HCV genome sequence. PCR products purified by the QIAquick gel extraction kit (Qiagen) were assayed for direct sequencing. The nucleotide sequences of the PCR products were determined using an ABI Prism BigDye Terminator ready reaction kit (Applied Biosystems). A blood serum sample from a healthy volunteer was used as a negative control.

Massively parallel ultradeep sequencing. Paired-end sequencing with multiplexed tags was carried out using the Illumina Genome Analyzer II. End repair of DNA fragments, the addition of adenine to the 3′-ends of the DNA fragments, adaptor ligation, and PCR amplification by Illumina-paired end PCR primers were performed as described previously (6, 7). Briefly, the viral genome sequences were amplified with high-fidelity PCR and sheared by nebulization using 32 lb/in^2 N2 for 8 min, and the sheared fragments were purified and concentrated using a QIAquick PCR purification kit (Qiagen). The overhangs resulting from the fragmentation were then converted into blunt ends using T4 DNA polymerase and Klenow enzymes, followed by the addition of terminal 3′-adenine residues. One of the adaptors containing six unique base pair (bp) tags, such as ATCACG and CGATGT (multiplexing sample preparation oligonucleotide kit; Illumina), was then 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 were performed for 64 cycles on the Illumina Genome Analyzer II according to the manufacturer's instructions. The obtained images were analyzed and base called using the GA pipeline software version 1.4 with default settings provided by Illumina. Validation of the multiplex ultradeep sequencing of the HCV genome was performed using a plasmid encoding full-length HCV as a template, as reported previously (6). The overall error rates were determined to be, on average, 0.0010 per base pair. We also confirmed that high-fidelity PCR amplification with HCV-specific primer sets followed by multiplex ultradeep sequencing resulted in no significant increase in the error rates of viral sequencing data (ranging from 0.0012 to 0.0013 per bp; per-nucleotide error rate, 0.12% to 0.13%) (6).

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 ~9,200 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 <20 and when they contained >3 uncalled nucleotides. Low-quality bases were trimmed from the reads when >3 consecutive bases fell below a quality score of 16. Based on the above criteria, reads were aligned if ≥90% of their bases matched a particular position of the reference sequence. Each position of the viral genome was assigned a coverage depth representing the number of times that nucleotide position was sequenced.

Detection of defective HCV clones. The methods for detecting defective HCV clones were reported previously (8). Briefly, reverse transcription-PCR (RT-PCR) was performed using the OneStep RNA PCR kit (TaKaRa) with the extracted RNA from liver and blood serum as a template and two pairs of primers, 5′-CGCCGACCTCATGGGGTACA-3′ and 5′-TGGTGTACATTTGGGTGATT-3′ for the first RT-PCR (HCV-P1) and 5′-TGCTCTTTCTCTATCTTCCT-3′ and 5′-GTGATGATGCA ACCAAGTAG-3′ for the second PCR (HCV-P2). The PCR products were analyzed by electrophoresis in 0.8% agarose gels stained with ethidium bromide. Each purified DNA sample was sequenced at least three times using an ABI Prism BigDye Terminator ready reaction kit (Applied Biosystems). To determine defects in the HCV genome, the sequence of each sample was compared with the registered HCV genome sequence.

Statistical analysis. The viral quasispecies nature was evaluated by analyzing the genetic complexity based on the number of different sequences present in the HCV population. The genetic complexity was determined by Shannon entropy index, calculated as follows:

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

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 (9, 10). Statistical comparisons of the complexity between two groups were made using the Wilcoxon rank sum test or the Mann-Whitney U test. P values of <0.05 were considered to be statistically significant.

RESULTS

Patient characteristics. The clinical and virological characteristics of the 5 patients are summarized in Table 1. Four of the 5 recipients were male, and the median age of the patients at the time of LDLT was 52 years (range, 47 to 65 years). All patients had decompensated cirrhosis caused by chronic hepatitis C, and 3 patients had hepatocellular carcinoma before liver transplantation. Right-lobe grafts were used for all patients. All patients had an HCV genotype 1b infection. The median blood serum HCV RNA load before transplantation was 5.5 log IU/ml (range, 4.6 to 6.6 log IU/ml) and was 5.9 log IU/ml (range, 5.8 to 6.4 log IU/ml) 1 month after liver transplantation; however, this difference was not significant ($P = 0.18$).

HCV population did not significantly differ between liver and serum samples. To clarify the landscape of HCV heterogeneity as a quasispecies, we determined the viral full-genome sequences in liver and serum samples collected from the 5 recipients before transplantation using multiplex ultradeep sequencing and compared the results with those obtained by the direct population Sanger sequencing method. The HCV nucleotide sequence reads obtained by ultradeep sequencing were aligned to the consensus viral sequences in the serum specimen of each individual that were determined by direct population Sanger sequencing. A mean of 1,548-fold coverage was achieved at each nucleotide site of the HCV sequences in each specimen. First, the nucleotide sequence complexities expressed as the Shannon entropy index of HCV in the liver were compared with those in the serum. The overall viral complexity determined by the Shannon entropy index did not significantly differ between the liver and serum samples of each individual (see Fig. S1 in the supplemental material). Moreover, the patterns and distributions of genetic heterogeneity of the viral nucleotide sequences in the liver tissue sample were similar to those observed in the serum sample of the same patient (see Fig. S2 in the supplemental material). Next, we compared the viral genome sequences in the liver tissue with those in the serum in the same patient at the sites of the reported mutations that are related to the efficacy of interferon treatment and drug resistance against

TABLE 1 Baseline characteristics of 5 patients with chronic HCV[a] genotype 1b infection

Patient characteristic[b]	Data for patient no[c]:				
	1	2	3	4	5
Age (yr)	65	52	47	58	48
Sex	Female	Male	Male	Male	Male
Existence of HCC	+	+	−	+	−
Child-Pugh score	10	10	9	10	10
MELD score	14	15	14	15	15
HCV viral load (log IU/ml)					
Pre-LDLT	4.6	6.6	4.9	5.5	5.9
Post-LDLT					
1 mo	5.9	6.1	5.8	5.8	6.4
22 mo					6.5
HCV infection					
Duration of hospital visit (yr)	37	18	3	24	13
Route of infection	Blood transfusion	Unknown	Unknown	Unknown	Unknown
Blood type	AB identical	A identical	A identical	A identical	A incompatible
Immunosuppressants	Tacrolimus, MMF	Tacrolimus, MMF	Tacrolimus, PSL	Tacrolimus, MMF	Tacrolimus, PSL

[a] HCV, hepatitis C virus.
[b] HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; LDLT, living donor liver transplantation.
[c] MMF, mycophenolate mofetil; PSL, prednisolone.

HCV protease and polymerase inhibitors (see Table S1 in the supplemental material). The prevalences of these mutations of the HCV genome in the liver were similar to those in the serum of the same patients. These findings suggested that a similar pattern of viral heterogeneity was maintained in the liver and serum of patients with chronic HCV infection.

Early dynamic decrease of viral complexity after liver transplantation. To clarify the changes in the viral quasispecies after liver transplantation, we investigated the change in the viral complexities of the serum specimens before and 1 month after liver transplantation in these 5 patients. The mean coverages of 1,284-fold and 1,141-fold were mapped to each reference sequence before and after liver transplantation, respectively. We then estimated the genomic complexity by calculating the Shannon entropy index for each nucleotide position before and after liver transplantation (Fig. 1A). The level of viral complexity of the blood serum HCV significantly differed between pretransplantation and posttransplantation (mean Shannon entropy index, 0.056 versus 0.029; $P = 0.043$), demonstrating that the viral quasispecies nature after reinfection and replication in the graft liver became more homogeneous than that before transplantation. To identify the specific regions in the HCV genome that were respon-

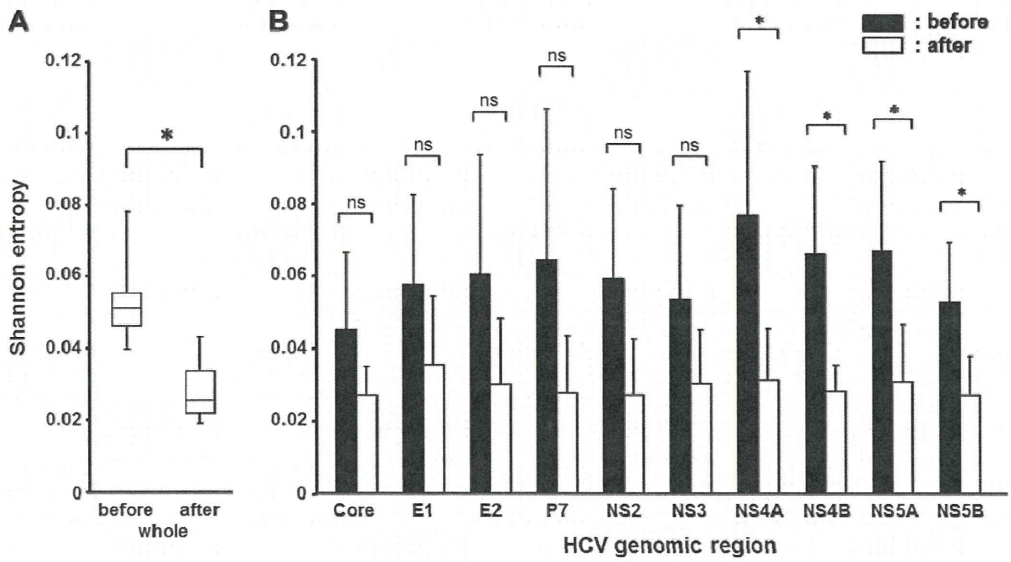

FIG 1 Changes in the genetic complexity of the HCV genome before and after liver transplantation. (A) Mean Shannon entropy index values for the overall HCV genome in 5 LDLT recipients before and after liver transplantation. (B) Mean Shannon entropy index values for each HCV genomic region before (black bars) and after (white bars) liver transplantation are shown. The error bars in panels A and B represent the standard deviation. *, $P < 0.05$; ns, nonsignificant.

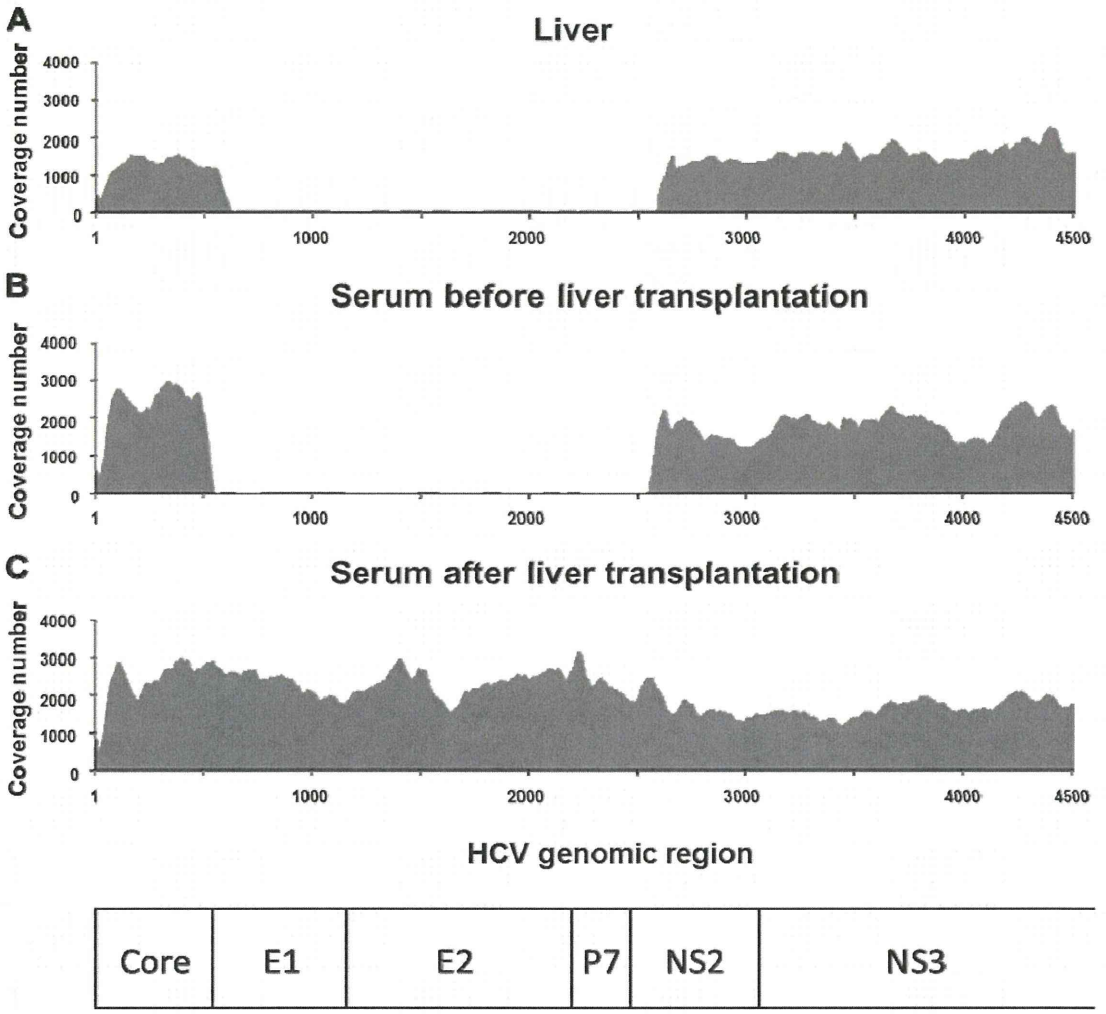

FIG 2 Dynamics of defective HCV clones indicated by coverage numbers of ultradeep sequence of HCV genome. Coverage of ultradeep sequence of HCV genome in liver (A), serum samples before liver transplantation (B), and serum samples after liver transplantation (C) for patient 1. The degree of coverage (fold) at each nucleotide site of the HCV sequence is shown. Nucleotide 1 indicates the first nucleotide of the core region of HCV RNA. Similar results were obtained in the samples from patients 2, 4, and 5.

sible for the selective increase in HCV after liver transplantation, we analyzed the changes in complexity of each region of the HCV genome (Fig. 1B). A decrease in the genetic complexity after liver transplantation was observed throughout the individual viral genetic regions. In particular, the complexity during pre- and post-transplantation was significantly different in the NS4A, NS4B, NS5A, and NS5B regions, suggesting that these regions are important for active proliferation of HCV at the early phase of reinfection in the graft liver. We then examined whether a specific nucleotide position was associated with a decrease in complexity after liver transplantation, but none of the specific nucleotide positions that changed by >50% after liver transplantation compared to before transplantation were commonly identified in the 5 patients (data not shown); this indicates that no association exists between a specific nucleotide position and the decrease in complexity after liver transplantation.

Defective HCV clones became undetectable immediately after liver transplantation. Using the ultradeep sequencing analyses, we found that the sequence coverage of viral genomic regions spanning from the end of the core to the middle of NS2 was smaller than those of the other regions in several liver and serum samples before liver transplantation, but this tendency was not observed in the samples after liver transplantation (Fig. 2). As we previously identified the defective HCV clones lacking the structural regions of the HCV genome in the serum samples of HCV-positive liver transplant recipients (8), we speculated that the presence of the defective HCV clones would result in the smaller coverage of E1-NS2 before transplantation, and the population of the defective clones would change after liver transplantation. Therefore, we next analyzed the population change of the defective HCV clones between before and after liver transplantation. Using RT-PCR analysis with the primers HCV-P1 and HCV-P2 (Fig. 3A), we detected both defective HCV clones and full-genome HCV clones before liver transplantation at various ratios in each sample, except for in patient 3 (Fig. 3B). The defective HCV clones became undetectable and the full-genome HCV clones became

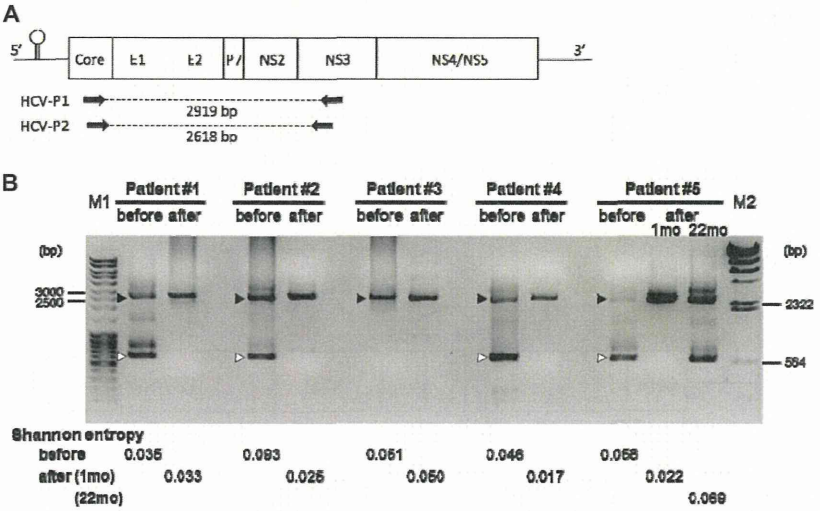

FIG 3 Dynamics of defective HCV clones based on RT-PCR analysis. (A) Schematic presentation of the HCV genome and the primer sets used in this study. (B) Results of RT-PCR analysis by using RNA samples as a template, which were extracted from blood serum before and 1 month after liver transplantation in all patients and 22 months after transplantation in patient 5. HCV-P1 and HCV-P2 (panel A) were used as primers. Lanes M1 and M2, the molecular weight markers MassRuler DNA ladder mix (Fermentas, Canada) and Lambda DNA-HindIII Digest (New England BioLabs, USA), respectively. The values shown indicate the sizes of the band in the molecular weight markers. Black arrowheads, full-length PCR fragment of 2,618 bp; white arrowheads, defective HCV clones that were confirmed by sequencing analysis. The Shannon entropy index values of these HCV specimens in the serum (before and after liver transplantation) are shown at the bottom.

dominant in the serum samples 1 month after liver transplantation, indicating that the defective HCV clones have less of a replication advantage than the full-genome clones. In patient 3, the defective HCV clones were undetectable both before and after liver transplantation.

To determine the internal structure of the deletions in the defective HCV genomes, major amplified fragments from each of the four patients with defective HCV clones before transplantation were subcloned for further sequence analyses. Schematic representations of the defective HCV RNA detected in the blood serum specimens of these patients are shown in Fig. 4. Sequence analyses revealed that the structural region was widely deleted in all of the defective HCV clones. The 3′-boundaries of the deletions were quite diverse in the clones, while the 5′-untranslated region and core regions were preserved in all four clones, as reported previously (8). Two distinct defective clones were found in patient 2. All of the deletions identified were in frame, implying that these defective HCV genomes have the potential for translation from the core to the authentic end of NS5B without a frameshift.

We then analyzed the dynamics of the defective HCV clones at the chronic hepatitis phase after liver transplantation in patient 5. As shown in the Patient 5 column in Fig. 3B, RT-PCR from a serum sample collected at 22 months after liver transplantation, when a liver biopsy specimen demonstrated findings of chronic hepatitis C with fibrosis (METAVIR score, A1 F1), showed that a defective HCV clone had reappeared. The size of the defective clone was the same as that found in the serum before transplantation, and we confirmed by sequence analysis that the deleted region of the defective HCV clone was identical to that in the pretransplant serum sample. The viral complexity analyzed by calculating the Shannon entropy index from ultradeep sequencing data also returned to the pretransplantation level at the chronic hepatitis phase (Shannon entropy values, 0.056 before transplantation, 0.022 at 1 month posttransplantation, and 0.069 at 22 months after liver transplantation). These findings indicated that the reconstitution of HCV heterogeneity occurs at the chronic hepatitis phase after liver transplantation, and the same defective HCV clone present before liver transplantation reappears at the chronic hepatitis phase after liver transplantation.

DISCUSSION

The present study revealed two major findings from ultradeep sequencing analyses of the HCV genome sequence in liver transplant recipients before and after liver transplantation. First, the viral heterogeneity of HCV significantly decreased after liver transplantation, indicating that the clones with advantages for infection and/or replication in hepatocytes rapidly increased after liver transplantation. Second, full-genome HCV clones selectively increased, while the defective clones did not increase in number during the period immediately after liver transplantation.

The discovery of differences in the populations of HCV quasispecies between the liver and serum of the same individuals has been controversial. Most previous studies examined the HCV sequencing mainly for the hypervariable region in E2 using the Sanger sequencing method (11–13) or single-strand conformation polymorphism (12, 14, 15), but the findings were conflicting. In the present study, we obtained full-genome HCV sequences using ultradeep sequencing analysis. Our results suggested that a similar HCV population exists in the liver and blood serum, at least at the specific sites related to interferon sensitivity and drug resistance. These results are clinically important because we confirmed that the serum samples, which are easily obtained from patients, reflect the HCV population in the liver and are thus useful for analyses of resistance and sensitivity to treatment.

Differences in the HCV population between individuals can be determined by multiple factors, such as the duration of hospital visit, route of HCV infection, fibrosis progression, degree of inflammation, and the presence of hepatocellular carcinoma. In our

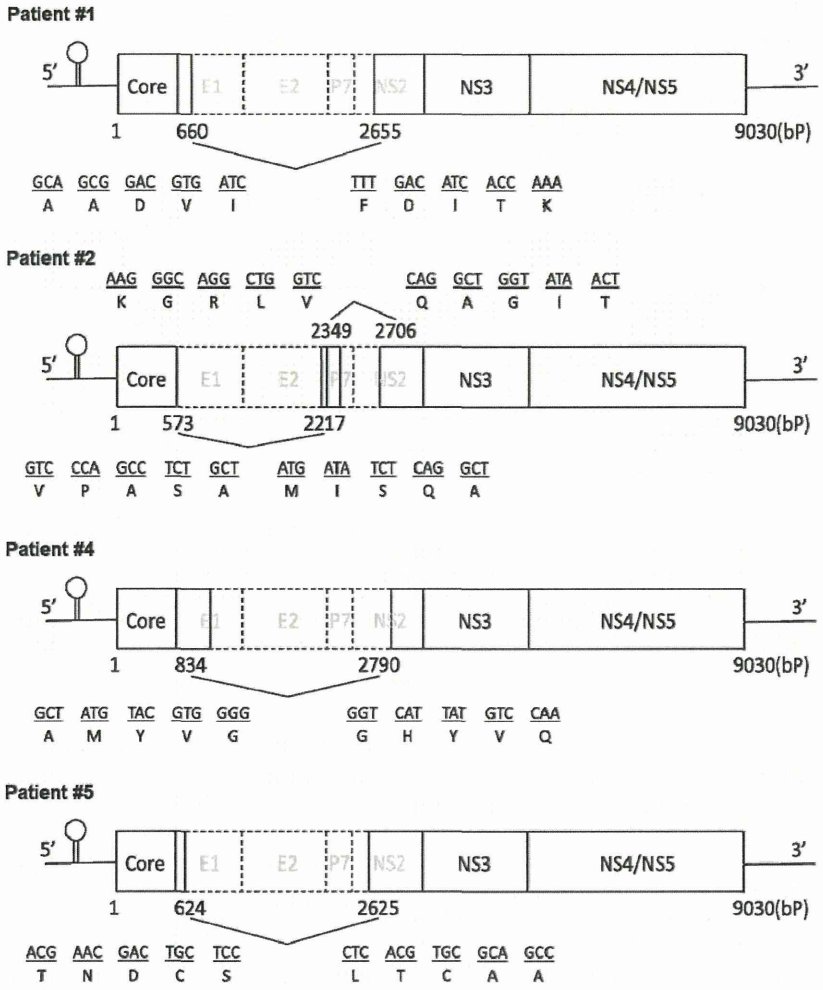

FIG 4 Schematic presentation of major defective HCV clones in 4 patients before liver transplantation. The values in the schema indicate the nucleotide numbers from the first ATG of the core region in HCV RNA. Nucleotide and amino acid sequences before and after the deleted region of the HCV genome are shown. E1, envelope glycoprotein 1; E2, envelope glycoprotein 2; NS, nonstructural protein.

analysis, we could not find an association between these clinical characteristics and the nature of the HCV population between patients. However, we speculated that undetectable defective HCV clones present before liver transplantation in patient 3 might be associated with a shorter duration of HCV infection. In patients 1 and 3, the difference in viral complexity as measured by the Shannon entropy index values between before and after liver transplantation was small. The reason is unclear at present, but differences in the clinical features of infected patients might affect the results. Further large-scale investigations may reveal the relationship between clinical features in patients and the nature of a specific HCV population.

Our large-scale analysis using ultradeep sequencing demonstrated that the complexity of all regions of the HCV genome was dramatically reduced 1 month after liver transplantation compared with the pretransplantation level of complexity. This finding is consistent with findings from previous reports using Sanger sequencing methods that showed that heterogeneity is decreased in the hypervariable region of E2 of HCV after liver transplantation (16, 17). Gretch et al. (16) analyzed HCV quasispecies before and after liver transplantation by comparing the differences in the hypervariable region of the HCV genome in 5 transplant recipients. They found that different HCV clones were present in pretransplant blood serum and relatively homogeneous quasispecies variants emerged after liver transplantation in all 5 cases. Hughes et al. (17) demonstrated that the viral complexity of the hypervariable region 1 in postperfusion liver tissue at 2.5 h after liver transplantation was significantly lower than that in explanted liver and in pretransplant serum, although there was no significant difference in the complexity between the explanted liver and pretransplant serum. Our present data confirmed the results of these previous studies and added new information from the full-genome ultradeep sequencing. In particular, our data demonstrated a new aspect of the analyses of full-genome and defective HCV clones, because the defective HCV clones lack hypervariable regions that were analyzed in previous studies. Interestingly, our analysis revealed significant decreases in complexity in the NS4A, NS4B, NS5A, and NS5B regions after transplantation, although a decreasing trend was detected in all regions of the HCV genome. Because the region from NS4A to NS5B has important roles in

HCV replication (18–20), a decrease in the complexity of the NS4A to NS5B sequence after liver transplantation might indicate the presence of the specific NS4A to NS5B sequence in the HCV genome that confers advantages in the reinfection and/or replication processes. Therefore, we attempted to identify the specific HCV genome sequences with such advantages. However, we could not identify a common feature of the HCV genomic changes in amplified HCV clones after liver transplantation among the 5 cases tested. This may be due to differences between individuals in the relative fitness of a viral subpopulation in its host, which is determined by multiple factors, including infection capacity, replication ability, and mechanisms by which to escape from immune pressure.

We previously identified defective HCV clones in the blood serum of patients after liver transplantation (8). Other groups also reported that defective HCV clones exist in the liver and serum of patients with chronic hepatitis C and patients with immunosilent infections (21–25). These reports demonstrated that deletions in the HCV genome were present mainly in the structural region, while the 5′-untranslated region, the core, and NS3 to NS5B regions were preserved, and that most of the deletions were in frame, indicating that the preserved regions can be translated to the authentic terminus. Indeed, Sugiyama et al. (24) recently demonstrated that the defective genome can be translated, self-replicated, and encapsidated as an infectious particle by *trans*-complementation of the structural proteins *in vitro*. Pacini et al. (23) also reported that defective HCV clones show robust replication, efficient *trans*-packaging, and infection of cultured cells. These data suggest that the abilities of defective HCV genomes to infect, replicate, and be encapsidated do not differ from those of full-genome HCV. The *in vivo* data reported here, however, clearly reveal that the amount of defective HCV clones was lower than that of full-genome HCV after liver transplantation, although the reason for this remains unknown. One possibility is that the capabilities to infect, replicate, or be encapsidated differ between defective HCV and full-genome HCV *in vivo*. It is noteworthy that an identical defective HCV clone that was detected before transplantation reappeared during the chronic hepatitis phase after transplantation in patient 5. This finding suggests that the defective clone in the blood serum also infected the graft liver, replicated, and was encapsidated in the graft liver after liver transplantation. Therefore, the speed of these steps would differ between defective HCV clones and full-genome HCV clones.

The present study revealed a limitation of the massively parallel ultradeep sequencing technology in the analyses of viral quasispecies. Because the massively parallel ultradeep sequencing platform is based on multitudinous short reads, it is difficult to separately evaluate the association between nucleotide sites that are 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 lengths of the shotgun sequencing approach.

In conclusion, after liver transplantation, viral heterogeneity decreased significantly and the number of full-genome HCV clones increased immediately, whereas the defective HCV clones began to increase in number over a longer period. Further analysis will reveal the significance of the changes in defective HCV clones after liver transplantation.

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Pretransplant Serum Hepatitis C Virus RNA Levels Predict Response to Antiviral Treatment after Living Donor Liver Transplantation

Yoshihide Ueda[1]*, Toshimi Kaido[2], Yasuhiro Ogura[2], Kohei Ogawa[2], Atsushi Yoshizawa[2], Koichiro Hata[2], Yasuhiro Fujimoto[2], Aya Miyagawa-Hayashino[3], Hironori Haga[3], Hiroyuki Marusawa[1], Satoshi Teramukai[4], Shinji Uemoto[2], Tsutomu Chiba[1]

1 Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **2** Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **3** Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan, **4** Division of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

Abstract

Background: Given the limited efficacy and high adverse event rate associated with treatment of recurrent hepatitis C after liver transplantation, an individualized treatment strategy should be considered. The aim of this study was to identify predictors of response to antiviral therapy for hepatitis C after living donor liver transplantation (LDLT) and to study the associated adverse events.

Methods: A retrospective chart review was performed on 125 hepatitis C virus (HCV)-positive LDLT recipients who received interferon plus ribavirin and/or peginterferon plus ribavirin therapy at Kyoto University between January 2001 and June 2011.

Results: Serum HCV RNA reached undetectable levels within 48 weeks in 77 (62%) of 125 patients, and these patients were defined as showing virological response (VR). Of 117 patients, 50 (43%) achieved sustained VR (SVR). Predictive factors associated with both VR and SVR by univariate analysis included low pretransplant serum HCV RNA levels, a non-1 HCV genotype, and low pretreatment serum HCV RNA levels. In addition, LDLT from ABO-mismatched donors was significantly associated with VR, and white cell and neutrophil counts before interferon therapy were associated with SVR. Multivariate analysis showed that 2 variables–pretransplant serum HCV RNA level less than 500 kIU/mL and a non-1 HCV genotype–remained in models of both VR and SVR and that an ABO mismatch was associated with VR. No variables with a significant effect on treatment withdrawal were found.

Conclusions: Virological response to antiviral therapy in patients with hepatitis C recurring after LDLT can be predicted prior to transplant, based on pretransplant serum HCV-RNA levels and HCV genotype. LDLT from ABO-mismatched donors may contribute to more efficacious interferon therapy.

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* E-mail: yueda@kuhp.kyoto-u.ac.jp

Introduction

Hepatitis C virus (HCV) infection, leading to liver cirrhosis and hepatocellular carcinoma, is the leading indications for liver transplantation in Japan, the United States, and Western Europe. However, almost all patients who undergo liver transplantation for HCV-related liver disease develop recurrent viral infection, and 70–90% of patients suffer from histologically proven recurrent hepatitis [1,2,3,4,5,6]. The progression of recurrent hepatitis C is often accelerated and, without appropriate antiviral therapy, 10–25% of patients develop cirrhosis within 5 years after transplantation, resulting in poorer prognoses for HCV-positive recipients than HCV-negative recipients [7]. To prevent the progression of hepatitis C after liver transplantation, interferon-based combination therapy is commonly administered [8,9]. However, its efficacy in liver transplant recipients is limited, with the mean sustained virological response (SVR) rate among patients with recurrent hepatitis C after liver transplantation being only 30% (range, 8–50%) [10]. One of the reasons for the low SVR rate is the high rate of treatment withdrawal. Several severe adverse events have been reported in transplant recipients after interferon therapy,

including chronic rejection and *de novo* autoimmune hepatitis [11,12,13].

To improve the efficacy of anti-HCV treatment in patients with hepatitis C after liver transplantation, an individualized treatment strategy based on efficacy prediction and adverse events should be attempted. In several studies, an analysis of predictors associated with SVR was conducted in patients with recurrent hepatitis C after deceased donor liver transplantation (DDLT) [10,14,15,16,17,18,19,20]. In these studies, variables most frequently associated with SVR were early virological response (EVR) at 3 months of therapy, HCV genotype 2, adherence to therapy, and baseline viremia [14,15,16,17,18,19,20]. Of these factors, EVR and adherence to therapy can only be recognized after the initiation of treatment. However, to enable decisions on treatment indications and strategy, predictors of response that are available before initiation of therapy are more valuable. Thus, an individualized treatment strategy could be based on the identification of baseline predictive factors before interferon therapy. Moreover, no study of factors predictive of response to the interferon therapy in patients with recurrent hepatitis C after living donor liver transplantation (LDLT) has been reported so far. Characteristics specific to LDLT, including blood-relative donors, post-transplant liver regeneration, and ABO-incompatible liver transplantation, might cause the antiviral effects of interferon therapy in these patients to differ from those who received DDLT.

The direct-acting antiviral agents telaprevir and boceprevir recently became available for clinical use. The results of clinical trials of these agents in combination with peginterferon plus ribavirin in nontransplant patients with HCV were promising [21,22,23,24]. SVR rates to telaprevir-based combination therapy were significantly higher than those to the peginterferon-ribavirin combination. The efficacy in the patients who had suffered a relapse after a previous treatment by peginterferon plus ribavirin was especially striking [21,24]. The SVR rate to telaprevir based-therapy in patients who had a previous relapse was more than 80%, while that in patients who had no response to previous treatment was around 30% [24]. These results suggest that patients who show a virological response (VR) to peginterferon plus ribavirin are expected to achieve SVR after telaprevir-based therapy. Therefore, identification of factors predictive of virological response to peginterferon plus ribavirin should also prove useful when making the clinical decision about telaprevir usage. In liver transplant recipients, the use of telaprevir and boceprevir poses risks because of their inhibitory action on the enzyme cytochrome P450 3A, responsible for the metabolism of both tacrolimus and cyclosporine. In fact, the phase I study of telaprevir in healthy individuals revealed that it significantly increased the blood concentrations of both tacrolimus and cyclosporine [25]. Therefore, the selection of the patients for whom telaprevir is prescribed is especially important in liver transplant recipients.

Recently, a polymorphism in the interleukin-28B (IL28B) gene region, encoding interferon-lambda 3, was identified as a strong predictive factor for response to antiviral treatment in nontransplant patients with hepatitis C [26,27,28]. In post-transplant patients, the IL28B polymorphism in both recipients and donors was shown to be associated with response to antiviral treatment [29,30]. In addition, HCV-RNA mutations, including those affecting amino acid (aa) residues 70 and 91 in the core region of HCV and those in the interferon sensitivity determining region (ISDR) in nonstructural protein 5A (NS5A), were also demonstrated to be predictors of response to interferon therapy in transplant recipients, as well as in nontransplant settings [31,32,33]. These factors could be used to predict response to antiviral therapy, but these are presently not part of a routine clinical examination and require special techniques not covered by health insurance. Moreover, probing individual genetic information poses potential ethical issues.

The aims of this study were, therefore, to identify noninvasively obtained regular baseline factors associated with VR, SVR, and treatment withdrawal, in order to elucidate the factors associated purely with response to interferon therapy, to identify the valuables related to final outcomes, and to clarify the factors associated with adverse events.

Methods

A retrospective chart review was performed for all HCV-positive liver transplant patients who received antiviral therapy with standard interferon and/or pegylated interferon in combination with ribavirin after liver transplantation at Kyoto University between January 2001 and June 2011.

Patients

Between March 1999 and June 2011, 214 HCV-positive recipients underwent LDLT at Kyoto University. Of these, 157 patients were followed up for more than 6 months after LDLT in our hospital. Anti-viral therapy was administered to 125 of the 157 patients with recurrent hepatitis C between January 2001 and June 2011. The remaining 32 patients did not receive anti-viral therapy for various reasons: serum HCV-RNA negative after LDLT (n = 4), no histological hepatitis C recurrence in the follow-up period (n = 13), no fibrosis seen by liver histology (n = 8), and ongoing treatment for the other complications (n = 7). HCV RNA concentrations and histological evidence were used to diagnose patients with recurrent hepatitis C after LDLT. These patients were given combination therapies with interferon plus ribavirin and/or peginterferon plus ribavirin at Kyoto University between January 2001 and June 2011. The study protocol was approved by the Ethics Committee at Kyoto University and performed in compliance with the Helsinki Declaration. Written informed consent for participation in this study was not obtained, because this study is an observational study without use of human specimen. Our institutional review board waived the need for written informed consent from the participants of the initial study.

Treatment Protocol and Definition of Responses to Treatment

Between January 2001 and April 2004, patients with recurrent hepatitis C after LDLT received treatment with interferon-α-2b (3 or 6 mega units, 3 times/week) plus ribavirin (400–800 mg/day orally), for the first 6 months. This was followed by interferon monotherapy for 6 months [34]. Forty patients received this treatment. Of the 40 patients, 14 patients achieved SVR and 9 withdrew from the treatment protocol. The remaining 17 patients, including 2 who relapsed and 15 nonresponders were retreated by the following protocol with peginterferon and rebavirin. Between May 2004 and June 2011, patients received combination therapy with peginterferon-α-2b (1.5 μg/kg) plus ribavirin (400–800 mg/day orally) [35]. Patients who acquired a negative serum HCV RNA status within 12 months after treatment initiation continued to receive the treatment for an additional 12 months before treatment termination. Total 102 patients, including 17 patients who had previously treated with standard interferon plus ribavirin and did not achieve SVR, were treated with this treatment protocol. Patients who were negative for serum HCV RNA for more than 6 months after completion of interferon therapy were defined as having achieved SVR. If serum HCV RNA was positive after 12 months of treatment, therapy was discontinued or

switched to maintenance therapy with low-dose peginterferon [36], and the patient was classified as having shown no response. Treatment was discontinued in patients with severe adverse events. Additionally, peginterferon treatment was discontinued when neutrophil and platelet counts fell below 500/μL and 30000/μL, respectively, and ribavirin was discontinued when hemoglobin levels fell below 8 g/dL.

We studied the final outcomes of the treatment with peginterferon plus ribavirin (n = 102) and with standard interferon plus ribavirin (n = 23).

Histological Assessment

Liver biopsies were performed when patients' alanine aminotransferase (ALT) levels were more than twice the normal upper limit, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by 2 pathologists (H.H. and A.M-H.) with extensive experience in the pathology of liver transplantation. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores [37,38]. Activity was graded as A0 (no activity), A1 (mild activity), A2 (moderate activity), or A3 (severe activity); Fibrosis was staged as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis).

Immunosuppression

Tacrolimus and low-dose steroid therapy were administered to induce immunosuppression in most patients [34]. Four patients received cyclosporine microemulsions instead of tacrolimus. Mycophenolate mofetil was administered to patients who experienced refractory rejection or required reduction of tacrolimus or cyclosporine doses due to adverse events. Patients who received ABO blood-type incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone [39].

Virological Assays

HCV genotype was determined using a genotyping system based on polymerase chain reaction (PCR) to amplify the core region using genotype-specific PCR primers [40]. Serum HCV RNA load was evaluated before LDLT, before interferon treatment, once a month during treatment, and 24 weeks after treatment, using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor, Roche Molecular Systems, Pleasanton, CA, USA) until April 2008, or a real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Molecular Systems, Pleasanton, CA, USA) from May 2008. Detection of amino acid substitutions in the HCV core region was performed using the method reported previously [31].

Statistical Analysis

To evaluate the association between the patient characteristics and the outcomes (VR, SVR, or withdrawal), the Wald test was performed based on a logistic regression model. Multivariate logistic regression analysis with backward variable selection was used to identify independent and significant predictors for the outcomes, and to estimate the odds ratio (OR) ant its 95% confidence interval (CI). A p-value of 0.05 was used for variable selection and was regarded as significant. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary NC).

Results

Patient Characteristics

This study included 125 HCV-infected liver transplant patients treated with standard interferon and/or pegylated interferon in combination with ribavirin for recurrent hepatitis C after LDLT. Of the 125 patients, 69 (55%) were male, and the median age was 57 years (range: 15–70) at the beginning of the therapy. Most patients were infected with HCV genotype 1b (n = 103, 82%). HCV genotypes of the remaining patients were 2a (n = 13), 2b (n = 5), 3a plus 3b (n = 1), not determined (n = 2), and not examined (n = 1). Median serum HCV RNA load was 410 kIU/mL (range: <0.5–5000<kIU/mL) before LDLT, and 3260 kIU/mL (range: 31–69000<kIU/mL) at the beginning of the interferon therapy after LDLT. The median donor age was 41 (range: 19–65) years. Seventy-two donors (58%) were male, and 86 (69%) were related to the recipients. The graft type was the right lobe in 109 patients (87%), and the left lobe in 16 patients (13%). The blood type combination was incompatible in 26 patients (21%). The median time to treatment initiation after LDLT was 9.0 months (1.1–85.3 months). Before treatment, the necroinflammatory activity was A1 or greater in all patients, and 104 patients (83%) had a fibrosis score of F1 or greater (METAVIR score). Tacrolimus-based immunosuppression was used in 116 patients (93%). Among patients receiving tacrolimus for immunosuppression, the mean serum trough level was 6.0 ng/mL (range: 2.0–12.7) at the initiation of interferon therapy. In addition to calcineurin inhibitors, mycophenolate mofetil and prednisolone were used at the initiation of the interferon treatment in 36 (29%) and 19 (15%) patients, respectively.

Efficacy of Interferon Therapy

Of the 125 patients who received interferon therapy, serum HCV RNA reached undetectable levels (less than 0.05 kIU/mL) within 48 weeks in 77 patients (62%) (Figure 1). These patients were defined as showing virological response (VR). Of the remaining 48 patients, 2 patients received treatment for less than 48 weeks, and 15 patients withdrew from the treatment protocol within 48 weeks because of worsening of liver function (n = 5), recurrent hepatocellular carcinoma (n = 2), ascites (n = 2), anemia (n = 1), leucopenia (n = 1), brain hemorrhage (n = 1), biliary complication (n = 1), sepsis (n = 1), or myocardial infarction (n = 1). The remaining 31 patients with detectable HCV RNA in the serum 48 weeks after the initiation of the treatment were placed in the non-VR group. All patients in the non-VR group received peginterferon plus ribavirin therapy, including 9 patients who had previously treated with standard interferon plus ribavirin and did not achieve SVR. Of the patients with VR, 11 discontinued the treatment protocol within 24 weeks after serum HCV-RNA became negative, and 6 patients are still under treatment. The reasons for discontinuation were biliary complications (n = 2), worsening of liver function (n = 2), general fatigue (n = 2), recurrent hepatocellular carcinoma (n = 1), leucopenia (n = 1), hemoptysis (n = 1), brain tumor (n = 1), and depression (n = 1). Of 60 patients who achieved VR and completed the treatment protocol, 50 achieved SVR and 10 relapsed. None of the non-VR patients achieved VR even after more than 48 weeks of treatment, and were classified as nonresponder (NR).

In summary, among the 117 patients in whom the final outcomes of the treatment could be evaluated, 50 patients (43%) achieved SVR, and the remaining 67 patients, including 10 who relapsed (9%), 31 NR (26%), and 26 withdrawals (22%), were classified as non-SVR.

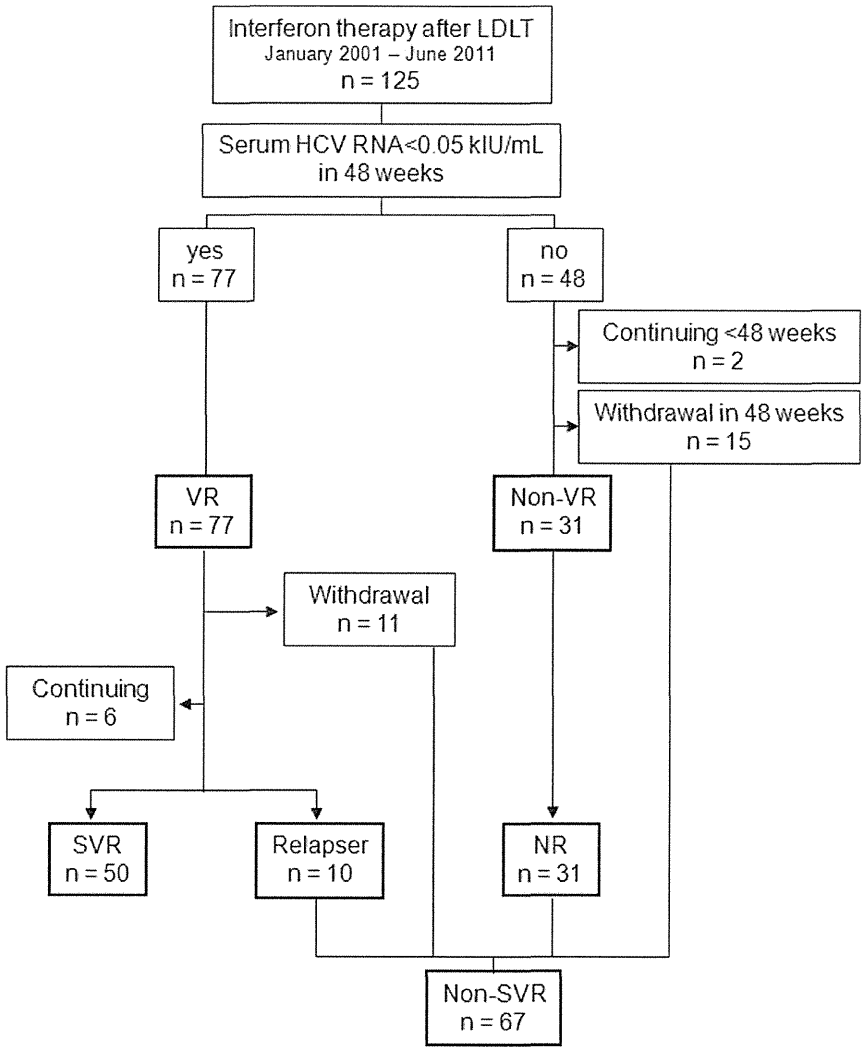

Figure 1. Flow diagram showing the outcome of interferon therapy in patients with recurrent hepatitis C after living donor liver transplantation (LDLT) and indicating the classification of patients in this study. N, number of patients; VR, virological response; SVR, sustained virological response; NR, nonresponder.
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Factors Predictive of Virological Response

Factors that could predict virological response were analyzed by comparing patients in the VR (n = 77) and non-VR (n = 31) groups (Table 1). Univariate analysis demonstrated that a low pretransplant serum HCV RNA level (less than 500 kIU/mL, $P<0.001$; and less than 1000 kIU/mL, $P<0.001$), an ABO-mismatched donor ($P=0.036$), HCV genotype (non-1, $P=0.001$), and a low pretreatment serum HCV RNA level (less than 5000 kIU/mL, $P=0.020$) were significantly associated with VR. There were no significant associations with any other variables, including donor factors. Multivariate analysis revealed that the 3 variables that retained a significant association in the model were a pretransplant serum HCV RNA level less than 500 kIU/mL [odds ratio (OR): 0.178, 95% confidence interval (CI): 0.054–0.535, $P=0.001$], a non-1 HCV genotype (OR: 0.087, 95% CI: 0.000–0.589, $P=0.008$), and an ABO-mismatched donor (OR: 5.492, 95% CI: 1.004–58.06, $P=0.049$) (Table 2). All 20 patients with a non-1 HCV genotype achieved VR, while VR rate in patients with the HCV genotype 1 was 65% (57 out of 88 patients). In the patients with HCV genotype 1, VR rate was 80% (36 of 45 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL and 42% (15 of 36 patients) when it was 500 kIU/mL or more. Among 22 recipients from ABO-mismatched donors, 20 patients (91%) showed VR, while 57 (66%) out of 86 patients who underwent LDLT from an ABO-matched (identical and compatible) donor achieved VR.

Factors Predictive of SVR

The same variables were analyzed to clarify factors that predicted SVR by comparing patients in the SVR (n = 50) and non-SVR (n = 67) groups (Table 1). By univariate analysis, the same variables that had a significant association with VR were identified as significant predictive factors for SVR–low pretransplant serum HCV RNA levels (less than 100 kIU/mL, $P=0.028$;

Table 1. Baseline predictive factors before liver transplantation (pre-LT), at liver transplantation (at LT), and before interferon therapy (pre-IFN) associated with virorogical response (VR) and sustained VR (SVR): Univariate analysis.

		VR	non-VR	*P*	SVR	non-SVR	*P*
		n = 77	n = 31		n = 50	n = 67	
Age at LT (years)		55 (8−67)	56 (37−69)	0.462	54.5 (8−67)	56 (30−69)	0.212
Gender	Male	45 (74%)	16 (26%)	0.518	30 (46%)	35 (54%)	0.404
	Female	32 (68%)	15 (32%)		20 (38%)	32 (62%)	
HCC pre-LT	No	29 (71%)	12 (29%)	0.919	18 (43%)	24 (57%)	0.984
	Yes	48 (72%)	19 (28%)		32 (43%)	43 (57%)	
MELD pre-LT		15.5 (3−51)	15 (6−25)	0.403	16 (3−51)	15 (0−43)	0.616
Child-Pugh pre-LT	A/B	35 (74%)	12 (26%)	0.488	25 (49%)	26 (51%)	0.192
	C	41 (68%)	19 (32%)		24 (37%)	41 (63%)	
	unknown	1	0		1	0	
Serum HCV RNA pre-LT	<100 kIU/mL	16 (89%)	2 (11%)	0.063	11 (65%)	6 (35%)	0.028
	100 kIU/mL≤	52 (65%)	28 (35%)		31 (35%)	57 (65%)	
	unknown	9	1		8	4	
Serum HCV RNA pre-LT	<500 kIU/mL	50 (85%)	9 (15%)	<0.001	30 (55%)	25 (45%)	0.002
	500 kIU/mL≤	18 (46%)	21 (54%)		12 (24%)	38 (76%)	
	unknown	9	1		8	4	
Serum HCV RNA pre-LT	<1000 kIU/mL	56 (81%)	13 (19%)	<0.001	34 (49%)	36 (51%)	0.013
	1000 kIU/mL≤	12 (41%)	17 (59%)		8 (23%)	27 (77%)	
	unknown	9	1		8	4	
HCV genotype	Non-1	20 (100%)	0 (0%)	0.001	15 (79%)	4 (21%)	0.002
	1	57 (65%)	31 (35%)		35 (36%)	62 (64%)	
	unknown				0	1	
Donor age at LT (years)		42 (20−63)	38 (21−61)	0.504	43 (20−60)	38 (19−63)	0.748
Donor gender at LT	Male	41 (67%)	20 (33%)	0.287	27 (40%)	40 (60%)	0.538
	Female	36 (77%)	11 (23%)		23 (46%)	27 (54%)	
Sex mismatch	Match	28 (72%)	11 (28%)	0.932	18 (43%)	24 (57%)	0.984
	Mismatch	49 (71%)	20 (29%)		32 (43%)	43 (57%)	
ABO mismatch	Match	57 (66%)	29 (34%)	0.036	38 (40%)	56 (60%)	0.310
	Mismatch	20 (91%)	2 (9%)		12 (52%)	11 (48%)	
Relation of donor	Nonrelated	24 (73%)	9 (27%)	0.827	16 (44%)	20 (56%)	0.803
	Related	53 (71%)	22 (29%)		34 (42%)	47 (58%)	
Graft type	Left lobe	13 (81%)	3 (19%)	0.347	8 (62%)	5 (38%)	0.155
	Right lobe	64 (70%)	28 (30%)		42 (40%)	62 (60%)	
Splenectomy	No	38 (68%)	18 (32%)	0.413	25 (39%)	39 (61%)	0.378
	Yes	39 (75%)	13 (25%)		25 (47%)	28 (53%)	
Age pre-IFN (years)		57 (15−68)	57 (41−70)	0.494	56 (15−68)	57 (32−70)	0.200
Months from LT to therapy		9.2 (1.1−85.3)	8.9 (1.8−59.0)	0.846	9.0 (1.3−85.3)	9.0 (1.3−72.4)	0.879
Trough level for tacrolimus (ng/mL) pre-IFN		5.9 (2.0−10.9)	6.4 (3.3−10.6)	0.323	6.2 (2.2−9.5)	5.9 (2.0−12.7)	0.933
MMF pre-IFN	No	55 (71%)	23 (29%)	0.772	36 (43%)	48 (57%)	0.966
	Yes	22 (73%)	8 (27%)		14 (42%)	19 (58%)	
Prednisolone pre-IFN	No	64 (70%)	28 (30%)	0.347	41 (41%)	60 (59%)	0.245
	Yes	13 (81%)	3 (19%)		9 (56%)	7 (44%)	
Serum HCV RNA pre-IFN	<1000 kIU/mL	17 (89%)	2 (11%)	0.064	8 (38%)	13 (62%)	0.583
	1000 kIU/mL≤	58 (67%)	29 (33%)		42 (45%)	52 (55%)	
	unknown	2	0		0	2	
Serum HCV RNA pre-IFN	<5000 kIU/mL	52 (78%)	15 (22%)	0.020	36 (50%)	36 (50%)	0.030
	5000 kIU/mL≤	18 (55%)	15 (45%)		10 (28%)	26 (72%)	
	unknown	7	1		4	5	

Table 1. Cont.

		VR	non-VR	P	SVR	non-SVR	P
		n = 77	n = 31		n = 50	n = 67	
White cell count (102/mL)		51 (13–114)	49 (17–98)	0.135	49 (18–114)	48.5 (13–99)	0.049
Neutrophil count (102/mL)		26 (8–89)	22 (11–58)	0.127	26 (11–89)	23 (8–61)	0.044
Hemoglobin (g/dL)		12.0 (9.2–17.2)	12.0 (8.9–17.9)	0.638	12.0 (9.4–17.2)	11.8 (8.9–17.9)	0.157
Platelet count (104/mL)		21.7 (4.7–58.1)	15.1 (4.3–40.0)	0.153	20.3 (5.0–58.1)	15.8 (4.3–45.8)	0.165
AST (IU/L)		78 (19–352)	72 (25–464)	0.677	85 (21–352)	75 (24–547)	0.887
ALT (IU/L)		93 (18–395)	82 (21–392)	0.544	106 (22–395)	82 (18–597)	0.251
ALP (IU/L)		461 (199–1985)	433 (168–2977)	0.345	470 (204–1985)	470 (168–2977)	0.610
g-GTP (IU/L)		118.5 (15–1623)	114 (20–1827)	0.856	141 (15–1623)	115 (20–1827)	0.356
Bilirubin (mg/dL)		0.9 (0.3–11.0)	0.9 (0.3–10.4)	0.827	0.9 (0.4–11.0)	1.0 (0.3–13.7)	0.611
Activity grade pre-IFN	A1	54 (75%)	18 (25%)	0.448	35 (47%)	40 (53%)	0.517
	A2	22 (65%)	12 (35%)		14 (36%)	25 (64%)	
	A3	1 (50%)	1 (50%)		1 (33%)	2 (67%)	
Fibrosis stage pre-IFN	F0	9 (60%)	6 (40%)	0.446	6 (32%)	13 (68%)	0.530
	F1	54 (75%)	18 (25%)		34 (46%)	40 (54%)	
	F2/3	14 (67%)	7 (33%)		10 (42%)	14 (58%)	
Steatosis (5%<) pre-IFN	No	40 (69%)	18 (31%)	0.609	27 (42%)	38 (58%)	0.633
	Yes	36 (73%)	13 (27%)		23 (46%)	27 (54%)	
	unknown	1	0		0	2	
Cholestasis pre-IFN	No	58 (71%)	24 (29%)	0.903	38 (42%)	53 (58%)	0.577
	Yes	18 (72%)	7 (28%)		12 (48%)	13 (52%)	
	unknown	1	0		0	1	

NOTE. Qualitative variables are shown in number; and quantitative variables expressed as median (range). P-values are calculated by Wald test for logistic regression analysis.

LT, liver transplantation; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; HCV, hepatitis C virus; MMF, mycophenolate mofetil; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; g-GTP, gamma-glutamyl transpeptidase.

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less than 500 kIU/mL, $P = 0.002$; and less than 1000 kIU/mL, $P = 0.013$), HCV genotype (non-1, $P = 0.002$), and low pretreatment serum HCV RNA levels (less than 5000 kIU/mL, $P = 0.030$). In addition, white cell count ($P = 0.049$) and neutrophil count ($P = 0.044$) before interferon therapy were significantly associated with SVR. Multivariate analysis showed that 2 variables were independently associated with SVR–a non-1 HCV genotype (OR: 0.182, 95% CI: 0.054–0.614, $P = 0.006$), and pretransplant serum HCV RNA levels lower than 500 kIU/mL (OR: 0.310, 95% CI: 0.130–0.742, $P = 0.009$) (Table 3). SVR rate among patients with a non-1 HCV genotype was 79% (15 of 19 patients) on average, 83% (10 of 12 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL, and 50% (2 of 4 patients) when it was 500 kIU/mL or more. In patients with HCV genotype 1, SVR rate was 36% (35 of 97 patients) on average, 47% (20 of 43 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL, and 22% (10 of 45 patients) when it was 500 kIU/mL or more.

Amino Acid Substitutions in Core Region of HCV

To determine the viral factors that predicted VR and SVR in patients infected with HCV genotype 1b, association of aa substitutions at aa 70 of arginine or glutamine/histidine and aa

Table 2. Predictive factors associated with virological response (VR): Multivariate analysis.

		Odds Ratio	95% confidence intervals	P-value
Serum HCV RNA pre-LT	<500 kIU/mL	1	-	-
	500 kIU/mL≤	0.178	0.054–0.535	0.001
HCV genotype	Non-1	1	-	-
	1	0.087	0.000–0.589	0.008
ABO mismatch	Match	1	-	-
	Mismatch	5.492	1.004-58.06	0.049

HCV, hepatitis C virus; LT, liver transplantation.

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