Dynamics of defective hepatitis C virus clones in reinfected liver grafts in liver transplant recipients; ultra-deep sequencing analysis

Shigeru Ohtsuru1,2, Yoshihide Ueda1(#), Hiroyuki Marusawa1, Tadashi Inuzuka1, Norihiro Nishijima1, Akihiro Nasu1, Kazuharu Shimizu3, Kaoru Koike2, Shinji Uemoto4 and Tsutomu Chiba1

1 1. Department of Gastroenterology and Hepatology, Graduate School of Medicine
2 2. Department of Primary Care and Emergency Medicine, Graduate School of Medicine
3 3. Department of Nanobio Drug Discovery, Graduate School of Pharmaceutical Sciences
4 4. Department of Surgery, Graduate School of Medicine
5 Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Running title: Defective HCV clones in liver transplantation

Corresponding author: Yoshihide Ueda, M.D., Ph.D.

Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.

Phone: +81-75-751-4302, Fax: +81-75-751-4303, E-mail: yueda@kuhp.kyoto-u.ac.jp
ABSTRACT

Hepatitis C virus (HCV) reinfects liver allografts in transplant recipients, replicating immediately after transplantation, followed by a rapid increase in serum HCV RNA levels. We evaluated dynamic changes in the viral genetic complexity after HCV reinfection of the graft liver and identified the characteristics of replicating HCV clones using a massive-parallel ultra-deep sequencing technique to determine full-genome HCV sequences in the liver and serum of five transplant recipients with genotype 1b HCV infection before and after liver transplantation. Recipients showed extremely high genetic heterogeneity before transplantation, and the HCV population was not significantly different between the liver and serum of each individual. Viral quasispecies complexity in the serum was significantly lower after liver transplantation than before, suggesting that specific HCV clones selectively proliferated after transplantation. Defective HCV clones lacking the structural region of the HCV genome did not increase and full-genome HCV clones selectively increased immediately after liver transplantation. Re-increase of the same defective clone existing before transplantation was detected 22 months after transplantation in one patient. Ultra-deep sequencing technology revealed reduced genetic heterogeneity of HCV after liver transplantation. Dynamic changes in defective HCV clones after liver transplantation indicate that these clones have important roles in the HCV life cycle.

Key words: hepatitis C, liver transplantation, living donor
INTRODUCTION

The hepatitis C virus (HCV) has an approximately 9.6-kb plus-strand RNA genome that encodes the viral core, E1, E2, and p7 structural proteins, and 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 be evolving 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. Serum HCV RNA levels then rapidly increase to levels 10- to 20-fold higher than pretransplant levels. It is thus hypothesized that specific HCV clones with growth advantages increase after liver transplantation. Although several studies have attempted to clarify the change in genetic heterogeneity after liver transplantation, the abundant diversity and complexity of HCV has been an obstacle to the detailed evaluation of viral genetic heterogeneity. The recent introduction of ultra-deep sequencing technology, 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 ultra-deep sequencing analyses to unveil the levels of viral quasispecies of genotype 1b HCV in the livers and the sera of 5 patients who underwent living donor liver transplantation (LDLT), and clarified the changes in the viral genetic complexity after reinfection of HCV to the graft liver. In the analysis, we found that the population of defective HCV clones that lack structural regions of the
HCV genome dynamically changed after liver transplantation. We then clarified the dynamics and characteristics of the defective HCV clones.
MATERIALS AND METHODS

Patients

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 liver transplantation and 1 month after liver transplantation. In addition, a serum sample of 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 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 number 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 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 developed by Ohno et al. (5).

Serum HCV RNA load was evaluated before LDLT, and at 1 month and then every 3 months after LDLT using PCR and an Amplicor HCV assay (Cobas 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) from May 2008.

Direct population Sanger sequencing

To define the representative reference sequences of full-length HCV in each clinical specimen, serum samples 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 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 (~5000 base pairs [bp]) and latter-half (~4500 bp) of the genotype 1b HCV genome sequences. PCR products purified by the QIAquick Gel Extraction kit (QIagen) were assayed for direct sequencing. Nucleotide sequences of PCR products were determined using an ABI Prism Big Dye Terminator Ready Reaction Kit (Applied Biosystems). Serum from 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 (6, 7). Briefly, the viral genome sequences were amplified with high-fidelity PCR and sheared by nebulization using 32 pounds per square inch N2 for 8 min and the sheared fragments were purified and concentrated using a 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. 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 following the manufacturer’s instructions. The obtained images were analyzed and base-called using GA pipeline software version 1.4 with default settings provided by Illumina. Validation of the multiplex ultra-deep sequencing of the HCV genome was performed using a plasmid encoding full-length HCV as a template, as reported previously (6). Overall error rates were determined to be a mean of 0.0010 per base pair. We also confirmed that high-fidelity PCR amplification with HCV-specific primer sets followed by multiplex ultra-deep 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%–0.13%) (6).
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. Low quality bases were trimmed from the 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.

Detection of defective HCV clones

The methods for detecting defective HCV clones were reported previously (8). Briefly, reverse transcription (RT)-PCR was performed using the One Step RNA PCR Kit (Takara) with the extracted RNA from liver and serum as template and two pairs of primers, 5’-CGCCGACCTCATGGGGTACA-3’ and 5’-TGGTGTACATTTGGGTGATT-3’ for first RT-PCR (HCV-P1), and 5’-TGCTCTTTCTCTATCTTCCT-3’ and 5’-GTGATGATGCAACCAAGTAG-3’ for the second PCR (HCV-P2). 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 Big Dye Terminator Ready Reaction Kit (Applied Biosystems). To determine the 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 population. Genetic complexity was determined by Shannon entropy values calculated as follows:

\[ S_n = -\frac{\sum_{i=1}^{n} f_i \ln f_i}{N} \]

where \( n \) is the number of different species identified, \( f_i \) 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 complexity between two groups were made using the Wilcoxon rank sum test or the Mann–Whitney U-test. P values of less than 0.05 were considered statistically significant.
RESULTS

Patient characteristics

The clinical and virologic 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 LDLT was 52 years (range, 47–65 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 were infected with HCV genotype 1b. Median serum HCV RNA load before transplantation was 5.5 logIU/mL (range, 4.6–6.6 logIU/mL), and these became 5.9 logIU/mL (range, 5.8–6.4 logIU/mL) 1 month after liver transplantation, showing no significant difference (p = 0.18).

HCV population did not significantly differ between liver and serum

To clarify the landscape of HCV heterogeneity as a quasispecies, we determined the viral full-genome sequences in liver and serum derived from the 5 recipients before transplantation by multiplex ultra-deep sequencing and compared the results with those obtained by the direct population Sanger sequencing method. HCV nucleotide sequence reads by ultra-deep 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 number of 1548-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 of HCV in the liver were compared with those in the serum. The overall viral complexity determined by Shannon entropy value did not
significantly differ between the liver and serum of each individual (Supplemental Figure 1). Moreover, the pattern and distribution of genetic heterogeneity of the viral nucleotide sequences in the liver tissue was similar to those observed in the serum of the same patient (Supplemental Figure 2). Next, we compared the sequences of viral genome 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 HCV protease and polymerase inhibitors (Supplemental Table 1). The prevalence of these mutations of the HCV genome in the liver was similar to that in the serum of the same patients. These findings suggested that 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 change in viral complexities of the serum before and 1 month after liver transplantation in these 5 patients. Mean coverage of 1284-fold and 1141-fold was mapped to each reference sequence before and after liver transplantation, respectively. We then estimated the genomic complexity by calculating the Shannon entropy for each nucleotide position before and after liver transplantation (Figure 1A). The level of viral complexity of serum HCV significantly differed between pre-transplantation and post-transplantation (mean Shannon entropy value 0.056 vs. 0.029, p=0.043), demonstrating that the viral quasispecies nature after reinfection and replication in the graft liver became more homogeneous compared with those before transplantation. To identify the specific regions in the HCV genome for the selective increase in HCV after
liver transplantation, we analyzed the changes of complexity in each region of HCV (Figure 1B). A decrease in the genetic complexity after liver transplantation was observed throughout the individual viral genetic regions. In particular, the complexity between pre- and post- transplantation was significantly different in the NS4A, NS4B, NS5A, and NS5B regions, suggesting these regions are important for active proliferation of HCV at the early phase of reinfection to the graft liver. We then examined whether a specific nucleotide position was associated with the decrease of complexity after liver transplantation, but none of the specific nucleotide positions that changed by more than 50% after liver transplantation compared to those before transplantation were identified commonly among the 5 patients (data not shown), indicating no association between the specific nucleotide position and the decrease in complexity after liver transplantation.

Defective HCV clones became undetectable immediately after liver transplantation

Using the ultra-deep 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 (Figure 2). As we previously identified the defective HCV clones lacking the structural regions of HCV genome in serum of HCV-positive liver transplant recipients (8), we speculated that 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 before and after liver transplantation.
Using RT-PCR analysis with primers HCV-P1 and HCV-P2 (Figure 3A), we detected both defective HCV clones and full-genome HCV clones before liver transplantation at various ratios in each sample except for that of patient #3 (Figure 3B). The defective HCV clones became undetectable, and the full genome HCV clones became dominant in serum samples 1 month after liver transplantation, indicating that the defective HCV clones have less of a replication advantage. In patient #3, defective HCV clones were undetectable both before and after liver transplantation.

To determine the internal structure of these deletions, 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 serum of these patients are shown in Figure 4. Sequence analyses revealed that the structural region was widely deleted in all of the defective HCV clones. The 3'-boundary of the deletions was quite diverse among 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 right-hand column for patent #5 in Figure 3B, RT-PCR from a serum sample collected at 22 months after liver transplantation, when liver biopsy demonstrated findings of chronic hepatitis C with fibrosis (A1 F1 in METAVIR score), showed that a defective HCV clone had reappeared. The size of the defective clone was the same as that shown 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 pre-transplant serum sample. The viral complexity analyzed by calculating the Shannon entropy from ultra-deep sequencing data also recovered to the pre-transplantation level at the chronic hepatitis phase (Shannon entropy value 0.056 before transplantation, 0.022 at 1 month, and 0.069 at 22 months after liver transplantation). These findings indicated that reconstitution of HCV heterogeneity occurs at the chronic hepatitis phase after liver transplantation, and the same defective HCV clone present before liver transplantation re-appears at the chronic hepatitis phase after liver transplantation.
DISCUSSION

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

Differences in the populations of HCV quasispecies between the liver and serum of the same individuals have 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), and the findings were conflicting. In the present study, we obtained full-genome HCV sequences using ultra-deep sequencing analysis. Our results suggested that a similar HCV population exists in the liver and 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 analysis of resistance and sensitivity to treatment.

Differences in the HCV population among individuals can be determined by multiple factors such as duration of hospital visit and route of HCV infection, fibrosis progression, degree of inflammation, and presence of hepatocellular carcinoma. In our analysis, we could not find an association between these clinical characteristics and
HCV population. However, we speculated that undetectable defective HCV clones present before liver transplantation in patient #3 might be associated with a shorter duration of the HCV infection. In patients #1 and #3, the difference of viral complexity presented by the Shannon entropy between before and after liver transplantation was small. The reason is unclear at present, but differences in the clinical features might affect the results. Further large-scale investigations may reveal the relation between clinical features and HCV population.

Our large-scale analysis using ultra-deep sequencing demonstrated that the complexity of all regions of the HCV genome was dramatically reduced 1 month after liver transplantation compared with the pre-transplantation level. 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. analyzed HCV quasispecies before and after liver transplantation by comparing the differences in the hypervariable region of HCV in 5 transplant recipients. They found that different HCV clones were present in pre-transplant serum and relatively homogeneous quasispecies variants emerged after liver transplantation in all 5 cases (16). Hughes et al. demonstrated that the viral complexity of the hypervariable region 1 in post-perfusion liver at 2.5 h after liver transplantation was significantly lower than that in explanted liver and in pre-transplant serum, although there was no significant difference in the complexity between explanted liver and pre-transplant serum (17). Our present data confirmed the results of these previous studies, and added new information from the full genome ultra-deep sequence. In particular, our data demonstrated a new aspect in the analysis of full genome and defective HCV clones, because the defective HCV clones lack
hypervariable regions that were analyzed in the previous papers. Interestingly, our
analysis revealed a significant decrease in complexity in the NS4A, NS4B, NS5A, and
NS5B regions, 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 preferential decrease in the complexity of the NS4A-NS5B
sequence after liver transplantation might indicate the presence of the specific
NS4A-NS5B sequence of the HCV genomes that has advantages for reinfection and/or
replication. Therefore, we attempted to identify the specific HCV genome sequences
with such advantages. A common feature of the HCV genomic change in amplified
HCV clones after liver transplantation could not be identified, however, among 5 cases
tested. This may be due to differences among individuals in the relative fitness of a viral
subpopulation in a host, which is determined by multiple factors, including infection
capacity, replication ability, and escape mechanism from immune pressure.

We previously identified defective HCV clones in the 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 were present mainly in the
structural region, while the 5’ untranslated region, the core, and NS3-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.
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 (24). Pacini et al. also reported that defective HCV clones show robust
replication, efficient trans-packaging, and infection of cultured cells (23). These data
suggest that the ability of defective HCV genomes for infection, replication, and encapsidation does not differ from that of full-genome HCV. The in vivo data reported here, however, clearly revealed 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 capability of infection, replication, or encapsidation differs between defective HCV and full-genome HCV in vivo. It is noteworthy that an identical defective HCV clone that was detected before transplantation reappeared in the chronic hepatitis phase after transplantation in patient #5. This finding suggests that the defective clone in the 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 massive-parallel ultra-deep sequencing technology in the analyses of viral quasispecies. 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.

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

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REFERENCES


10. Wolinsky SM, Korber BT, Neumann AU, Daniels M, Kunstman KJ, Whetsell AJ,
immunodeficiency virus type 1 during the natural course of infection. Science
272:537-542.

2000. Nucleotide and amino acid complexity of hepatitis C virus quasispecies in

hepatitis C virus 5' untranslated region quasispecies in serum and liver. J Gen Virol
80 (Pt 3):711-716.

hepatitis C virus in serum and in three different parts of the liver of patients with

1998. Comparison of serum and liver hepatitis C virus quasispecies in HCV-related

I, Murakami T, Marumo F, Sato C. 1995. Comparison of the hypervariable region of

Tracking hepatitis C virus quasispecies major and minor variants in symptomatic

17. Hughes MG, Jr., Rudy CK, Chong TW, Smith RL, Evans HL, Iezzoni JC, Sawyer RG,
Pruett TL. 2004. E2 quasispecies specificity of hepatitis C virus association with
allografts immediately after liver transplantation. Liver Transpl 10:208-216.

proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA

19. Moradpour D, Brasca V, Bieck E, Friebe P, Gosert R, Blum HE, Bartenschlager R,

2004. Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C


Figure 1. Changes in the genetic complexity of the HCV genome before and after liver transplantation.

(A) Mean Shannon entropy values for the overall HCV genome in 5 LDLT recipients before and after liver transplantation are shown. (B) Mean Shannon entropy values for each HCV genomic region before (black bars) and after (white bars) liver transplantation are shown. Error bars represent the standard deviation. *: p<0.05, ns: nonsignificant.

Figure 2. Dynamics of defective HCV clones indicated by coverage numbers of ultra-deep sequence of HCV genome.

Coverage of ultra-deep sequence of HCV genome in liver (A: upper panel) and serum samples before liver transplantation (B: middle panel), and serum sample after liver transplantation (C: lower panel) for patient #1. Number of coverage (fold) at each nucleotide site of the HCV sequence is shown. Nucleotide number 1 indicates the first nucleotide of the core region of HCV RNA. Similar results were obtained in the samples of patients #2, #4, and #5.

Figure 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 templates, which were extracted from serum before and 1 month after liver transplantation in all patients, and 22 months after transplantation in patient #5. HCV-P1 and HCV-P2 shown in Figure 3A.
were used as primers. Lanes M1 and M2 indicate molecular weight markers, MassRuler DNA Ladder Mix (Fermentas, Canada) and Lambda-DNA Hind III Digest (BioLabs, USA), respectively. Values indicate the sizes of the band in the molecular weight markers. Black arrowheads indicate a full-length PCR fragment of 2618 bp, and white arrowheads indicate defective HCV clones that were confirmed by sequencing analysis. Shannon entropy values of these HCV specimens in the serum are shown at the bottom.

**Figure 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.
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<th>2</th>
<th>3</th>
<th>4</th>
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<td>(22 mo)</td>
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HCV: hepatitis C virus, HCC: hepatocellular carcinoma, MELD: model for end-stage liver disease, LDLT: living donor liver transplantation, MMF: mycophenolate mofetil, PSL: prednisolone
Figure 1. Ohtsuru et al
Figure 2. Ohtsuru et al

A. Liver coverage number

B. Serum before liver transplantation coverage number

C. Serum after liver transplantation coverage number

HCV genomic region

<table>
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<tr>
<th>Core</th>
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<th>E2</th>
<th>P7</th>
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<th>NS3</th>
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Figure 3. Ohtsuru et al

A

\[
\begin{array}{ccccccc}
& \text{Core} & t_1 & t_2 & P & NS2 & NS3 & NS4/NS5 \\
5' & \uparrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & 3' \\
\text{HCV-P1} & \text{2919 bp} & \text{HCV-P2} & \text{2618 bp} &
\end{array}
\]

B

Shannon entropy
before after before after before after before after before after

| Patient #1 | 0.036 | 0.033 |
| Patient #2 | 0.093 | 0.026 |
| Patient #3 | 0.081 | 0.060 |
| Patient #4 | 0.046 | 0.017 |
| Patient #5 | 0.086 | 0.022 |

(22mo)
Figure 4. Ohtsuru et al