CD_{81} Binding Regions of Hepatitis C Virus Remain Conserved After Liver Transplantation

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CD_{81} is a surface-associated protein expressed in the membranes of mammalian cells. It has been suggested that CD_{81} interacts with hepatitis C virus E2 protein, and thus might facilitate the entry of HCV into hepatocytes. The envelope-binding site appears to involve amino acids (aa) 480-493 and 544-551 within the E2 glycoprotein. Little is known about the quasispecies genetic diversity of these two regions. We studied four patients who underwent transplantation for HCV-related cirrhosis and who developed recurrent hepatitis C. We evaluated HCV quasispecies diversity in serum samples obtained at the time of transplantation and at several time points thereafter. Quasispecies diversity was assessed by cloning and sequencing of viral isolates, with computer analysis of evolution models. The genetic distance in the region that spans aa 480 to 493 was 0.019 ± 0.004 before the transplant, and 0.039 ± 0.014 after the transplant (p=0.324). In the aa 544 to 551 region, the pre-transplant genetic distance was 0.012 ± 0.008 and the post-transplant distance, 0.010 ± 0.007 (p=0.890). There was also no significant difference between the number of nonsynonymous substitutions per nonsynonymous site before and after transplantation. In conclusion, the HCV genetic sequences of putative CD_{81} binding regions aa 480-493 and aa 544-551 did not diversify significantly after liver transplantation. This may favor HCV re-infection of the allograft after liver transplantation.

Key Words: Hepatitis C virus, CD_{81}, diversity.

Abbreviations: HCV = hepatitis C virus; OLT = orthotopic liver transplantation; AA = amino acids.

Hepatitis C virus (HCV) is a positive-strand RNA virus member of the Flaviviridae family, and it has been recognized as a major causative agent of chronic liver disease, including chronic active hepatitis, cirrhosis and hepatocellular carcinoma [1]. The HCV genome is subject to considerable variability, which may lead to the appearance of the quasispecies population, HCV variants with closely related genetic codes whose sequences differ only by a few nucleotides [2].

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Chronic hepatitis C infection is also a major indication for liver transplantation worldwide [3] and re-infection of the allograft by the virus invariably occurs [4,5]. The mechanisms by which HCV enters target cells are not yet well known.

CD_{81} is a widely expressed cell membrane-associated protein that belongs to the tetraspanin family [6]. It contains four transmembrane domains and two extracellular loops. Recently, it has been demonstrated that CD_{81} interacts with E2 protein [7,8] and thus it might be the cellular receptor for HCV. Binding of the hepatitis C virus E2 glycoprotein to CD_{81} may be strain specific [9] and could inhibit natural killer cell functions [10].

Flint et al. have suggested that the HCV envelope-binding site is of a conformational nature and involves aa 480 to 493 and 544 to 551 within the E2 glycoprotein [11].

We evaluated and compared the genetic diversity of these two putative CD_{81} binding sites within E2, before and after liver transplantation. Our hypothesis is that these regions should remain conserved after organ transplant, which may facilitate the binding of the virus to the CD_{81} protein in the hepatocytes and re-infection of the liver after transplantation.

Material and Methods

The study group was comprised of four patients who underwent liver transplantation for HCV-related cirrhosis at Saint Louis University (Table1). These four cases were selected because all were infected with viral genotype 1 and there were stored serial serum samples available for analysis. Informed written consent was obtained from all subjects and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Serum samples in each patient were obtained on the day of the transplant (time point 0) and for at least one time point thereafter.

Total RNA was extracted from 100 µl serum using phenol chloroform extraction, as previously described [12], and resuspended in 60 to 80 µl of water.

HCV genotype was determined by restriction fragment length polymorphism (RFLP) assay of PCR products of the 5' UTR, as previously described [12], and HCV genotypes were classified according to the nomenclature of Simmonds et al. [13]

RNA samples were subjected to a nested RT-PCR amplification with primers for E1 and E2 region (Table 2) [14]. One of the following three primers was used for the reverse transcription reaction. Primer DPR1 was utilized for both subtypes 1a and 1b, primer EAR1 for subtype 1a, and EBR1 for subtype 1b. If primer DPR1 failed to amplify the HCV RNA, the others were used. Five microliters of the extracted RNA in water was added to 15 µl of an RT-PCR solution. The final concentration of this 20 µl reaction contained 1 X PCR buffer, 4.0 mM/L $MgCl_2$, 5.0 mM/L DTT, 1.5 mM/L of each of 4 dNTPs, 1.0 µM/L of primer, 16 U RNAsin, and 80 U Moloney Murine Leukemia Virus enzyme (MMLV) (Promega, Madison, WI). The reaction was carried out at 42°C for 60 minutes followed by 94°C for 5min. Subsequently, the first round of PCR was performed after adding 30 µL of a PCR mix containing 1 X PCR buffer, 1.5 mM/L MgCl2, 0.7 mM/L dNTPs, 0.7µM/L primer, and 1.25 U of Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) to the 20µl of the RT-reaction. Sense primer CF1 (Table 1) was utilized for this reaction. The PCR cycles consisted of one cycle of 94°C for 4 min, followed by 5 cycles (95°C, 1 min; 55°C, 1 min; 72°C, 2 min), and then 30 cycles (95°C, 30 sec; 55°C, 1 min; 72°C, 2 min), with a final 7 min extension (72°C). For the second amplification, 5 µl of the first PCR product was added to 45 µl of the PCR mix. The final concentration of the 50 µl reaction contained 1 X PCR buffer, 2.5 mM/L of MgCl2, 1.0 mM/L of each of the 4 dNTPs, 0.4µM/L of each primer, and 1.25 U of Taq polymerase enzyme (Perkin-Elmer-Cetus, Norwalk, CT). The oligonucleotides used for the second round of PCR were sense primer CF2 and anti-sense primers EAR2 for subtype 1a, and EBR2 for subtype 1b (Table 2). Identical cycle parameters were utilized for the second round of amplification. The expected amplicon was 1.38 kb in length and spanned most of E1 and part of E2, including two putative CD_{81} binding regions.

The 1.38 kb E1/E2 amplicon was digested with Eco RI and Bgl II, gel-purified and ligated into a digested pUC 19 vector containing the appropriate restriction sites. The plasmid with the HCV insert was transformed into competent *E. coli* JM109 cells and plated on LB agar plates containing ampicillin (100 µg/ml), and incubated overnight at 37°C. Four to ten clones were picked, and grown in LB medium containing 100 µg/ml of ampicillin. Plasmid DNA from the cultures were purified by the alkaline lysis method using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA).

All purified plasmids were sequenced using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Reactions were analyzed with an automated sequencer (ABI model 377-96).

Sequences were aligned and edited using CLUSTAL W [15] and GCG package (Oxford Molecular Group, Inc., version 10.0), and length

Table 1. Clinical and virological features of four liver transplant patients

Patient	Genotype	Serum time points evaluated for quasispecies		Stage of fibrosis after OLT
1	1a	Time 0	day of transplant	
		Time 1	60.3 months	1
2	1a	Time 0	day of transplant	
		Time 1	32.4 months	1
		Time 2	70 months	3
3	1b	Time 0	day of transplant	
		Time 1	11.6 months*	3
		Time 2	14.5 months **	2
		Time 3	26.8 months **	2
4	1a	Time 0	day of transplant	
		Time 1	8.7 months	3

* Patient was subjected to a re-transplant; ** After first liver transplantation.

Table 2. Primers utilized for PCR amplification (Fan et al., 2001)

Primer	Polarity	Sequence 5' 3'
DPR1	Antisense	AGCAGRAGTTTGGTGATGTC
EAR1	Antisense	TCCAGTTGCAGGCAGCWTCCAGCC
EBR1	Antisense	TCCARTTGCATGCRGCATYGAGCC
CF1	Sense	GACGGCGTGAACTATGCAACAGG
CF2	Sense	GTACTGAATTCGGTACCGGTTGCTCTTTCTCTATCTTCC
EAR2	Antisense	ACTCGAAGCTTAGATCTTTGATGGTACAAGGRTAATGCC
EBR2	Antisense	ACTCGAAGCTTAGATCTTTGASRGTGCARGGGTAGTGCC

polymorphisms were corrected after both alignment and visual inspection of all sequences from variants within each time point. Final fragments, 411 bp in length, spanning two putative CD_{81} binding regions within the E2 glycoprotein (aa 480 to 493 and 544 to 551) were analyzed utilizing the MEGA program [16]. The total number of nucleotide substitutions per site was estimated using the two-parameter method described by Kimura [17]. The number of nonsynonymous and synonymous nucleotide substitutions per nonsynonymous and synonymous site, respectively, was estimated using the Jukes-Cantor one-parameter method [18].

Quasispecies nucleotide intra-sample diversity or genetic distance was defined as the total number of nucleotide substitutions per site among all sequences analyzed at one time point. Similar definitions were utilized for synonymous and nonsynonymous nucleotide substitutions per synonymous and nonsynonymous site, respectively.

Table 3. Diversity of putative CD_{81} binding regions aa 480-493 and aa 544-551

Region	Before OLT	After OLT	P value
aa 480-493 (42 bp)			
Genetic distance*	0.019 ± 0.004	0.039 ±0.014	.324
Nonsynonymous†	0.010 ± 0.004	0.018 ± 0.010	.599
Synonymous‡	0.037 ± 0.025	0.115 ± 0.036	.166
aa 544-551 (24 bp)			
Genetic distance*	0.012 ± 0.008	0.010 ±0.007	.890
Nonsynonymous†	0.004 ± 0.004	0.004 ± 0.003	.866
Synonymous‡	0.044 ± 0.044	0.032 ± 0.021	.776

* Nucleotide substitutions per site.
† Nonsynonymous nucleotide substitutions per nonsynonymous site.
‡ Synonymous nucleotide substitutions per synonymous site.

Continuous variables were expressed as the mean ± SEM. They were analyzed using an unpaired, two-tailed Student T-test, with equal or unequal variances, depending on the distribution of each set based on analysis by the F-test (Levene's test for equality of variances). All analyses were performed utilizing the SPSS package (SPSS for windows release 10.0. SPSS Inc. Chicago, IL). A P value of <0.05 was considered to be statistically significant.

Results

A total of 73 clones from four patients were sequenced and analyzed. Fifteen of these clones were from samples obtained after a re-transplant in one patient. The GenBank accession numbers for these sequences are AF431816 to AF431888. All four patients were infected with HCV genotype 1. Three of them were infected with subtype 1a and one with subtype 1b. The mean time of follow-up after first transplantation for all patients was 38 months. After the first transplant, one patient had stage 2 fibrosis and three patients had stage 3 fibrosis, noted in the liver biopsy at the last time point in which quasispecies were analyzed (Table 2). One patient was subjected to a second liver transplantation after 11.6 months because of fibrosing cholestatic hepatitis. Fifteen months after this re-transplant he had fibrosis stage 2, noted at liver biopsy.

The mean values of nucleotide intra-sample diversity or genetic distance at time 0 of all patients in both putative CD_{81} binding regions was not significantly different from the mean values of intra-sample diversity for all time points combined after transplant (Table 3). There was also no significant difference between the number of nonsynonymous and synonymous nucleotide substitutions per nonsynonymous and synonymous site, respectively.

In fact, there were only a few differences in nucleotides and amino acids between sequences before and after transplantation in all patients (Figures 1 and 2). After re-transplantation of patient 3, both putative CD_{81} binding regions remained conserved up to approximately 15 months of follow-up (Figures 1 and 2).

Discussion

We found little diversity in the sequences of two putative CD_{81} binding regions located at aa 480-493 and aa 544-551, before and after liver transplantation. In fact, both regions were relatively conserved and only a few HCV variants differed by a few amino acids, confirming our initial hypothesis.

Figure 1. Hepatitis C virus amino acids sequences of putative CD_{81} binding-region aa 480 to 493, from all time points of all four patients

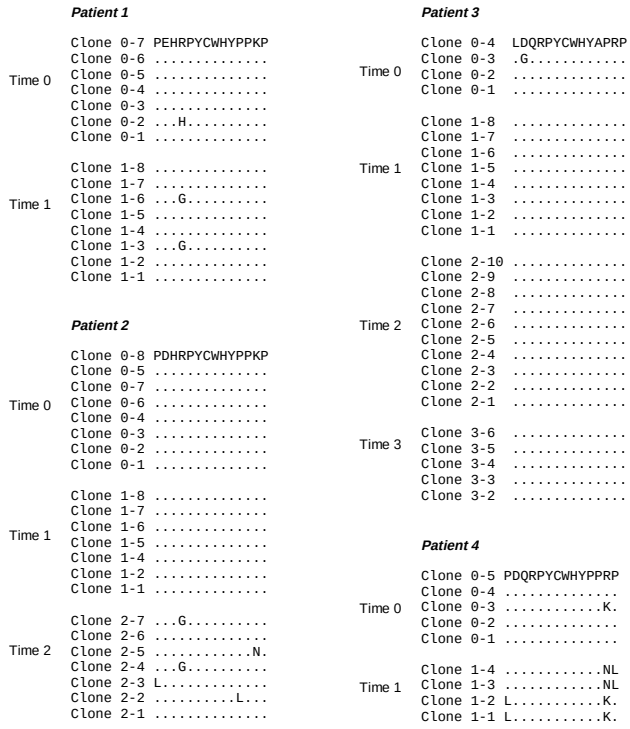
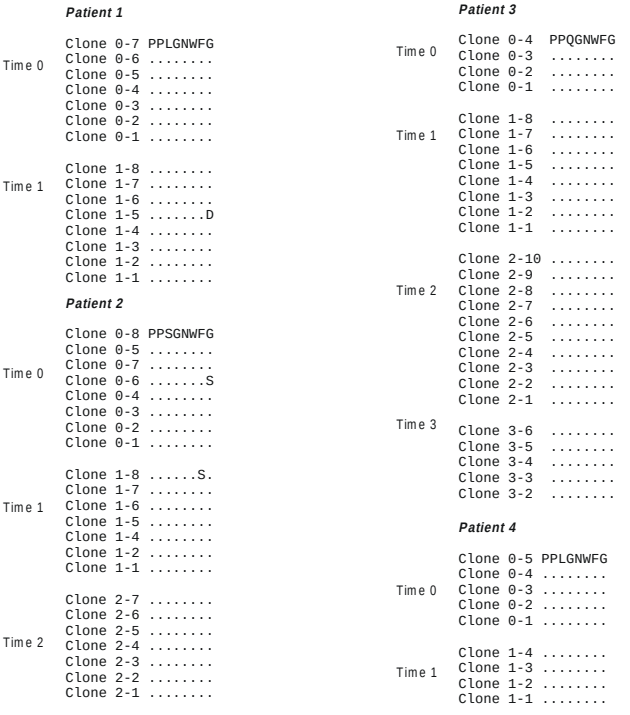


Figure 2. Hepatitis C virus amino acids sequences of putative CD_{81} binding-region aa 544 to 551, from all time points of all four patients



Liver transplantation appears to be a valuable model for evaluating HCV molecular evolution because the virus needs to infect another liver. Our results show that in the context of a new environment and transplant related factors, which include immunosuppression and a new liver, the genetic codes of both CD_{81} binding regions are kept relatively stable. Other regions of the HCV genetic code, such as hypervariable region 1 (HVR1), NS2 and NS3 have been reported to undergo considerable variability as soon as a few months after liver transplantation [19-21].

CD_{81} is a protein that is expressed in cell membranes. Several studies have suggested that CD_{81} interacts with E2 protein of hepatitis C virus [9,10], and thus, it is a potential cellular receptor or co-receptor for HCV entry into hepatocytes. Therapy with interferon alpha, in combination with ribavirin, appears to down-regulate cell surface-associated CD_{81} in peripheral blood lymphocytes of patients infected with HCV [22]. This further supports the concept that this protein is functionally involved in the interaction between the host and the virus. Thus, it is important to study the genetic diversity of the regions of the hepatitis C virus genome involved in CD_{81} binding, and its potential clinical implications. The envelope-binding site appears to be of conformational nature and has been suggested to involve aa 480 to 493 and 544 to 551 within the E2 glycoprotein of HCV [11]. The fact that these regions are conserved probably favors the binding of the virus to the CD_{81} protein in the hepatocytes and thus, the re-infection of the allograft after liver transplantation.

HCV quasispecies complexity and diversity may be evaluated by several means. Among the techniques used are the heteroduplex mobility assay (HMA) [23], which evaluates diversity by providing an estimation of the Hamming distances, PCR-single strand conformation polymorphism (PCR-SSCP) [12,24], which estimates the complexity of the virus, and cloning and sequencing. The latter is believed to be the "gold standard", and its usage permits measuring of the rate of nucleotide and amino acid substitutions in several ways. The disadvantages of this method are that it is labor intensive, and costly. We utilized cloning and sequencing, and evolutionary models, including the Kimura two-parameter method for estimation of the total number of nucleotide substitutions, and the Jukes-Cantor one parameter method for synonymous and nonsynonymous nucleotide substitutions. Both models have been used for analysis of HIV and HCV quasispecies [25], and they seem to be valuable tools for evaluating HCV molecular evolution.

Of note, in spite of using regular Taq polymerase for PCR amplification, which is prone to proof-reading errors, we found little variability in the CD_{81} binding regions.

In summary, we evaluated the genetic diversity of putative CD_{81} binding-regions aa 480 to 493 and aa 544 to 551 within the HCV E2 protein, before and after orthotopic liver transplantation, in four patients, by cloning and sequencing. We found that both regions are relatively conserved and the mean genetic distance and mean number of nonsynonymous substitutions per nonsynonymous site did not change significantly after the transplant.

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