

Sequence evolution of putative cytotoxic T cell epitopes in NS3 region of hepatitis C virus

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Abstract

AIM: Quasispecies of hepatitis C virus (HCV) are the foundation for rapid sequence evolution of HCV to evade immune surveillance of hosts. The consensus sequence evolution of a segment of HCV NS3 region, which encompasses putative cytotoxic T cell epitopes, was evaluated.

METHODS: Three male patients, infected with HCV through multiple transfusions, were identified from clinical symptoms and monitored by aminotransferase for 60 months. Blood samples taken at months 0, 32, and 60 were used for viral RNA extraction. A segment of HCV NS3 region was amplified from the RNA extraction by RT-PCR and subjected to subcloning and sequencing. HLA types of these three patients were determined using complement-dependent microlymphocytotoxic assay. CTL epitopes were predicted using MHC binding motifs.

RESULTS: No patient had clinical symptoms or elevation of aspartate/alanine aminotransferase. Two patients showed positive HCV PCR results at all 3 time points. The other one showed a positive HCV PCR result only at month 0. A reported HLA-A2-restricted CTL epitope had no alteration in the HLA-A2-negative carrier over 60 months. In the HLA-A2-positive individuals, all the sequences from 0 month 0 showed an amber mutation on the initial codon of the epitope. Most changes of consensus sequences in the same patient occurred on predicted cytotoxic T cell epitopes.

CONCLUSION: Amber mutation and changes of consensus sequence in HCV NS3 region may be related to viral immune escape.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the main pathogens of

transfusion-associated hepatitis. After acute transfusion-associated HCV infection, about 70-80% of the patients may progress to chronicity. Although many patients with chronic hepatitis C have no symptoms, cirrhosis may develop in 20% within 10 to 20 years after acute infection. The risk for hepatocellular carcinoma is increased in patients with chronic hepatitis C and almost exclusively in patients with cirrhosis^[1-15].

HCV is a linear, single-stranded positive-sense, 9400-nucleotide RNA virus. HCV constitutes its own genus in the family *Flaviviridae*. The HCV genome contains a single large open reading frame that codes for a virus polyprotein of approximately 3000 amino acids. Due to the high mutation rate of RNA dependent RNA polymerase, there are genotype and quasispecies diversity of HCV^[16-19]. The high mutation rate may interfere with effective immunity and cause the progression to chronicity^[20, 21].

Of the components of adaptive immunity, cytotoxic T cells play an important role in eliminating intracellular infections^[22]. They recognize body cells infected with viruses by detecting peptide fragments derived from viruses bound to MHC class I molecules on the infected body cells. Then, they kill the infected cells before viral replications complete. In this study, 3 patients with transfusion-associated hepatitis C were followed-up for 60 mo to evaluate the evolution of cytotoxic T cell epitopes in the HCV NS3 region.

MATERIALS AND METHODS

Patients

Patients C, Z and W, being 43, 48 and 49 years old Chinese males, were infected with HCV through multiple transfusions. They were followed-up for 60 mo after identification. During the follow-up period, no elevation of aspartate/alanine aminotransferase was found. Their peripheral blood was collected at mo 0 (the time of identification), 32 and 60, and stored at -70 °C. Patients C and Z were positive for HCV RNA. Patient W was positive for HCV RNA only in the blood sample taken at mo 0 and consistently negative after that.

HLA typing

HLA types of the three patients were assessed by using the Tasaki HLA class I dry tissue typing tray (One Lambda, Canoga Park, CA). Briefly, blood samples were drawn and lymphocytes were isolated immediately. After antibody and 2×10^6 lymphocytes were mixed in each well, 1 μ L of complement was added into each well to incubate at room temperature for 1 hour. After incubation, the cells were stained with eosin and fixed with formaldehyde. Positive (dead) lymphocytes appeared dark and non-refractiles with eosin dye.

RNA extraction and RT-PCR

Single step guanidine thiocyanate-chloroform method^[23] was used to extract HCV RNA from 50 μ L of plasma. RNA extracted was reverse-transcribed using random primers. Nested PCR (primers see Table 1) was used to amplify the HCV NS3 region that spanned a reported cytotoxic T cell

epitope (-KLVALGINAV-)^[24], which is HLA-A2-restricted. The first round PCR was run for 35 cycles with denaturing at 94 °C for 1 min, annealing at 53 °C for 1 min, and elongating at 72 °C for 1 min. The second round of PCR was run for 35 cycles with denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongating at 72 °C for 1 min.

Cloning and sequencing of amplified segment of HCV NS3

PCR products were subcloned into M13mp19 phage. For each blood sample, 5 clones were selected and amplified. The single strand DNA produced by the M13mp19 phage was purified by QIAprep Spin 13 kit (Qiagen, Valencia, CA) and sequenced using sequence version 2.0 sequencing kit (USB, Cleveland, OH).

Sequence analysis

DNA sequences were translated and aligned. Consensus sequence was produced for every 5 clones of each blood sample. Cytotoxic T cell epitopes for each consensus sequence were predicted based on the HLA type of the patients and MHC molecule binding motifs^[25].

RESULTS

HLA types

Patient C was (A11, 30; B13, -; Bw4, -). Patient Z was [A2, 11; B60 (40), 70; Bw6, -]. Patient W was [A2, 11; B40, 55 (22); Bw6, -].

Nucleotide sequences of HCV

Five clones of NS3 sequences were ascertained for each blood sample. Since all the blood samples of patients C and Z were positive for HCV RNA, 15 sequences were obtained from each one of them. Due to the negative result of HCV RNA in the later 2 blood samples in patient W, only 5 cDNA sequences

were obtained. The GenBank accession numbers for all the sequences are in Table 2. The translated amino acid sequences are aligned in Figure 1.

Table 2 Assigned accession number for each nucleotide sequence of three patients

Time point measured	Patient W	Patient C	Patient Z
0 mo	AF051270	AF051261	AF051270
	AF051270	AF051261	AF051270
	AF051271	AF051261	AF051270
	AF051272	AF051260	AF051270
	AF051273	AF051259	AF051270
32 th mo	NA	AF051254	AF051265
		AF051255	AF051266
		AF051256	AF051267
		AF051257	AF051268
		AF051258	AF051269
60 th mo	NA	AF051253	AF051262
		AF051253	AF051262
		AF051253	AF051263
		AF051253	AF051263
		AF051253	AF051264

NA: not applicable as the sample was PCR negative for HCV RNA.

Sequence variation on reported cytotoxic T cell epitope

Our consensus sequences showed (K/*)LSSLGLNAV (*: stop codon) on the site of the reported HLA-A2-restricted cytotoxic T cell epitope^[24]. In patient C, who was not HLA-A2-restricted, all the 15 sequences were KLSSLGLNAV. In patient W, who was HLA-A2-restricted, all the 5 sequences showed a stop codon at the beginning of this peptide (four sequences showed

Table 1 Oligonucleotides used to amplify the NS3 region*

	Primer sequences	Strand	Nucleotide position in HCV genome
First round primers (5' -3')	CCCCATCAC(A/G)TACTC(C/T)ACCTA	+	4 197
	ACA(C/T)GT(A/G)TT(G/A)CAGTC(T/G)ATCAC	-	4 681
Second round primers (5' -3')	CGAGGATCCGTCCT(T/G)GACCAAGC(A/G)GAGAC	+	4 315
	GCAACTGCAGCT(G/A)G(T/A)(C/T)GG(G/T)ATGAC(A/G)GACAC	-	4 597

*Degenerate primers were used to cover the variable HCV genomes.

Table 3 Predicted CTL epitopes by MHC binding peptide motifs while incorporating persistent substitutions

Patient	HLA subtype	MHC binding peptide motif ^[38, 39]	Genome position	Predicted epitope	Time point measured
C	A11	X[MLIVSATGNCDF]XXXXXX[KRHY]	1 349	ATPPGSITVPH*	0 mo
			1 378	ATPPGSVTVPH*	32 th , 60 th mo
				KAIPIEAIR	0 mo
Z	A11	X[MLIVSATGNCDF]XXXXXX[KRHY]	1 381	KAIPIEAIK	32 th , 60 th mo
				PIEAIKGGGR	0 mo
				PIEAIKGGGR	32 th , 60 th mo
	A2	X[LMIVAT]XXXXXX[LVIAMT]	1 347	ATATPPGSV	0 mo
			1 348	ATATPPGSI	32 th , 60 th mo
			1 349	TATPPGSVT	0 mo
			TATPPGSIT	32 th , 60 th mo	
			ATPPGSVTV	0 mo	
			ATPPGSITV	32 th , 60 th mo	

*These two peptides fitted the MHC binding motif because proline and glycine residues allowed flexibility which made the peptides accommodate to the motif by kinking in the peptide backbone^[40].

*LSSLGLNAV and one sequence showed *LSPLGLNAV). In patient Z, who was also HLA-A2-restricted, all the sequences from 0-mo showed the stop codon at the beginning of the epitope (*LSSLGLNAV), nine sequences from blood samples at mo 32 and 60 showed KLSSLGLNAV, one sequence from blood sample at mo 32 showed KLSSLGLKPV.

Sequence variation on predicted cytotoxic T cell epitopes

Using MHC binding motifs to predict cytotoxic T cell epitopes, we found that most sites which showed changes of consensus sequences between successive blood samples were on the predicted cytotoxic T cell epitopes (Table 3).

DISCUSSION

Due to errors of the RNA-dependent RNA polymerase, RNA genomes had a relatively high mutation rate^[25,26]. RNA viruses evolve as complex distributions of mutants termed viral quasispecies. These coexisting mutant genomes always have a consensus or master sequence. Despite the potentially high mutation rate and variability of RNA viruses, changes in the consensus sequence of a viral population would occur only if some selection mechanism acted on the population and caused a shift in the population equilibrium^[27]. Immune response of the host can influence the distribution between different viral variants and will consequently cause a change in the consensus

Patient W

0 mo
 1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSVTVPHPNIQEVGLSNTGEIPFYGKAIP IEA IKGGRHLIFCHSKKKCDELA A*LSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4P.....
 Clone 5P.....

Patient Z

0 mo
 1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSVTVPHPNIQEVGLSNTGEIPFYGKAIP IEA IKGGRHLIFCHSKKKCDELA A*LSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4
 Clone 5

32th mo

1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSITVPHPNIQEVALSNTGEIPFYGKAIP IEA IRGGRHLIFCHSKKKCDELA AKLSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4P.....
 Clone 5K.....

60th mo

1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSITVPHPNIQEVALSNTGEIPFYGKAIP IEA IRGGRHLIFCHSKKKCDELA AKLSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4
 Clone 5KP.....

Patient C

0 mo
 1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSITVPHPNIQEVALSNTGEIPFYGKAIP IEA IKGGRHLIFCHSKKKCDELA AKLSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4R.....
 Clone 5

32th mo

1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSVTVPHPNIQEVALSNTGEIPFYGKAIP IEA IKGGRHLIFCHSKKKCDELA AKLSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4
 Clone 5T.....

60th mo

1340 1350 1360 1370 1380 1390 1400 1410 1420
 Consensus AGARLVVLATATPPGSVTVPHPNIQEVALSNTGEIPFYGKAIP IEA IKGGRHLIFCHSKKKCDELA AKLSSLGLNAVAYYRGLD
 Clone 1
 Clone 2
 Clone 3
 Clone 4
 Clone 5

Figure 1 Alignment of HCV amino acid sequences from three patients. Consensus sequences were given for the 5 sequences from the same blood sample.

sequence. A cellular immune-driven selection pressure has been demonstrated by the existence of HCV escape mutants in relation to cytotoxic T cell epitopes^[28]. In the HCV-infected human, the NS3 protein seems to be fairly immunogenic. T cell activation in response to NS3 has been detected in a number of studies of patients with acute or chronic HCV infection^[24, 29]. It was proposed that a strong *in vitro* T cell reaction to NS3 correlated with clearance of acute HCV infection whereas a less vigorous, or absent, NS3-specific T cell reactivity was observed in those who progressed to chronicity^[30]. Thus, in this study, we chose a segment of HCV NS3 region as our focus on sequence evolution.

T lymphocytes recognize their antigens in context of MHC-encoded molecules, a phenomenon called MHC restriction. Our sequence segment encompassed a cytotoxic T cell epitope, which was restricted by HLA-A2 and reported by Rehmann *et al*^[24]. In patients with HLA-A2 allele, their viral consensus sequences showed stop codons at the initial part of this epitope. On the contrary, in patients without HLA-A2 allele, their viral consensus sequences did not show the stop codon. Normally, stop codons are generated by random non-sense mutations in RNA virus and they are expected to occur randomly throughout the entire coding region. Viruses with stop codon in the open reading frame have been found to be defective viruses which usually make a small fraction of the RNA virus quasispecies^[31,32]. Here, stop codons were unusually concentrated at the beginning of the reported epitope, in the sequences of patients with HLA-A2 allele, suggesting that they are specifically selected by some pressure, probably by cytotoxic T cells. We would suppose that HCV specific and HLA-A2-restricted cytotoxic T cells, which recognize and kill the infected hepatocytes to prevent replication and proliferation of the viruses, were generated in patients W and Z. Under this immune pressure, viral quasispecies in these two patients would have shifted toward a new equilibrium to avoid the immune attack. In patients W and Z, the defective viruses, which did not express the reported cytotoxic T cell epitope, dominated the viral quasispecies at month 0. This may reflect the strong immune pressure at that time. Thirty-two months later, in patient W, the viruses were cleared and the patient was recovered. In patient Z, the viruses were not cleared at month 32 or 60, suggesting that the viral quasispecies escaped from the immune pressure and survived.

Cytotoxic T cells could recognize peptides loaded on the MHC class I molecules^[33]. The solution of the crystal structure of MHC class I molecules could reveal peptide-binding groove made up by $\alpha 1$ and $\alpha 2$ domains of heavy chains^[34,35]. Naturally occurring processed peptides have been isolated from purified MHC class I molecules. Analyzing their sequences revealed the presence of simple amino acid sequence motifs that were specific to particular allelic forms of class I molecules^[36]. Based on the sequence motifs, we found that most sites, with changes of the consensus sequences, were on the putative cytotoxic T cell epitopes in the corresponding patients, implying the possible underlying immune impetus for sequence evolution.

In summary, by molecular sequencing, the quasispecies nature and sequence evolution of HCV NS3 region can be revealed. By HLA typing and epitope prediction, the non-sense mutation and changes of consensus sequences might be the result of immune pressure. This study has paved the way for further cytotoxicity assay^[37] to confirm the possible immune target sites of HCV.

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