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Hepatitis C virus quasispecies in cancerous and noncancerous hepatic lesions: the core protein-encoding region.

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Abstract

We have shown that highly proofreading DNA polymerase is required for the polymerase chain reaction in the genetic analysis of hepatitis C virus (HCV). To clarify the status of HCV quasispecies in hepatic tissue using proofreading DNA polymerase, we performed a genetic analysis of the HCV core protein-encoding region in cancerous and noncancerous lesions derived from 4 patients with hepatocellular carcinoma. In contrast to the previously published data, we observed neither deletions nor stop codons in the analyzed region and no significant difference in the complexity of HCV quasispecies between cancerous and noncancerous lesions. This result suggests that the HCV core gene is never structurally defective in hepatic tissues, including cancerous lesions. However, in 3 of the patients, the consensus HCV species differed between cancerous and noncancerous lesions, suggesting that the predominant replicating HCV species differs between these 2 types of lesions. Moreover, during the course of the study, we obtained several interesting variants possessing a substitution at codon 9 of the core gene, whose substitution has been shown to induce the production of the F protein synthesized by a - 2/+1 ribosomal frameshift.

KEYWORDS: hepatitis C virus, core gene, hepatocellular carcinoma, quasispecies, proofreading DNA polymerase

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Original Article

Hepatitis C Virus Quasispecies in Cancerous and Noncancerous Hepatic Lesions: The Core Protein-encoding Region

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We have shown that highly proofreading DNA polymerase is required for the polymerase chain reaction in the genetic analysis of hepatitis C virus (HCV). To clarify the status of HCV quasispecies in hepatic tissue using proofreading DNA polymerase, we performed a genetic analysis of the HCV core protein-encoding region in cancerous and noncancerous lesions derived from 4 patients with hepatocellular carcinoma. In contrast to the previously published data, we observed neither deletions nor stop codons in the analyzed region and no significant difference in the complexity of HCV quasispecies between cancerous and noncancerous lesions. This result suggests that the HCV core gene is never structurally defective in hepatic tissues, including cancerous lesions. However, in 3 of the patients, the consensus HCV species differed between cancerous and noncancerous lesions, suggesting that the predominant replicating HCV species differs between these 2 types of lesions. Moreover, during the course of the study, we obtained several interesting variants possessing a substitution at codon 9 of the core gene, whose substitution has been shown to induce the production of the F protein synthesized by a −2/+1 ribosomal frameshift.

Key words: hepatitis C virus, core gene, hepatocellular carcinoma, quasispecies, proofreading DNA polymerase

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (CH) [1, 2] and progresses to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [3, 4]. HCV is an enveloped positive single-stranded RNA (9.6-kilobases) virus belonging to the family *Flaviviridae* [5, 6]. To date, a large number of genetic analyses of HCV have been reported, and the viral genome structure has been elucidated [for review, see 7, 8]. The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH2-core-envelope (E1)-E2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH. The most characteristic feature of the HCV genome is its remarkable genetic heterogeneity. To date, at least 6 major HCV genotypes have been identified, and these have been further grouped into more than 50 subtypes. Furthermore, HCV may show quasispecies distribution in an infected individual. These genetic features of HCV are considered to be one of the most important factors regarding maintenance of the chronic viremic state and have hampered the development of vaccines [for review, see 7, 8].

The HCV core protein is a 21-kDa protein produced from the N-terminal portion of the precursor protein by

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host-cell signal peptidase(s) [9], and resides primarily in the cytoplasm [10]. To date, it has appeared that the HCV core protein not only functions in viral replication as a viral capsid protein, but also affects a variety of cellular functions, including gene expression, signal transduction, and apoptosis [11]. The core protein is thus a multifunctional protein and may play an important role in hepatocellular carcinogenesis. To understand the relationship between the progression of hepatic disease and the genomic characteristics of the HCV core protein-encoding region, genetic analyses of HCV derived from patients with acute hepatitis, CH, LC and HCC have been performed [12, 13]. In addition, several groups have carried out comparative sequence analyses of the HCV core gene in cancerous and noncancerous hepatic lesions [14–16]. The results obtained from these studies have shown that the intra-patient variation in nucleotides increases with the progression of liver disease, and that deletions and mutations in the core gene occur more frequently in cancerous than in noncancerous lesions. However, because nonproofreading DNA polymerase was used for the polymerase chain reaction (PCR) in these studies, the possibility remains that some of these observations were experimental artifacts. Therefore, to accurately elucidate the status of quasispecies of the HCV core gene, we consider the use of highly proofreading DNA polymerase for amplification of the HCV genomes to be indispensable. In the present study, we have shown that nonproofreading DNA polymerases give artifactural quasispecies representations, and that there appears to be no difference in core-gene genetic diversity between cancerous and noncancerous hepatic lesions, although different sequences are clearly evident. In addition, during the course of the study, we obtained several interesting variants possessing a substitution at codon 9 of the core gene.

Materials and Methods

Patients. Four Japanese patients with HCC were selected for this study. Patient 1 was a 68-year-old man with CH and HCC, and a serum alanine aminotransferase (ALT) level was 49 IU/l. Patient 2 was a 69-year-old man with LC and HCC and a serum ALT level of 120 IU/l. Patient 3 was a 67-year-old female with LC and HCC and a serum ALT level of 22 IU/l. Patient 4 was a 58-year-old man with LC and HCC and a serum ALT level of 48 IU/l. The results of the pathogenic examination of these HCCs were as follows: patient 1, highly differentiated HCC; patient 2, a mixture of highly and moderately differentiated HCC; patient 3, moderately differentiated HCC; patient 4, undifferentiated HCC. All patients were positive for antibodies against HCV but negative for hepatitis B surface antigen. The HCV genotype in these patients was determined to be genotype 1b by a previously described method [17]. Informed consent was obtained from each patient before the study. Fresh tissue samples (including a cancerous and noncancerous portion) were collected intraoperatively from the resected specimens, immediately frozen, and stored overnight at −80 °C.

RNA extraction and reverse transcription (RT)-nested PCR. RNAs from hepatic tissues were prepared using the ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan). These RNA samples (1 μg of RNA) were used as templates for the RT-nested PCR to amplify the core gene. An antisense primer, 968R, 5'-GTTGGAGCAGTCGTTCGTGAC-3' (corresponding to position 948–968 of HCV-JS [18]), was used to prime cDNA synthesis by SuperScript II (Life Technologies, Rockville, MD, USA). One-tenth of the synthesized cDNA was used for the first round of PCR. Primers 201A, 5'-GCCTGATAGGGTGCTTGCGA-3' (corresponding to position 291–310 of HCV-JS [18]), and 968R were employed in the first round of PCR (35 cycles in 50 μl). Two microliters of the first reaction mixture was used for the second round of PCR. An internal primer pair, 338(B), 5'-ATTATGGATCCGGAGGTCTCGTAGACCGTGC-3' (corresponding to position 319–338 of HCV-JS [18] and containing a *Bam*HI recognition site (underlined) in the 5' region), and 948R(H), 5'-ATTATAAGCTTCATGGTATATCCCGGACGCGTT-3' (corresponding to position 927–948 of HCV-JS [18] and containing a *Hin*dIII recognition site (underlined) in the 5' region) was used for the second round of PCR (30 cycles in 50 μl). KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used as a proofreading enzyme for PCR, and each PCR cycle consisted of annealing at 55 °C for 45 sec, primer extension at 74 °C for 1 min and denaturation at 94 °C for 30 sec. Taq DNA polymerase (Sawady, Tokyo, Japan) was

Footnotes: The nucleotide-sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide-sequence databases under accession numbers AB061927 to AB062026 (pTL1～pTL4 series), AB062173 to AB062272 (pTH1～pTH4 series).

used as a nonproofreading enzyme for PCR under the same conditions as those used with the KOD-plus DNA polymerase. The second PCR reaction of the HCV core gene yielded a 652-bp amplified product.

cDNA cloning and sequencing. PCR products containing the HCV core protein-encoding region were cloned into the *Bam*HI and *Hin*dIII sites of pTZ19R, as described previously [19]. Plasmid inserts were sequenced in both the sense and antisense directions using Big Dye terminator-cycle sequencing on an Applied Biosystems 310 automated sequencer (Applied Biosystems, Inc., Norwalk, CT, USA).

Results

To examine the influence of using the different kinds of DNA polymerase in RT-nested PCR, we used KOD-plus DNA polymerase and Taq DNA polymerase as highly proofreading and nonproofreading enzymes, respectively. A RNA specimen derived from a cancerous lesion in patient 1 with HCC was used for cDNA synthesis with SuperScript II. The obtained cDNA was used as a template for amplification of the core gene by KOD-plus or Taq DNA polymerase. We compared the nucleotide sequences of each of 20 clones (pTH1 and pTH1S series) obtained from the PCR products amplified by the KOD-plus and Taq DNA polymerases, respectively. We obtained the same consensus sequence from both series. However, the level of nucleotide substitution in clones of the pTH1 series was drastically lower than that of clones of the pTH1S series, as shown in Table 1. In particular, the number of nonsynonymous substitutions with a transition in the pTH1 series (3 positions) was 7 times lower than that in the pTH1S series (21 positions), although the number of synonymous substitutions with transition was 1.5 times lower in the pTH1 series than in the pTH1S series (Table 1). The number of synonymous and nonsynonymous substitutions with transversion in the pTH1 series was also 7 times lower than that in the pTH1S series (Table 1). Furthermore, we obtained 6 clones possessing one nucleotide deletion at different positions in the pTH1S series, but no such clones were obtained in the pTH1 series. The distribution pattern of clones diverging from the consensus sequences was also rather different between series at both the nucleotide and amino acid-sequence levels. In the pTH1 series, 6 and 17 out of 20 clones were the same as the consensus sequences at the nucleotide and amino acid levels, respectively, but in the pTH1S series, only 1 and 5 out of 20 clones were the same as the consensus clones at the nucleotide and amino acid levels, respectively. These results suggest that the genetic differences detected between the pTH1 and pTH1S series were due to the differences in proofreading activity between the DNA polymerases used. Therefore, we judged that use of a proofreading DNA polymerase is required for the PCR in genetic analysis of HCV.

To elucidate the status of HCV quasispecies in cancerous and noncancerous lesions, RNA specimens derived from 4 HCV-positive patients with HCC were used for amplification of the core gene by RT-nested PCR using KOD-plus polymerase. We determined the nucleotide sequences of 20 independent clones obtained from each of two specimens of patients 1–3 (n = 120 clones in total). In the case of patient 4, we analyzed 40 clones from the cancerous lesion and 40 from the noncancerous lesion, as several interesting clones were obtained (these will be described later in this report). Based on these sequence data, we analyzed the status of the sequence diversity of the core gene in cancerous (pTH series) and noncancerous (pTL series) lesions. As shown in Table 2, the consensus sequences obtained in each of the specimens from the cancerous and noncancerous lesions showed 1.4–2.6%

Table 1 Sequence diversity of PCR products amplified with proofreading and nonproofreading DNA polymerases

Series	No. of clones sequenced	No. of nt substituted from consensus sequences						No. of deleted nt
		Transition			Transversion			
		Synonymous	Nonsynonymous	Average/clone	Synonymous	Nonsynonymous	Average/clone	
pTH1	20	23	3	1.30	1	0	0.05	0
pTH1S	20	36	21	2.85	5	2	0.35	6

The pTH1 and pTH1S series were obtained by amplification using proofreading (KOD-plus) and nonproofreading (Taq) DNA polymerases, respectively.

nucleotide-sequence diversity compared with the consensus sequence of genotype 1b. The amino acid sequences deduced from the consensus sequences also differed from the consensus sequences of genotype 1b by 1–6 amino acids (Table 2), although these sequences did not differ in the pTH2 series. These values were within the sequence variation in genotype 1b. However, we found that the consensus sequences differed between the cancerous and noncancerous lesions in 3 of 4 patients. The consensus sequences of the pTH4 and pTL4 series differed by 9 nucleotides and 1 amino acid. 5 nucleotides and 2 amino acids were different between the consensus sequences of the pTH2 and pTL2 series (Table 2). We further found that, except in the case of patient 3, the distribution of HCV species substituted from each of the consensus sequences also differed between cancerous and noncancerous lesions, particularly at the nucleotide level (Table 3). Fifty percent of clones in the pTH4 series (cancerous lesion) were identical to the consensus sequence, but none of the clones in the pTL4 series (noncancerous lesion) were the same. Forty percent of clones in the pTL2 series (noncancerous lesion) showed sequences identical to the consensus sequences; however, there were no clones possessing the consensus sequences in the pTH2 series

Table 2 Comparison of the consensus sequences obtained from cancerous and noncancerous lesions

Consensus sequences	Differences from consensus sequence of genotype 1b		Differences between consensus sequences of pTH and pTL series	
	nt/573 nts	aa/191 aa	nts/573 nts	aa/191 aa
pTH1	14	6	0	0
pTL1	14	6		
pTH2	12	0	5	2
pTL2	15	2		
pTH3	12	2	1	0
pTL3	11	2		
pTH4	11	2	9	1
pTL4	8	1		

Table 3 Distribution of HCV clones diverged by substitution from each consensus sequence

No. of substitutions differing from each consensus sequence	No. of clones															
	pTH1		pTL1		pTH2		pTL2		pTH3		pTL3		pTH4		pTL4	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
0	6	17	1	13		4	8	12	4	14	4	17	20	22		10
1	8	3	7	4	1	11	1	6	2	5	6	2	1	3		15
2	2		8	2		5	4	1	7	1	5		1	7		8
3	2		2	1	2		3		3		2	1		6	2	4
4	1		2		1		3	1	2		2			2	1	3
5	1				11				2		1				1	
6					2								1		11	
7					2								3		6	
8															8	
9					1										6	
10													2		3	
11							1								2	
12													2			
13													2			
14													3			
15													1			
16													4			

(cancerous lesion). In the case of patient 1, who also showed identical consensus sequences between the cancerous and noncancerous lesions, only 1 clone from the noncancerous lesion was identical to the consensus sequence, while 6 clones possessing the consensus sequence were obtained from the cancerous lesion (Table 3). These results suggest that the HCV quasispecies proliferating in cancerous and noncancerous lesions are rather different in each case.

Table 4 summarizes the numbers of nucleotides deviating from each of the consensus sequences. In both patients 2 and 3, the frequencies of substitutions with transition or transversion were higher in cancerous than in noncancerous lesions, while in patients 1 and 4, noncancerous lesions showed higher frequencies of substitutions than the cancerous lesion. These results do not support the conclusion in previous reports [14–16] that the complexity of quasispecies on the core gene in cancerous lesions is generally higher than that in noncancerous lesions. Furthermore, in contrast to previous reports [14–16], no clones possessing the deletion or stop codon were obtained in the core gene. Nonsynonymous substitutions were 2.4 to 8 times less frequent than synonymous ones, and transitions were 7.8 to 40 times more frequent than transversion, particularly at the third position of the codon.

During the course of this study, we obtained 6 interesitng clones from the noncancerous lesion of patient 4 (pTL4 series); all of these had AGA (Arg) to AAA (Lys) substitutions at codon 9, resulting in an A stretch (A_8 to A_{10}) at codons 8–11 (Fig. 1). Although the 4 major clones possessed an A_9 stretch, one possessed an A_{10} stretch due to an additional substitution (ACC to AAC) at codon 11, while another possessed an A_8 stretch due to an additional substitution (ACC to CCC) at codon 11. To date, only the prototype HCV-1 strain has been reported to possess the same A_{10} stretch at codons 8–11 [20]. It has also been reported that the HCV-1 genome produces a small 17-kDa protein with a 21-kDa core protein in *in vitro* transcription and translation experiments [21]. Furthermore, codon 9 (AAA) has been shown to play an important role in determining production of the 17-kDa protein [21]. Recently, this

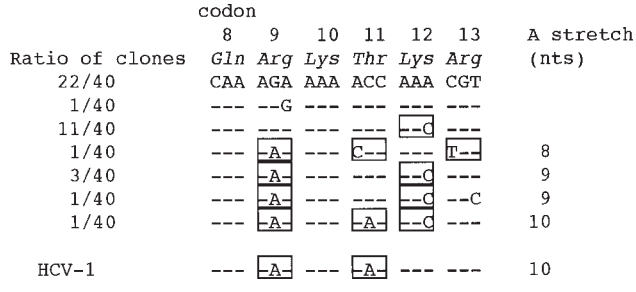

Fig. 1 Nucleotide sequences of codons 8–13 in the pTL4 series. The status of the quasispecies on codons 8–13 is shown. Boxed codons indicate the nonsynonymous substitutions. The known HCV-1 strain possessing an AAA sequence at codon 9 is also shown.

Table 4 Sequence diversities of the HCV core protein-encoding region derived from cancerous and noncancerous lesions

Series	No. of clones sequenced	No. of nt substituted from each consensus sequences						Synonymous/ nonsynonymous	Transition/ transversion
		Transition			Transversion				
		Synonymous	Nonsynonymous	Average	Synoymous	Nonsynonymous	Average		
pTH1	20	23	3	1.30	1	0	0.05	8.0	26.0
pTL1	20	26	7	1.65	0	4	0.20	2.4	8.3
pTH2	20	80	16	4.80	0	5	0.25	3.8	19.2
pTL2	20	28	12	2.00	1	0	0.05	2.4	40.0
pTH3	20	33	6	1.95	3	1	0.20	5.1	9.8
pTL3	20	27	4	1.55	3	1	0.20	6.0	7.8
pTH4	40	173	25	4.95	0	19	0.48	3.9	10.3
pTL4	40	233	34	6.67	5	20	0.62	4.4	10.8
cancerous lesion*		223	37.5	3.25	4.0	15.5	0.24	4.2	13.3
noncancerous lesion**		198	40.0	2.97	6.5	15.0	0.27	3.9	11.0

*One half of the total number of clones from the pTH4 series was added to the calculation. **One half of the total number of clones from the pTL4 series was added to the calculation.

17-kDa protein, named the F protein, has been clarified to be produced by a ribosomal frameshift into the −2/+1 reading frame [22]. The obtained 6 mutants possessing a substitution at codon 9 may also produce the F protein.

Discussion

In this study, we have shown the importance of using proofreading DNA polymerase in the genetic analysis of HCV. Regarding this point, it has recently been shown that the use of nonproofreading Taq DNA polymerase to assess viral diversity could yield an incorrect quasispecies spectrum [23]. In this previous study, sequence analysis of hypervariable region 1 of the E2 envelope gene and the interferon sensitivity-determining region of the NS5A gene revealed a great number of minor variants in the PCR products amplified by nonproofreading DNA polymerase, 80% of which were not observed in the PCR products amplified by proofreading DNA polymerase [23]. These findings thus support our present results. Our assertion of the importance of using a proofreading DNA polymerase also seems reasonable in light of the finding that the fidelity of proofreading DNA polymerase is more than 10 times greater than that of Taq DNA polymerase [24, 25].

A number of sequence analyses have been carried out on quasispecies of 5' UTR and the envelope gene [for review, see 7, 8]; however, there is much less information on quasispecies of the core gene. In this study, we compared the status of quasispecies of the core gene between cancerous and noncancerous lesions by sequence analysis of the PCR products amplified with highly proofreading thermostable DNA polymerase. Previous studies [13–16] have revealed the appearance of in-frame stop codons and deletions leading to frame-shifts within the core gene, especially in the case of RNA specimens from cancerous lesions. In contrast to these published data, neither stop codons nor deletions were observed among the 200 HCV clones derived from cancerous and noncancerous lesions in the present study. Because we also observed several one-nucleotide deletions with a frequency of 30% when nonproofreading Taq DNA polymerase was used for the PCR, it may be concluded that such discrepancy depends on the fidelities of thermostable DNA polymerases used for PCR.

Although previous studies [13–16] have shown a significantly higher genetic variability of HCV in cancerous than in noncancerous lesions, our results demonstrated that the quasispecies diversity of HCV is not dependent on the status of hepatic diseases. This discrepancy is also probably due to differences in the DNA polymerases used for PCR. To obtain a conclusion regarding this point, analysis of several additional specimens will be required.

In this study, we obtained several core variants possessing an AAA sequence at codon 9, resulting in an A stretch (A_8 to A_{10}) at codon 8–11. It is unlikely that detection of the A stretch was due to an error of DNA polymerase or reverse-transcriptase, as such an A stretch was reproducibly obtained from other RT-PCR products derived from patient 4. Although the frequency of clones possessing an A stretch of more than 8 nucleotides was 15% in the noncancerous lesion of patient 4, no such clones were obtained from the cancerous lesion of this patient, indicating that the frequency of such clones in cancerous lesion is less than 2.5%. However, it will be necessary to clarify the prevalence of such core variants, which may produce an F protein synthesized by a −2/+1 ribosomal frameshift, because it has been estimated that the F protein plays an important role in the HCV life cycle [22].

Finally, the present data obtained using a proofreading DNA polymerase showed that none of the HCV species obtained from hepatic tissues - including cancerous lesions - were structurally defective at least in the core protein-encoding region. Genetic analyses of other HCV protein-encoding regions will be needed to confirm this observation.

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