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Characterization of hypervariable region in hepatitis C virus envelope protein during acute and chronic infection

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Summary. Hepatitis C virus (HCV) causes persistent infection in most patients. To clarify the mechanisms underlying establishment of this persistent infection, nucleotide sequences of the E1/E2 region were characterized in 5 patients with acute and chronic HCV infection. We used direct DNA sequencing methods to identify the major sequence of HCV in each patient. Each HCV genome displayed a high frequency of nucleotide sequence variation in the hypervariable region (HVR) of E2. However, patient-specific conserved nucleotide sequences were identified in the E1/E2 region during the course of infection and conserved the higher-order protein structure.

In the acute phase HCV infection, amino acid substitution in HVR-1 as the monthly rate of amino acids substitution per site (%) between each point exceeded 10.2%. In the chronic phase HCV infection, a significantly lower rate of amino acid substitution was observed in patients. The host immune responses to HVR-1 of each HCV isolates from all clinical courses were characterized using synthetic peptides and ELISA. One chronic patient serum (genotype 1b) did not react at all to its own HVR-1 peptides, however another patient (genotype 2b) reacted to all clinical course. These results indicated that HVR-1 might not always exhibit

Note: DDBJ/EMBL/GenBank accession numbers of E1/E2 sequences reported in this paper are AB107929-AB107949.

neutralizing epitopes of HCV infection. The sequence variation in HVR-1 may instead indicate the existence of various clones in acute phase infection and the adaption of these clones is thought to have caused persistent and chronic infection in each patient.

Introduction

Hepatitis C virus (HCV) possesses a genome of single-strand RNA with positive polarity (about 9.6 kb), and is classified in the family *Flaviviridae*, genus *Hepacivirus* [24]. HCV is the major causative agent of post-transfusion-associated non-A, non-B hepatitis, and it an estimated 170 million people are infected worldwide. Persistent HCV infections often progress to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3, 25]. Previous reports have suggested that variability of the HCV genome is likely to play crucial roles in facilitating escape from host immune surveillance [9, 12, 29]. In particular, high degrees of sequence variability have been observed in regions coding the E2 envelope protein, designated as hypervariable regions (HVR)-1 [13, 28] and 2 [22]. HVR-1 has been suggested as a dominant neutralizing epitope for HCV infection in chimpanzees [6]. Despite the confirmed presence of HCV-specific antibodies and cytotoxic T lymphocytes [1, 7], HCV causes frequently persistent infection. These results suggest that variation occurring in neutralizing epitopes within HVR-1 could produce escape variants able to elude the host immune system. Recent reports have indicated that the evolution of viral quasispecies may predict clinical course in viral hepatitis [8].

Although HCV preferably infects hepatocytes, as confirmed by the existence of negative-strand RNA [15], the mechanisms of adsorption into hepatocytes and transcription and replication of viral RNA in the cell remain unclear. The possibility of low-density lipoprotein (LDL)-receptor has been suggested as a virus receptor for HCV infection [2, 18]. CD81 belongs to a family of molecules called tetraspanins, characterized by four transmenbrane domains forming two extracellular loops [17], and interacts with E2 protein as a putative viral receptor [23]. So far, six hepatocyte-binding regions have been defined in the E1/E2 region using synthetic peptides [11]. Inhibition of natural killer cells through engagement of CD81 by E2 protein has been reported [5]. Moreover, no polymorphisms in CD81 amino acid (a.a.) sequences on peripheral blood mononuclear cells (PBMCs) have been observed between healthy volunteers and patients during HCV infection [10].

The mechanisms of adaptation and selection allowing HCV to establish chronic infection during the first phase of acute infection remain unclear. The present study characterized patient-specific conserved original nucleotide sequences of the E1 and E2 regions, and deduced amino acid (a.a.) substitutions during the course of HCV infection for acute and chronic phase using direct DNA sequencing methods and humoral immunity of patients to HVR-1 peptides during the course of chronic HCV infection.

Materials and methods

Patients and sera

Two patients displaying acute infection with hepatitis C virus by transfusion (patients A and B; Table 1, Fig. 1) were selected retrospectively, along with three randomly selected patients with chronic hepatitis C in which high levels of serum alanine aminotransferase (ALT) were maintained for more than six months after first medical examination (patients C–E; Table 1, Fig. 1). All serum samples were utilized to determine nucleotide sequences of the HCV E1 and E2 regions during disease progression, and deduced a.a. sequences were predicted. These selected sera were aliquoted and stored below −80 °C until characterization. Two patients were infected with HCV by transfusion: patient A (a 58-year-old woman) when she donated a kidney; and patient B (a 55-year-old man) during hip joint surgery. Patients A and B were followed up for 11 and 13 years, respectively. In patients A and B, serum ALT levels remained abnormal during the entire follow-up period. Patients C (54-year-old man), D (26-year-old man), and E (67-year-old man) displayed histological evidence of chronic active hepatitis C.

Informed consent was obtained from all patients in accordance with the Helsinki Declaration.

Detection of anti HCV antibody and HCV RNA

Second-generation enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Raritan, NJ) was used to detect HCV antibody in sera from the five patients during disease progression. Serum HCV RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform (AGPC) method [4], and detected by reverse transcription and nested polymerase chain reaction (PCR) using primers for the 5-noncoding region of the HCV genome [19]. Results

Table 1. Clinical evaluation of patients and time points of characterization. Randomly selected patients with hepatitis C were analyzed

Patients	HCV genotype	Age (years)	Sex	Points[a]	Duration[b] (months)
(Acute)					
A	1b	58	F	1 to 2	3
				2 to 3	7
B	1b	55	M	1 to 2	4
				2 to 3	8
(CH)					
C	1b	54	M	1 to 2	12
				2 to 3	3
D	1b + 2a	26	M	1 to 2	8
				2 to 3	7
E	2a	67	M	1 to 2	10
				2 to 3	11

[a]Points, points of analysis

[b]Duration, duration between points of analysis

Acute: acute infection with hepatitis C virus by transfusion, with ALT and viral RNA levels rapidly decreased immediately after infection, then subsequently increased

CH: chronic hepatitis patient, with high levels of ALT maintained for more than six months after first medical examination

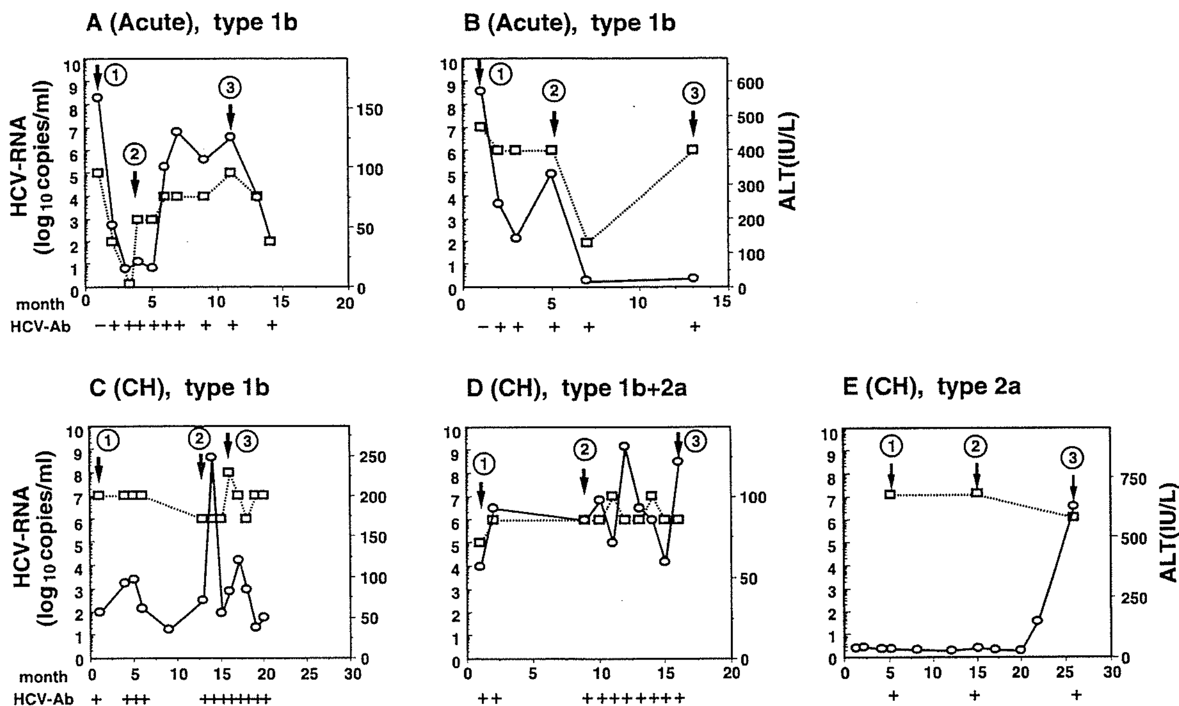

Fig. 1. Clinical course of hepatitis C patients. Changes in HCV RNA titer (broken line with open square), ALT level (black line with open circle) and HCV antibodies (− or +) for each patient. Numbers *1–3* in open circles indicate DNA sequencing points. In acute patients **A** and **B**, time point 1 represents onset by transfusion. In chronic patients **C** and **D**, time point 1 represents first medical examination. In patient **E**, time point 1 represents five months after first medical examination. *Acute*: acute HCV infection; *CH*: chronic HCV infection

were quantified using competitive PCR assay [30] in which cloned DNA (with a 15-bp deletion in the middle portion) used as a competitor [29].

Nucleotide sequence analysis in E1/E2 region

Nucleotide sequences for the E1/E2 region were analyzed according to direct DNA sequencing methods for PCR products using λ exonuclease (Gibco-BRL, Rockville, MD). HCV genome was extracted using AGPC methods [4], then amplified by reverse transcriptase and nested-PCR from serum for overlapping regions using two sets of primer pairs. PCR primers and amplified regions utilized for each patient are listed in Table 2. The second PCR product which was amplified by 5′-phosphorylated primer. PCR products were purified by 3% Nusieve 3:1 agarose gel electrophoresis (FMC BioProducts, Rockland, ME). One to four units of λ exonuclease was added to purified PCR products, including 67 mM glycine-KOH (pH 9.4) and 2.5 mM $MgCl_2$, and incubated at 37 °C for 1 h to form single-stranded DNA. Reaction mixtures were precipitated with ethanol and dried for DNA sequencing. Aliquoted DNA fragments were used for identification of nucleotide sequences in the E1/E2 region [27]. Nucleotide sequences of the E1/E2 region were determined for all five patients during disease progression. Characterization of nucleotide sequences and phylogenetic analyses of HVR-1 were performed using GENETYX version 10 software (Software Development, Tokyo, Japan). The phylogenetic tree for HVR-1 was constructed using the neighbor-joining (NJ) method [26].

Table 2. List of primer sequences for PCR of the HCV genome

Patients (Point*) [nt no.**]	Primer name	sequence
A (1, 3)	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1265]	1st anti-sense; (b)	5′-TAGATTGAGCAATTGCAATCTTGN-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1265]	2nd anti-sense; (b)	5′-TAGATTGAGCAATTGCAATCTTGN-3′
A (2), B	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1385]	1st anti-sense; (d)	5′-GCCACCATGTCCACGACAGCTTGGTGG-3′
	2nd sense; (e)	5′-TGGTAAGGTCATCGATACCCTCACN-3′
[697–1365]	2nd anti-sense; (f)	5′-TTGTGGGATCCGGAGTAACTGCGACAC-3′
A, C	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1385]	1st anti-sense; (d)	5′-GCCACCATGTCCACGACAGCTTGGTGG-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1365]	2nd anti-sense; (f)	5′-TTGTGGGATCCGGAGTAACTGCGACAC-3′
D-2a, E	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1387]	1st anti-sense; (p)	5′-CTAATGATGTCTATGATGACCTCGGGAACG-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1357]	2nd anti-sense; (q)	5′-CGCATCACGTACGCCAGAATCATGG-3′
D-1b	1st sense; (h)	5′-ATGGCTTGGGATATGATGATGAACTGGTC-3′
[1290–1867]	1st anti-sense; (i)	5′-TGAAACAATACACTGGACCACACAC-3′
	2nd sense; (j)	5′-ATTCCATGGTGGGGAACTGGGCTAA-3′
[1424–1813]	2nd anti-sense; (k)	5′-TAGGTGCGTAGTGCCAGCAATAAGG-3′
B	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1887]	1st anti-sense; (m)	5′-ACTACAACAGGGCTCGGAGTGAAN-3′
	2nd sense; (n)	5′-ATGGCTTGGGATATGATGATGAACTGGTCN-3′
[1291–1867]	2nd anti-sense; (o)	5′-TGAAGCAATACACTGGACCACACACN-3′
D-2a	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1887]	1st anti-sense; (m)	5′-ACTACAACAGGGCTCGGAGTGAAN-3′
	2nd sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1867]	2nd anti-sense; (o)	5′-TGAAGCAATACACTGGACCACACACN-3′
A, C, E	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1867]	1st anti-sense; (i)	5′-TGAAACAATACACTGGACCACACAC-3′
	2nd sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1813]	2nd anti-sense; (k)	5′-TAGGTGCGTAGTGCCAGCAATAAGG-3′

*Point, point of analysis; **nt no., nucleotide number on HC-R6, accession no. AY045702

Protein structure and amino acid substitution speed analyses in E1/E2 region

The a.a. sequence of the E1/E2 region was deduced from corresponding nucleotide sequences for all five patients. Protein structural analyses (hydrophobic profile, antigenic index and surface probability) were performed using MacVector sequence analysis software (International Biotechnologies, New Haven, CT). Protein secondary structure (Chou-Fas) was determined using GENETYX version 10 software (Software Development). Amino acid substitution speed was analyzed for HVR-1 (27 a.a.), HVR-2 (7 or 9 a.a.), another region of

Table 3. Reactivities of patient sera to HVR-1 peptides

	HVR peptide		
	Point: 1	2	3
Patient C			
Serum:			
1	–	–	–
Point: 2	–	–	–
3	–	–	–
Patient E			
Serum:			
1	+	+	+
Point: 2	+	+	+
3	+	+	+

Patient point	HVR-1 peptide sequences
C-1	HTHVIGGAQTQTTGSFASLFTPGASQK
C-2	**R**THVIGG**V**QTQTTGS**L**ASLFTPGASQK
C-3	**R**THV**T**GG**V**Q**SR**TTGS**LV**SLFTPGASQK
E-1	STHTIGGCTARSAAGFTRLFTQGARQN
E-2	STHTIGG**S**TARSAAGFTRLFTQGARQN
E-3	STHT**V**GG**S**TARSAAGFT**K**LFT**R**GA**H**QN

E2 (between HVR-1 and HVR-2; 63 a.a.) and E1 as the monthly rate of a.a. substitutions per site (%) between each point during disease progression (points 1–3; Fig. 1).

Test of host immune response to HVR-1 peptide

Synthetic peptides of HVR-1 for patients C and E were synthesized for each point in the clinical course (points 1–3; Fig. 1, Table 3). Peptides were tested using ELISA to characterize host immune responses to HVR-1 during chronic infection.

Results

Characterization of HCV-RNA, anti-HCV antibody and ALT levels in acute and chronic infection of hepatitis C virus

To clarify the mechanisms of genetic variation during persistent HCV infection, 5 patients were retrospectively analyzed (Table 1, Fig. 1). Patients A and B displayed acute infection with HCV genotype 1b, with progression from first phase of acute infection to chronic infection, and persistent viremia (Fig. 1). In the first phase of acute infection, antibody to HCV became positive (after point 1; Fig. 1). In patient A, HCV-RNA and ALT levels in serum decreased immediately after infection (point 1 to 2; Fig. 1), then elevated in the second phase of acute infection (point 2 to 3; Fig. 1). In patient B, HCV-RNA and ALT levels in serum decreased immediately

after infection, with an elevation of HCV-RNA levels occurring only in the second phase of acute infection (point 2 to 3; Fig. 1). Patients C, D and E displayed chronic hepatitis and persistent infection of HCV. Patient C was infected with genotype 1b, Patient E was infected with genotype 2a, and Patient D displayed co-infection with genotypes 1b and 2a (Table 1). In Figure 1, quantity of HCV-RNA in patient D indicates combined total RNA for both genotypes. These three patients displayed continuously high levels of ALT for more than six months after first medical examination and did not display marked changes in HCV-RNA levels (points 1–3; Fig. 1). A peak in ALT value was detected between points 2 and 3 for patients C and D, while elevation of ALT values was detected between points 2 and 3 for patient E.

Nucleotide sequence variation and patient-specific nucleotide sequence in E1/E2 region during clinical course of hepatitis C

To clarify the predominant sequence of E1/E2 region during progression of hepatitis C, 5 patients (2 patients with acute hepatitis, 3 patients with chronic hepatitis) were retrospectively selected and sequences (nucleotides 620 ~ 1867; Table 2) from the sera of these patients were analyzed at three points (points 1–3; Fig. 1) using direct DNA sequencing methods as described. Analyzed HCV DNA sequences of the E1/E2 region for each patient were registered to Genbank (accession numbers AB107929–AB107949). Alignment of nucleotide sequences on one-third of the E2 region (nucleotide 1492 ~ 1785) is indicated in Fig. 2. Sequences categorized as patient-specific conserved nucleotide sequences displayed the following characteristics: 1) identical nucleotide sequences at each of the three points; 2) sequences that are not conserved within the same genotypes (Fig. 2A). Consistent with previous results [29], numerous nucleotide sequence variations in HVR-1 and 2 were identified in these HCV isolates from acute and chronic infection patients. However, patient-specific conserved nucleotide sequences were observed in this E2 region even within HVR-1 and -2 for each patient (boxed region; Fig. 2A). In the E1 region, patient-specific conserved nucleotide sequences were also observed in the five patients (data not shown).

Sequences categorized as substituted nucleotide sequences displayed (Fig. 2B). Substituted nucleotide sequences were present in this E1/E2 region for all 5 patients during the clinical course of infection.

Amino acid sequence variations in the E1/E2 protein region during the clinical course of hepatitis C

Deduced amino acid sequences of the E1/E2 region (a.a. 192 ~ 480) were compared in 5 patients (2 acute patients, 3 chronic patients) at points 1–3 (Fig. 3). Sequences categorized as patient-specific amino acid sequences displayed the same characteristics as those of patient-specific nucleotide sequences. Variations in a.a. sequence were particularly concentrated in HVR-1 and -2 for HCV genotype 1b isolates (patients A–C and 1b isolate from patient D; Fig. 3) and in HVR-1 alone

for HCV genotype 2a isolates (patient E and 2a isolate from patient D; Fig. 3) during the clinical course of infection. Although patient-specific conserved amino acid sequences were present in this E1/E2 region for all 5 patients during the clinical course of infection, the impact was not as strong as that of patient-specific conserved nucleotide sequences (Fig. 2).

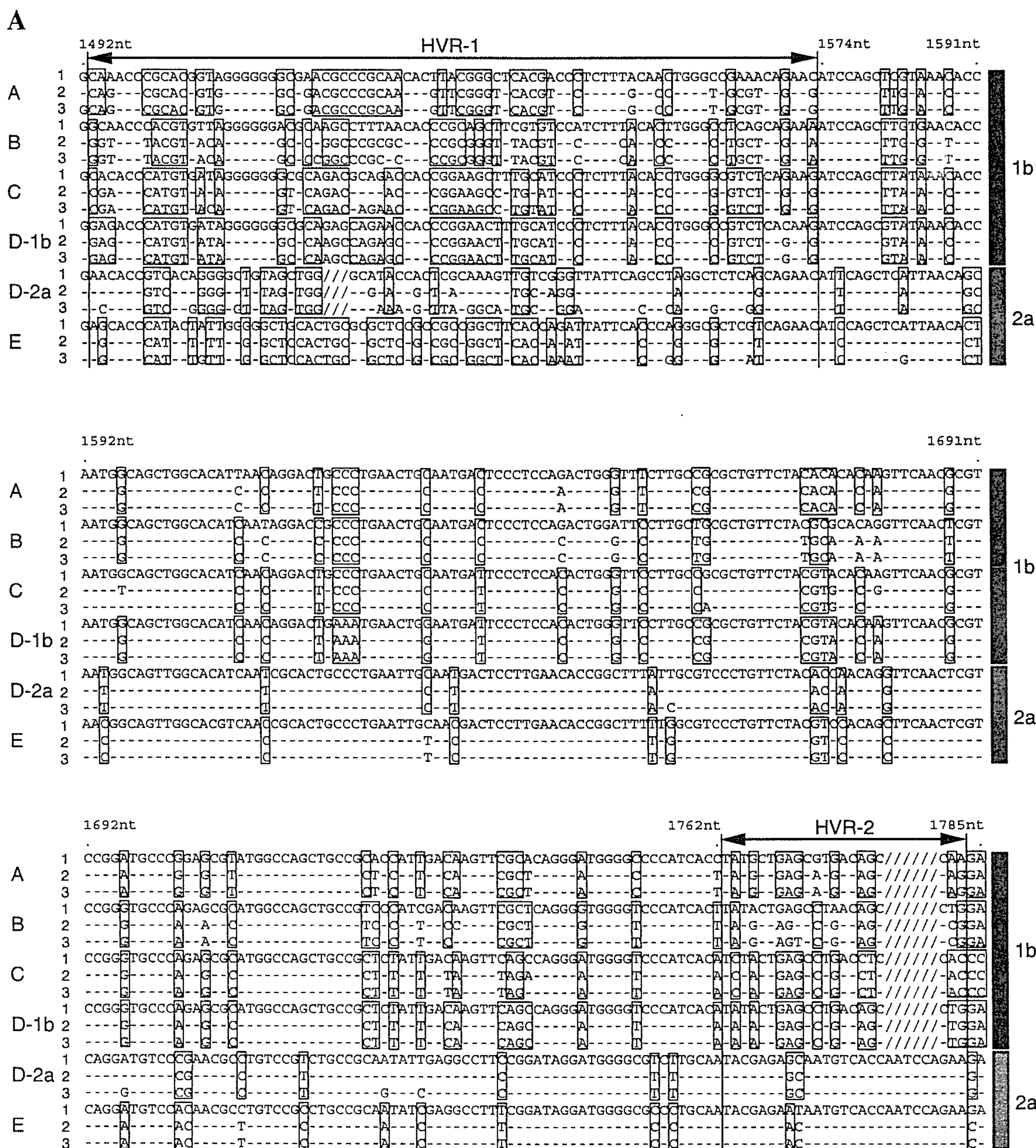

Fig. 2 (*continued*)

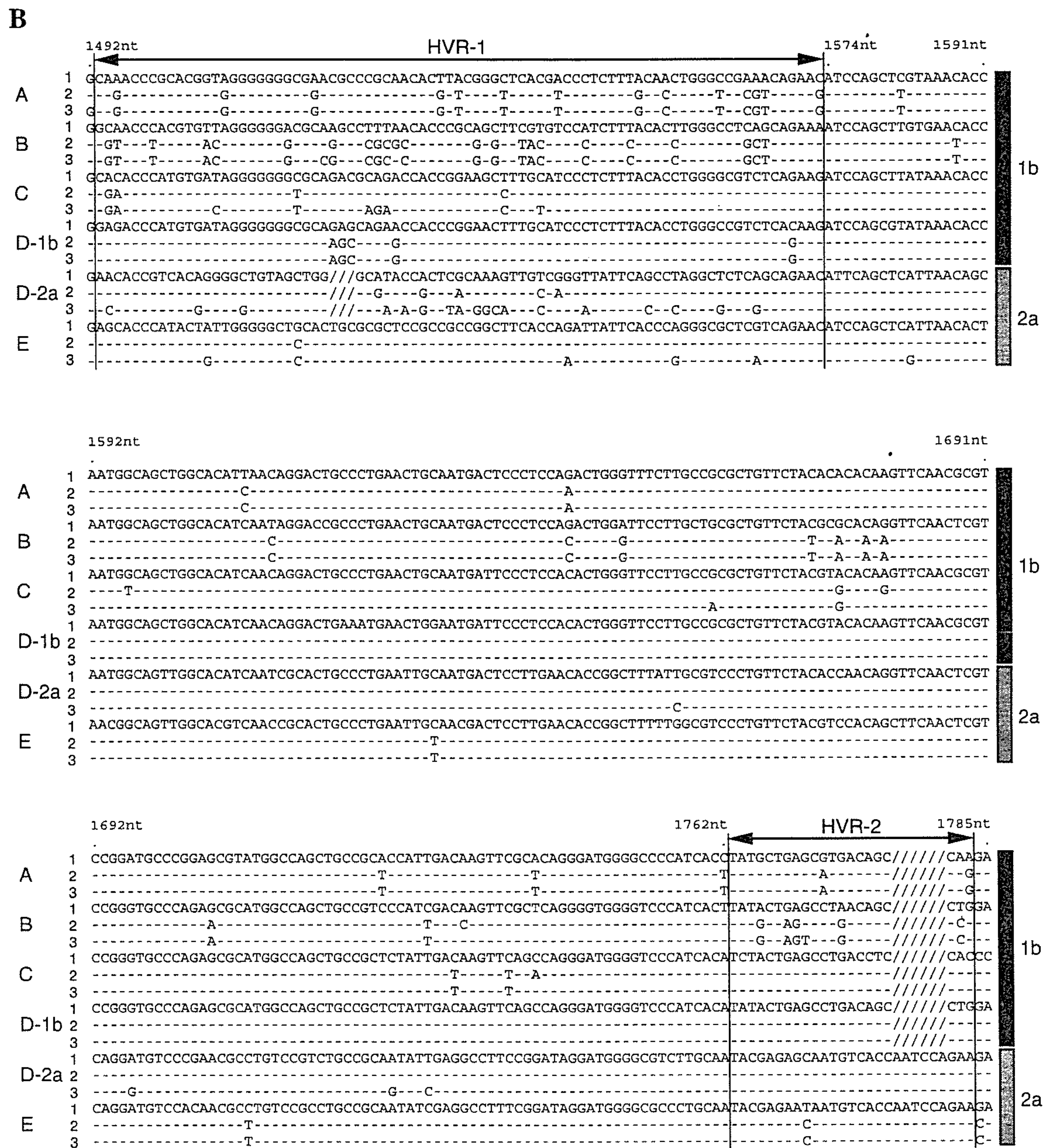

Fig. 2. Predominant nucleotide sequence comparison between each sequence from HCV patients at points 1–3. Region including nucleotides 1492–1785, including HVRs, was compared. **A** Patient-specific conserved sequences are enclosed in boxes. Each sequence column number indicates DNA sequencing point for each patient. Dash (–) indicates the same nucleotide as the first column sequence for each patient. Slash (/) indicates nucleotide deletion point. Column marked with a black box on the right side indicates HCV genotype 1b isolate. Column marked with a hatched box indicates HCV genotype 2a isolate. **B** Sequences categorized as substituted nucleotide sequences displayed

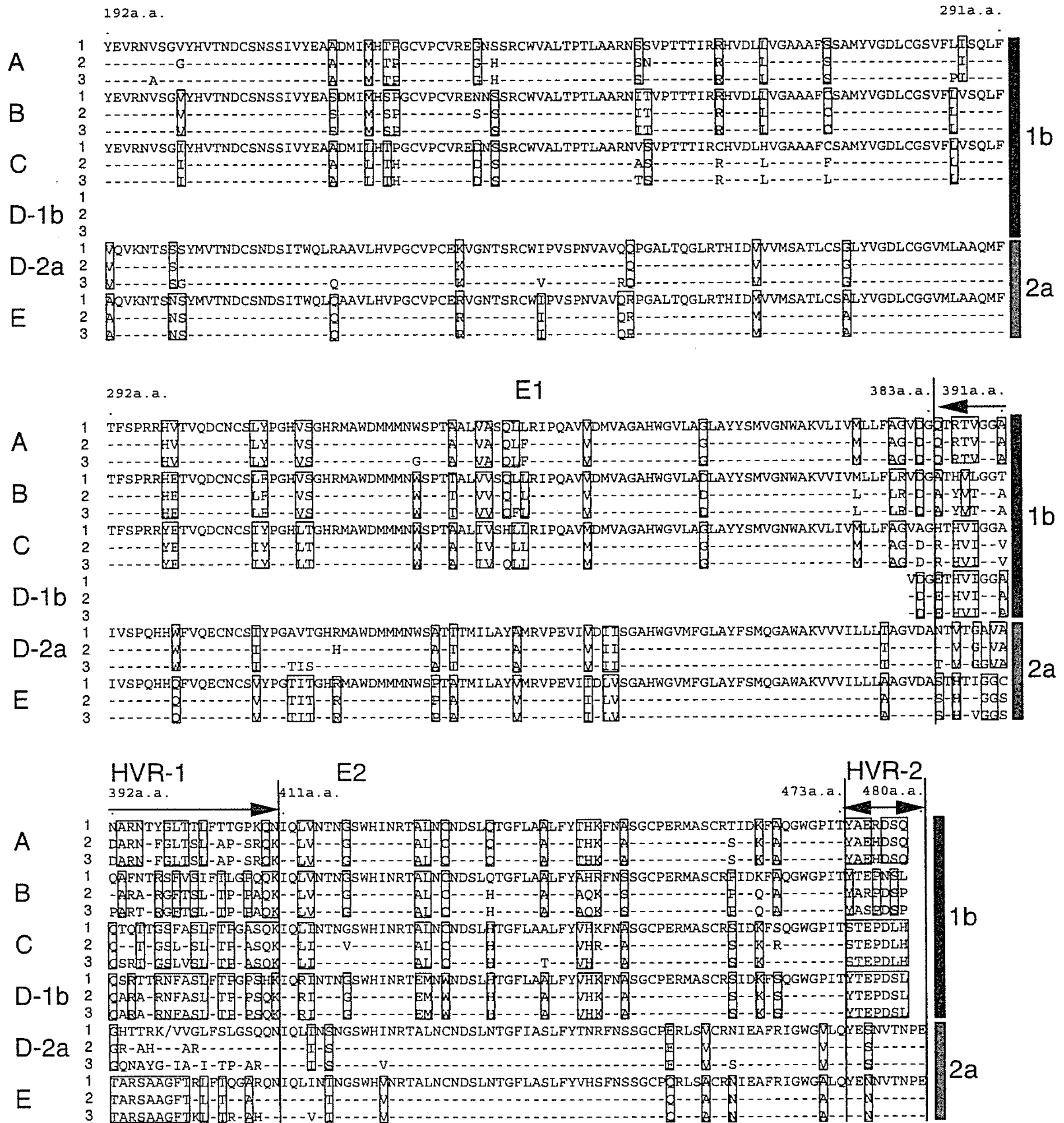

Fig. 3. Comparison of predicted amino acid sequences between each dominant HCV isolate from patients at points 1–3. E1/E2 protein sequences of HCV (a.a. 192–480) were compared. Sequence column number indicates DNA sequencing point for each patient. Patient-specific conserved sequences are enclosed in boxes. Dash (–) indicates the same a.a. residue as the first column sequence for each patient. Slash (/) indicates a.a. deletion point. Column marked with a black box on the right side indicates HCV genotype 1b isolate. Column marked with a hatched box indicates HCV genotype 2a isolate

In genotype 2a isolates from patient D (D-2a; Fig. 3), one a.a. deletion was identified in HVR-1 (residue 398, presented as a slash in Fig. 3). In genotype 2a isolates (patients D and E), two additional a.a.s in HVR-2 were noted, as reported elsewhere [21]. The deduced amino acid sequence of the E1 region (corresponding to a.a. 192 ~ 380) in genotype 1b isolates from patient D (D-1b; Fig. 3) could not be amplified by PCR at any time point (points 1–3; Figs. 1, 3).

Amino acid substitution speed in E1/E2 protein region and phylogenetic analysis of HVR-1 during progression of hepatitis C

To elucidate status of the HCV genome during infection, a.a. substitution speed between each point (point 1 to 2 and point 2 to 3; Fig. 1) was calculated as

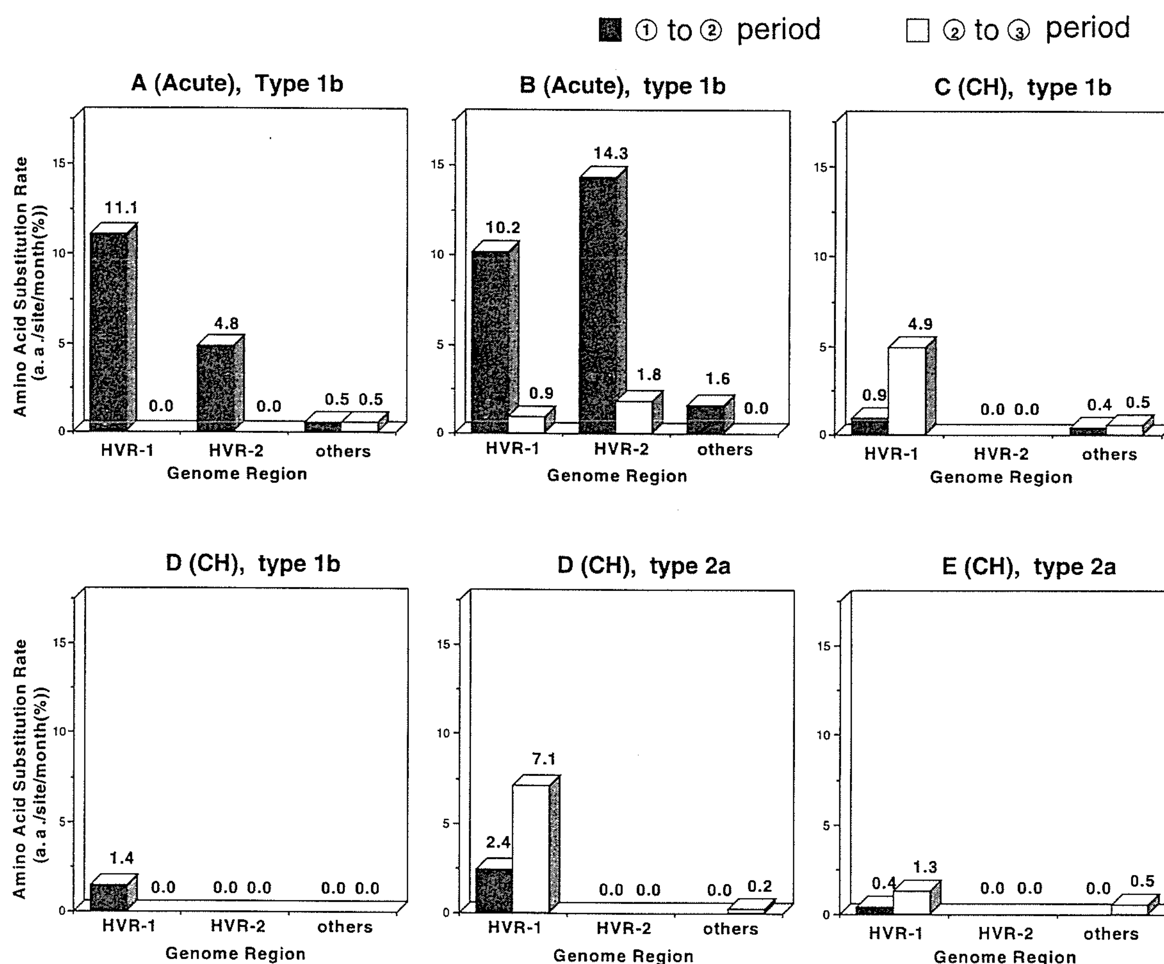

Fig. 4. Amino acid substitution speed in E1/E2 protein within HVR-1, HVR-2 and other regions. Amino acid substitution speed from DNA sequencing points 1 to 2 is indicated by black bars, and speed from points 2 to 3 is indicated by white bars. Regions HVR-1, HVR-2 and others represent a.a. 384–410, 474–480, and 411–473, respectively. *Acute*: acute HCV infection; *CH*: chronic HCV infection

the monthly rate of a.a. substitutions within each region (%; Fig. 4). In the first phase of acute infection (point 1 to 2), a.a. substitution speed in HVR-1 and HVR-2 was significantly faster than in the any other region of E1 and E2 in patients A and B (11.1% and 10.2% for HVR-1; 4.8% and 14.3% for HVR-2, respectively). In the second phase (point 2 to 3) of acute infection, a.a. substitution speed in HVR-1 and HVR-2 was slower than the first phase of acute infection in patients A and B (0% and 0.9% for HVR-1; 0% and 1.8% for HVR-2; 0.5% and 0% for other regions, respectively). In contrast, a.a. substitution speed in chronic patients was 0% in HVR-2 and below 0.5% in other regions (patients C–E; Fig. 4). Amino acid substitution speed in HVR-1 was fast during chronic HCV infection of ALT or when virus RNA levels underwent substantial transitions (patients C–E; Figs. 1, 4). In phylogenic tree analysis of HVR-1, sequence diversity of HVR-1 in the first phase of acute infection was phylogenetically distant from the original sequence (patients A and B), and the phylogenic tree of HVR-1 displayed clusters for each of the five patients (data not shown).

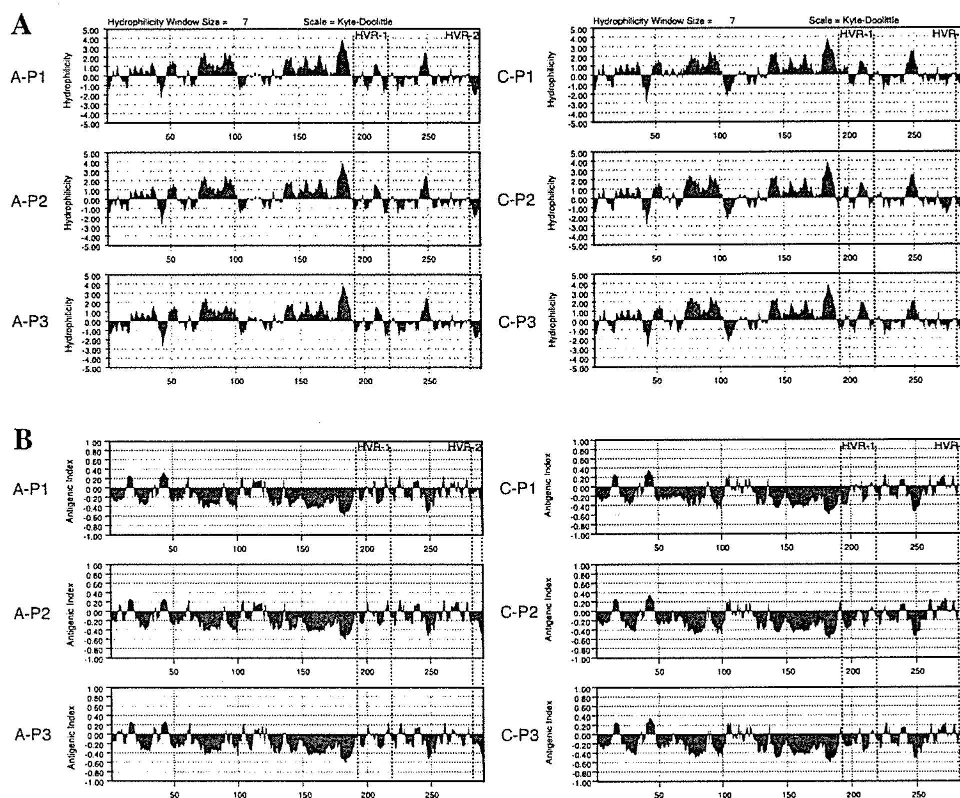

Fig. 5. Structural analysis of E1/E2 protein during HCV infection. Hydrophilicity profiles and antigenic indices of E1/E2 protein as predicted from direct DNA sequencing data were compared between points 1–3 for patients A and C. **A** Hydrophilicity profile. **B** Antigenic index. The presented region for HCV E1/E2 corresponds to a.a. 192–480

Comparison of protein characteristics for HVR-1 and -2 in the E1/E2 region

To examine the possibility that structural variation was generated in the E1/E2 region (a.a. 192～480) during disease progression in all five patients (Table 1), hydrophilicity, surface probability and antigenic indices were calculated from deduced a.a. sequences at each point. Figure 5 indicates the results of hydrophilicity (Fig. 5A) and antigenic index analyses (Fig. 5B) in patients A (acute infection) and C (chronic infection). These structural profiles displayed no significant changes during disease progression in patients A and C. The results of surface probability analysis in patients A and C likewise remained basically unchanged during disease progression. These three structural profiles demonstrate no significant changes in E1/E2 protein during the progression of HCV infection. Likewise, the remaining 3 patients (1 acute infection, 2 chronic infections; Table 1) displayed no significant changes in E1/E2 protein during disease progression. Moreover, the predicted secondary structure (chou-Fas) did not show any drastic changes between time points in any of the five patients (data not shown). Although some a.a. substitutions were observed in the E1/E2 region during disease progression in each patient, the major a.a. structure seems likely to have remained conserved in each case.

Humoral immune responses to each synthetic peptide from HVR-1

Synthetic HVR-1 peptides from chronic patients C and E were tested to characterize host immune responses during progression points using ELISA (points 1, 2, and 3; Fig. 1, Table 3). Patient C did not display antibody-positives against their own 3 HVR-1 peptides (C-1, C-2, C-3; Table 3) at any time point. In contrast, patient E displayed antibody-positives against their own 3 HVR-1 peptides (E-1, E-2, E-3; Table 3) at every time point (Table 3).

Discussion

The present study characterized nucleotide sequences of the E1/E2 protein region during clinical course from sera of 2 patients with acute HCV infection and 3 patients with chronic HCV infection using direct DNA sequencing methods. Furthermore, amino acid sequences and protein structures of the E1/E2 protein region (a.a. 192～480) were deduced during disease progression.

Nucleotide sequence variation in the E1/E2 region was mainly observed in HVR-1 and -2 for the 2 acute phase patients, and in only HVR-1 for the 3 chronic phase patients during clinical course. In the E1 protein region, a.a. substitution speed was below 0.69% in all five patients (2 acute patients, 3 chronic patients). This result indicates the possibility that E1 and HVR-2 may not be involved in escape mutation for chronic infection.

Previous reports have suggested that HVR-1 could serve as a target for neutralization of antibody and generation of escape mutants from humoral immune

responses, potentially contributing to the establishment of persistent HCV infection [14, 16, 20]. In our experiment, host immune responses to HVR-1 peptide during the course of chronic infection differed substantially between 2 patients (Table 3). For patient C, no antibody responses against 3 HVR-1 peptides (C-1, C-2 and C-3; Table 3) were observed at any time point. These data suggest two possible explanations. One is that antibodies were not produced in these stages, while the other is that the positions of C-1, C-2 and C-3 peptides might not be included in linear epitopes, instead being included in conformational epitopes. Patient E displayed a consensus sequence in each HVR-1 peptide (peptide sequence, TARSAAGFT; Table 3). For this reason, sera from patient E might react positively for E-1, E-2 and E-3 peptides at each time point. These results indicate the possibility that HVR-1 might not represent a significant epitope region for neutralization of HCV escape mutants in some cases.

Our results indicate the existence of patient-specific conserved nucleotide sequences in the E1/E2 region during clinical course of all HCV patients (Fig. 2). This finding may be useful for identifying HCV vertical transmission and other infection pathways. Furthermore, the existence of these patient-specific nucleotide sequences indicate the possible adaptation of the virus in patients and escape from the host immune surveillance systems in the early phase of HCV infection.

Rate of amino acid substitution speed between each point (point 1 to 2 and point 2 to 3; Fig. 1) during clinical course was calculated as the monthly rate of a.a. substitutions per site (%; Fig. 4). The data indicate that high a.a. substitution speed in HVR-1 and -2 was present in the first phase of acute infection, and that a.a. substitution speed in HVR-1 was elevated in chronic patients during major transitions in viral RNA or ALT levels. This phenomenon should support the understanding of HCV adaptation to host immune pressures and the establishment of persistent HCV infection.

The acute and secondary structures of the E1/E2 protein region (a.a. 192 ~ 380) from patients with hepatitis C displayed no significant change during clinical course. This observation suggests that HCV clones in hepatitis C patients may conserve a E1/E2 protein structure during persistent infection.

In conclusion, our observations suggest that the rapid substitution of amino acid sequences in the first phase of acute phase of infection may be involved the HCV adaptation to host immune pressures and the development of persistent HCV infection.

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A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase

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***Background/Aims*: Long-term follow-up study was performed to identify the candidates for antiviral therapy for hepatitis C virus (HCV) infection among carriers with persistently normal aminotransferase (ALT ≤ 30 U/L) levels (PNAL).**

***Methods*: One hundred and twenty-nine HCV carriers with PNAL who underwent liver biopsy and had platelet count over 150,000/μl were entered and 69 were followed for over 5 years. Thirty-five patients underwent serial liver biopsies. Serum ferritin and thioredoxin levels were also determined.**

***Results*: Seventeen patients had normal liver histology, 10 had moderate chronic hepatitis and the remainder 102 had mild hepatitis. Serum ferritin and thioredoxin levels were normal. The mean follow-up period for the 69 patients was 8.5 years. Of these 69 patients, 10 had persistently normal ALT levels (group A), 39 had transient elevation of ALT (group B), and 20 changed to symptomatic chronic hepatitis (group C). The rate of progression of fibrosis for groups A, B, and C were 0.05, 0.04, and 0.08, respectively. Hepatocellular carcinoma was not diagnosed in any of the patients.**

***Conclusions*: Around 90% of HCV carriers with PNAL have normal to mild liver histology. This long-term follow-up study demonstrated that 30% of such carriers became candidates for antiviral therapy within 5 years.**
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***Keywords*: Hepatitis C virus; Chronic hepatitis C; Asymptomatic HCV carrier; Normal serum ALT; Interferon**

1. Introduction

An estimated 170 million individuals are infected with hepatitis C virus (HCV) worldwide and chronic hepatitis C has recently become the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) in many countries including Japan. Most HCC develop in patients with advanced staged chronic hepatitis or cirrhosis, and rarely from mild chronic hepatitis type C.

It is thought that type C liver cirrhosis and HCC develop over 20–35 years following HCV infection [1], however, around 25% of patients with chronic HCV infection have normal serum aminotransferase (ALT) levels [2,3]. We reported previously that asymptomatic HCV carriers were predominant among females and that most of them had histologically minimal to mild chronic hepatitis [4]. In that paper, we defined asymptomatic HCV carriers as persistently HCV RNA positive patients with normal serum ALT levels (≤30 U/L) over 1 year. However, it has been reported that HCV carriers with normal serum ALT level had more advanced liver histology compared to HCV carriers with elevated serum ALT [5]. This discrepancy might be attributed to differences in the definition of the normal range of serum ALT used by various centers, however, it is very important to clarify whether HCV carriers with persistently normal ALT level (PNAL) are candidates for antiviral therapy.

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The current normal limit of serum ALT is 40 U/L, however, a recent report from an Italian group demonstrated that the healthy ranges for serum ALT were 30 U/L for men and 19 U/L for women, respectively [6], which are lower than the current values that have been used over the past 15 years. This criterion of normal serum ALT might be reasonable because a few cirrhotic patients have from 30 to 40 U/L of ALT [7].

In Japan, the number of HCC patients with HCV infection has increased since 1975. Antiviral treatment for chronic hepatitis C resulted in the inhibition of hepatic inflammation and progression of hepatic fibrosis and as a consequence the inhibition of the development of HCC [8–13]. Thus, inhibition of HCC is a very important issue in the treatment of patients with chronic hepatitis C. It remains controversial whether asymptomatic HCV carriers are candidates for antiviral therapy because of the low efficacy of treatment and flare-ups post treatment. However, taking into consideration the recent progress in antiviral therapy for chronic hepatitis C patients, the National Institute of Health Consensus Development Conference reported that patients with hepatitis C with normal serum ALT levels are candidates for interferon and ribavirin therapy [14]. Recently, a multicenter, randomized, controlled study for the treatment of patients with chronic hepatitis and persistently normal ALT levels with pegylated interferon alpha and ribavirin for 48 weeks led to eradication in 40% of patients infected with genotype 1b patients [15], which is similar to the results for symptomatic chronic hepatitis C patients [16,17]. However, most HCV carriers with PNAL have minimal to mild liver histology and their prognosis might be very good. Thus, there is some doubt, whether they are candidates for antiviral treatment to inhibit the progression of liver disease and hepatocarcinogenesis.

Recently, it has been reported that oxidative stress is an important factor in the development of HCV-related HCC [18–22] and the HCV core protein may generate oxidative stress via mitochondrial injury [23,24]. It is also demonstrated that iron overload generates oxidative stress, resulting in hepatic injury, and DNA damage and consequently this becomes an important factor for hepatocarcinogenesis [22,25,26].

We report here the biochemical and histological results of 8.5 years of follow-up of HCV carriers with PNAL. The data were analyzed according to the definitions of normal range (≤30 U/L) of serum ALT and platelet count (PLT) over 150,000 μl/ml. We also analyzed the status of oxidative stress using serum ferritin and thioredoxin levels. These results demonstrate the importance of the normal range of serum ALT, oxidative stress and follow-up study to decide the indication for antiviral therapy of HCV carriers with PNAL.

2. Patients and methods

2.1. Eligibility and definition

This study was conducted from January 1990 to August 2004.

HCV carriers with persistently normal ALT levels (PNAL) were defined as those patients who were HCV RNA positive by reverse transcriptase polymerase chain reaction (RT-PCR), had normal serum ALT levels (≤30 U/L) over a 12-months period and on least three different occasions and platelet count of over 15×10^4 μl/ml. Patients positive for hepatitis B surface antigen (HBsAg), previous interferon (IFN) treatment, a history of heavy alcohol abuse, anti-nuclear antibody (ANA) and anti-smooth muscle antibody (ASMA) positivity, patients with overt Diabetes mellitus and obesity (body mass index; over 30 kg/m^2) were excluded from this study.

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki, and approved by the Ethics Committee of Kyoto Prefectural University of Medicine. Informed consent was obtained from every patient.

2.2. Quantification and determination of HCV RNA and genotyping

Frozen-stored sera from 129 individuals were tested. Serum HCV RNA levels was determined using the AMPLICOR GT HCV MONITOR (Roche Diagnostic Systems, Tokyo, Japan). The detection range of this assay was between 0.5 and 850 KIU/ml, and each sample was measured again after dilution with distilled water. HCV genotypes 1 and 2 were determined by a serologic genotyping assay [27]. Genotypes 1 and 2 in this assay correspond to genotype 1 (1a, 1b) and 2 (2a, 2b) proposed by Simmonds et al. [28].

2.3. Study design

Of the 129 patients who underwent liver biopsy, 69 patients enrolled in this study and followed over 5 years (8 males, 61 females). These patients received blood tests every 4 months for an initial 2 years and then received blood tests every 6 months when they remained still normal ALT. αfetoprotein (AFP) was measured every years in all patients, and all patients underwent ultrasonography every year to detect HCC.

All patients submitted to a liver biopsy using a Menghini needle guided by ultrasonography prior to entry. Formalin-fixed liver specimens were stained with hematoxylin and eosin for morphological evaluation, with Masson's trichrome stain for assessment of fibrosis, and with Perls' Prussian blue stain (from February 1998) for assessment of iron loading. Histological follow-up studies were carried out for 35 patients 3.4–13.4 years (mean: 6.8 years) after the first biopsy.

The histological findings of HCV carriers with PNAL were interpreted and scored according to the classification proposed by Desmet et al. [29] and Ishak et al. [30]. Steatosis is defined having fat droplets in over 10% of hepatocytes. The degree of iron loading was assessed using a Perls' score of 0 to 4+, based on the scoring system of MacSween et al. [31].

Fasting blood samples were collected in the morning. Serum ALT, blood glucose level, serum ferritin, platelet count (PLT), serum HCV RNA level and HCV genotype were examined in the laboratory of our university hospital, using the standard analytical method; the ULA ALT value was 30 U/L. Serum thioredoxin (TRX) levels were measured with a sensitive sandwich ELISA kit (Fujirebio, Inc., Tokyo, Japan) as described previously [26,32] and of the 129 patients 47 were available for this assay. Blood chemistry was done every 4–6 months during the follow-up period.

2.4. Statistical analysis

Data values are expressed as medians with interquartile ranges. We compared continuous variables using the Mann–Whitney *U*-test. The Kruskal–Wallis test was used for multiple group comparisons, and Spearman correction coefficients were used to examine the relationship between groups. Frequency analysis was performed with the χ^2 test, and Fisher's exact test. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Demographic and clinical features

The demographic and clinical features of the 129 HCV carriers with PNAL are shown in Table 1. Twenty-four were male and 105 were female. No significant differences were noted in age, serum ALT, PLT, and follow-up period between males and females. Serum ferritin levels were 76.1±53.4 ng/ml in male and 60.0±43.3 ng/ml in female. Serum HCV RNA levels were significantly ($P=0.0012$) higher in G1 compared with G2 (648.7±622.5 KIU/ml vs 356.2±628.8 KIU/ml (Table 1).

Characteristics of the 69 patients followed over 5 years are also shown in Table 1. Their mean follow-up period was 8.5±2.4 years.

Of the 105 female patients, 44 had serum ALT levels≦19 U/L and 61 had serum ALT levels of 20–30 U/L at entry. There were no significant differences in their ages, platelet count, serum ferritin levels, serum HCV RNA levels, or BMI (Table 2).

Serum thioredoxin (TRX) levels in these patients were within the normal range, and significantly lower than those of patients with chronic hepatitis and cirrhosis (Table 3).

Table 1
Characteristics of 129 HCV carriers with persistently normal ALT who underwent liver biopsy

	N=129	Followed over 5 years (*N*=69)
Follow-up period (years)	5.7±3.6	8.5±2.4
Age (years)	48 (21–77)	45 (29–71)
Male (*N*=24)	49.8±16.4	42.3±14.9
Female (*N*=105)	47.2±12.5	46.63±11.6
Sex (M/F)	24/105	8/61
ALT (U/L)	8–30	9–30
Male (*N*=24)	22.5±5.7	21.1±5.4
Female (*N*=105)	21.6±4.8	22.3±5.1
PLT (×10⁴/ml)	15–31	15–31
Male (*N*=24)	20.3±4.4	20.9±5.3
Female (*N*=105)	21.8±4.4	21.2±4.0
Ferritin (ng/ml)	5–225	5–225
Male	76.2±53.5	84.6±59.2
Female	60.0±43.3	66.6±52.5
HCV RNA (KIU/ml)	6–3350	22–2100
G1 (*N*=58)	648.9±622.57*	595.1±561.1** (*N*=32)
G2 (*N*=45)	356.2±628.8	211.0±219.2 (*N*=27)
Mixed and unclassified	6–1994	
BMI (kg/m²)	16–27	16–27
Male	22.2±1.7	21.9±1.9
Female	21.3±2.2	21.0±2.4

Values were expressed as mean±SD. *P* values were calculated by Mann–Whitney *U*-analysis with correction for tie. *$P=0.0012$ (G1 vs G2); **$P=0.0006$ (G1 vs G2).

Table 2
Baseline of female patients between HCV carriers having ≦19 U/L of ALT and HCV carriers showing 20–30 U/L of ALT

	ALT≦19 (U/L)	20<ALT≦30 (U/L)	*P* value
Number of patient	44	61	
Age (y.o)	44.9±12.5	48.8±12.2	
ALT (U/L)	16.0±2.4	24.3±2.9	<0.0001
PLT (×10⁴/μl)	22.0±4.4	21.6±4.3	
HCV RNA (KIU/ml)	400.2±555.1	500.7±541.1	0.3896
BMI (kg/m²)	21.2±2.3	21.4±2.2	

Values were expressed as mean±SD. *P* values were calculated by Mann–Whitney *U*-analysis with correction for tie.

3.2. Liver histology

The results of liver histology for the first biopsy are described in Table 4. Normal liver histology was noted in 17 (14%) subjects, 102 (79%) showed minimal to mild chronic hepatitis, 10 (8%) had moderate chronic hepatitis.

Steatosis was seen in nine patients (7%) and iron loading was noted in 6/50 (12%).

3.3. Follow-up study of laboratory data

Of the 69 patients followed over 5 years (mean±SD: 8.5±2.4 years), 10 (14%) had continuously normal ALT (group A), 39 (57%) showed transient elevation of ALT (group B), and 20 (29%) changed to chronic hepatitis with continuously abnormal serum ALT (group C) (Table 5). Of the 61 female patients, eight were group A, 34 were group B, and 19 were group C. There were no significant differences in age, ferritin levels, serum HCV RNA levels, or BMI among the three groups. However, serum ALT levels were significantly lower in group A compared with group B and C (Table 6). The number of patients having ALT levels≦19 IU/L in these three groups were seven (7/8:87.5%) in group A, 12 (12/34:35.3%) in group B, and three (3/19:15.8%) in group C.

Table 3
Serum thioredoxin (TRX) levels in 47 HCV carriers with PNAL at liver biopsy

	Serum thioredoxin (ng/ml)
HCV carriers with PANL (*n*=47)	27.7 [9.1–38.5]
Chronic hepatitis (*n*=124)	34.5 [8.6–135.6][a]$^{+}$
Liver cirrhosis (*n*=24)	42.5 [21.4–97.2][a]$^{++}$
Control (*n*=15)	24.9 [1.3–50.7][a]

*$P=0.0012$ when compared with G2. The overall significance of differences between four groups according to non-parametric Kruskal–Wallis analysis of variance was $P<0.001$. Therefore, the significance of differences between groups was determined by Scheffe's method: $^{+}P<0.01$; $^{++}P<0.001$, compared to HCV carriers with PNAL.

[a] These data were reported in J Hepatol 2000; 33: 616–622.