**Nucleotide Sequence Diversity of Hypervariable Region 1 of Hepatitis C Virus in Japanese Hemophiliacs With Chronic Hepatitis C and Patients With Chronic Posttransfusion Hepatitis C**

By Hidenori Toyoda, Yoshihide Fukuda, Yasuo Koyama, Isao Nakano, Moritoshi Kinoshita, Toru Hadama, Junki Takamatsu, and Tetsuo Hayakawa

Hemophiliac patients with chronic hepatitis C might be exposed to and become infected with multiple hepatitis C virus (HCV) strains by means of frequent use of blood products, even if they are infected with a single subtype of HCV. To test this hypothesis, we analyzed the genetic diversity of hypervariable region 1 (HVR1) of HCV in chronically infected hemophiliacs and in patients with chronic posttransfusion hepatitis with a single HCV inoculation. The diversity of nucleotide sequences in HVR1 of serum HCV RNA was compared between 21 hemophiliacs infected with a single HCV subtype and 16 patients with posttransfusion HCV infection. The number of HCV quasispecies was determined by fluorescence single-strand conformation polymorphism (SSCP) analysis. Direct sequencing was performed to determine the diversity in HVR1. The number of HCV quasispecies in the blood was 5.2 ± 2.0 clones in hemophiliacs and 4.0 ± 2.3 clones in posttransfusion patients, a nonsignificant difference (P = .0943). The number of sites at which the nucleotide was not homogenous in all quasispecies was significantly higher in hemophiliacs (13.0% ± 7.4%) than in posttransfusion hepatitis patients (2.7% ± 2.8%; P < .0001). In conclusion, there was a high degree of genetic variation in HVR1 of HCV specimens isolated from hemophiliacs compared with posttransfusion patients. These findings indicate the possibility that multiple infections of a single HCV subtype may occur among patients frequently exposed to blood products; single HCV subtypes may therefore derive from multiple origins.

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NUCLEOTIDE DIVERSITY AND MULTIPLE INFECTIONS

Table 1. Clinical Profiles

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hemophiliacs With HCV Infection</th>
<th>Patients With Chronic Posttransfusion Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>Male:female</td>
<td>22:1</td>
<td>11:6</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>27.8 ± 9.1</td>
<td>60.4 ± 12.4</td>
</tr>
<tr>
<td>Median duration of HCV carriage, yr (range)</td>
<td>22.5 (11-40)</td>
<td>18.6 (4-40)</td>
</tr>
<tr>
<td>Biochemistry (mean ± SD)</td>
<td></td>
<td></td>
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<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>70.2 ± 47.0</td>
<td>68.6 ± 51.3</td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>59.5 ± 26.7</td>
<td>59.7 ± 37.9</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>4.29 ± 0.48</td>
<td>4.26 ± 0.37</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>3.34 ± 0.69</td>
<td>3.45 ± 0.42</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>134.4 ± 77.7</td>
<td>177.4 ± 55.2</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.81 ± 0.58</td>
<td>0.75 ± 0.24</td>
</tr>
<tr>
<td>Serology (on admission)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HCV* (second-generation)</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>HCV RNA* (nested PCR)</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>HBs antigen*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HBs antibody*</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Subtype</td>
<td></td>
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<tr>
<td>1a</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>2a</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2b</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic persistent hepatitis</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td>20</td>
<td>16</td>
</tr>
</tbody>
</table>

*Since the first elevation of alanine aminotransferase above the normal limit.
†Liver biopsy specimens were obtained after selection of the patients. They were taken after patients had given informed consent.
‡P < .0335.
§P < .0001.

HCV RNA extraction. RNA was extracted from serum HCV by the acid-guanidium isothiocyanate-phenol-chloroform method. It was extracted from a 100-μL aliquot of serum from a patient and then subjected to reverse transcription.

Quantification of HCV. Quantification of HCV RNA was performed by both competitive PCR methods and branched DNA (bDNA) probe assay. In patients whose HCV RNA was undetectable by bDNA probe assay, which could not detect less than 0.5 × 10⁶ Eq/mL, HCV RNA concentrations by bDNA probe assay were considered equal to 0.5 × 10⁶ Eq/mL.

Oligonucleotide primers for PCR amplification of HVR1. HVR1 of HCV was PCR-amplified using a newly developed primer set. This primer set was designed by analyzing 128 variations of reports of the HCV RNA genome so that the sequences of the primers were greater than 80% homologous to every described isolate. The primer sequences of the region were as follows: HVR1 (sense primer for the first PCR), TGCCGAGCCAGATTGAGAGAC-TGGG; HVR2 (sense primer for the second PCR), TACTTCTCATGGTGCGGACTTGTTG; HVR3 (antisense primer for the second PCR), GATTTGTCGTCGAGGCTTTTG; and HVR4 (antisense primer for the first primer and reverse transcription), CGTGCTGGTTGTGCTGCAAAC-TGCC.

These primers were obtained with an LKB Gene Assembler Plus DNA Synthesizer (Pharmacia, Uppsala, Sweden) using a protocol described previously. The HVR2 primer was labeled with fluorescein isothiocyanate, and the HVR3 primer was labeled with biotin.

Reverse transcription and PCR amplification. Reverse transcription from HCV RNA to cDNA was performed with a protocol described previously. All nested PCR reactions were performed in a thermal cycler (Lobocycler 40; Stratagene La Jolla, CA; and Nippon Genetics, Tokyo, Japan) over 29 cycles with 1 μmol/L primers and a Gene Amp kit (Perkin-Elmer, Foster City, CA) under conditions recommended by the kit supplier. Each PCR cycle consisted of denaturation at 94°C for 1 minute, annealing at 56°C for 1.5 minutes, and extension at 72°C for 1.5 minutes.

To determine the degree of sequence variation of the HCV-amplified sequences and the diversity of quasispecies, we performed an assay based on PCR amplification of viral sequences.

Fluorescence single-strand conformation polymorphism (SSCP) analysis. The number of quasispecies was determined by a fluorescence SSCP analysis. The sequencing reactions were performed using an auto-read sequencing kit (Pharmacia) under the conditions recommended by the kit supplier, with a protocol based on the dideoxy-mediated chain-termination method. The reaction products were electrophoresed in 6% acrylamide gel (Ready Mix Gel; Pharmacia) using the ALF II DNA Sequencer (Pharmacia). Identification of the mutation site was performed using Alignment software (Fujitsu, Tokyo, Japan).

Entire clones were analyzed directly, and the sites at which nonconserved nucleotides were located among HVR1 of HCV from each individual were detected by autosequencing. We sequenced 75 nucleotides of HVR1, and the percentage of sites at which nucleotides were not conserved in the entire population was calculated as an indication of diversity.

Statistical analysis. Statistical analyses were performed using the x² test and Mann-Whitney U test where appropriate. Pearson’s test was used in the correlation analysis. A P level less than .05 was considered statistically significant.

RESULTS

Comparison between HCV-infected hemophiliacs and patients with chronic posttransfusion hepatitis C. HCV RNA concentration tended to be lower in hemophiliacs than in posttransfusion chronic hepatitis patients when measured by a competitive PCR method (4.7 ± 1.3 log(copies)/mL in hemophiliacs vs 5.4 ± 1.2 log(copies)/mL in posttransfusion hepatitis patients, P = .0514) and when measured by bDNA probe assay (4.1 ± 3.8 × 10⁶ Eq/mL in hemophiliacs vs 6.2 ± 9.4 × 10⁶ Eq/mL in posttransfusion hepatitis patients, P = .9449), but it was not statistically significant. The distribution of patients in each group according to HCV RNA concentration is shown in Table 2.

In 21 of 23 hemophiliacs with chronic hepatitis C and 16 of 17 posttransfusion hepatitis patients, HVR1 was amplified by PCR. Both groups were similar regarding the number of viral quasispecies identified by SSCP analysis: 5.2 ± 2.0
Table 2. Distribution of Patients in Each Group According to HCV RNA Concentration

<table>
<thead>
<tr>
<th></th>
<th>Competitive PCR (copies/mL)</th>
<th>bDNA Probe Assay (x 10^6 Eq/mL)</th>
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<tbody>
<tr>
<td></td>
<td>&lt;10^4</td>
<td>10^4-10^5</td>
</tr>
<tr>
<td>Hemophiliacs with HCV infection</td>
<td>4/23 (17.4%)</td>
<td>14/23 (60.9%)</td>
</tr>
<tr>
<td>Chronic posttransfusion hepatitis patients</td>
<td>1/17 (5.9%)</td>
<td>9/17 (52.9%)</td>
</tr>
</tbody>
</table>

clones in hemophiliacs and 4.0 ± 2.3 clones in posttransfusion hepatitis patients (P = .0943; Fig 1).

Analysis by direct sequencing showed that the rate of sequences of nonhomology among quasispecies studied from a given patient (diversity) was 13.0% ± 7.4% (9.8 ± 5.5 of 75 nucleotides) in hemophiliacs and 2.7% ± 2.8% (2.0 ± 2.1 of 75 nucleotides) in posttransfusion hepatitis patients, the difference being statistically significant (P < .0001; Fig 2). In addition, the degree of diversity tended to increase with the duration of HCV infection in the posttransfusion hepatitis group (r = .6187, P = .0106), but not in the hemophiliac group (r = .0525, P = .8210).

Comparison among HCV-infected hemophiliacs. In HCV-infected hemophiliacs, the number of viral quasispecies identified by SSCP analysis was similar between patients who had received blood products both from Japan and from the United States (13 patients) and patients who had received only Japanese-made blood products (eight patients): 4.9 ± 2.0 clones in the former and 5.9 ± 2.1 clones in the latter (P = .2773). The diversity in both groups of patients was also similar: 12.7% ± 7.7% (9.5 ± 5.8 of 75 nucleotides)

![Fig 1. Number of populations in hemophiliacs and posttransfusion hepatitis patients measured by SSCP analysis. No significant difference was observed between these 2 groups, which indicated the existence of the same number of quasispecies in the serum of a given patient. (○) Patients with coinfection of HIV; (●) patients without coinfection of HIV.](image1)

![Fig 2. Diversity of HVR1 in E2/NS1 gene in hemophiliacs and posttransfusion hepatitis patients analyzed using direct sequencing. The diversity of hemophiliacs with HCV infection was significantly larger than for patients with posttransfusion chronic hepatitis C, which suggests the coexistence of HCV strains from multiple origins. (○) Patients with coinfection of HIV; (●) patients without coinfection of HIV.](image2)
in patients who had received blood products both from Japan and from the United States and 13.5% ± 7.4% (10.1 ± 5.5 of 75 nucleotides) in patients who had received only Japanese-made blood products (P = .8848).

In addition, the number of viral quasispecies and the diversity of patients coinfected with HIV in HCV-infected hemophiliacs (11 patients) were 4.7 ± 2.1 clones and 13.9% ± 7.7% (10.5 ± 5.8 of 75 nucleotides), and those of patients infected only with HCV (10 patients) were 5.8 ± 1.9 clones and 12.0% ± 7.3% (9.0 ± 5.5 of 75 nucleotides). There were no differences between these two groups with regard to the number of viral quasispecies and the diversity (P = .2050 and P = .5035, respectively).

**DISCUSSION**

Populations of RNA virus particles from a given patient consist of heterogeneous mixtures of genetically different but closely related variants called quasispecies.29,30 This heterogeneity has been attributed to the high error frequency in RNA replication.29,31,32 The genome of HCV itself has also been characterized by marked sequence heterogeneity33-35 and is suggested to have the quasispecies nature throughout the entire genome.36-38 HCV quasispecies have recently been demonstrated to be relevant to persistent infection,39 clinical features,40 viral titer,41 disease progression,42 and response to interferon therapy.43-45

Previous studies of HCV heterogeneity have generally consisted of cloning PCR-amplified sequence products,36,37,39,46-48 and some clones of which were subject to direct sequencing.48-52 However, cloned cDNA molecules do not always represent the true composition of HCV quasispecies.

By the methods used in this study, all possible quasispecies from individual patients were analyzed by direct sequencing, and therefore, diversity within the HVR is more reflective of the entire population of quasispecies.

We compared the diversity of HVR1 between hemophiliacs with chronic hepatitis C and posttransfusion hepatitis C patients. Because hemophiliac patients have a frequent use of blood products, we considered them the model cases that consisted of cloning PCR-amplified sequence products.46-52 However, cloned cDNA molecules do not always represent the true composition of HCV quasispecies.

The hemophiliacs' great number of quasispecies might be caused by the promotion of genetic variation. Kao et al1 reported a superinfection of genotype III (subtype 2a) in a patient with persistent HCV infection of genotype II (subtype 1b), and observed the disappearance of HCV of genotype III from serum and the promotion of the genetic variation of HCV of genotype II. In our study, it could also be suggested that the great sequence variability in the HVR of hemophiliacs might be caused by the promotion of genetic variation by means of repeated inoculation and disappearance of HCV of different subtypes.

Also, the tendency for a lower HCV RNA concentration in hemophiliacs with chronic hepatitis C versus chronic posttransfusion hepatitis C patients, especially as measured by competitive PCR, might be explained by the frequent immune response associated with repeated inoculation of HCV of different or even the same subtypes, which might be considered to promote the genetic variation.

In conclusion, it appears that hemophiliacs with chronic HCV infection have particles with considerable sequence variation in the HVR1 of the E2/NS1 gene, due to spontaneous mutation and to acquisition of new infecting strains. These results indicate the possibility of the coexistence of a single HCV subtype from another origin. The effect of multiple infection on the diversity of the HVR should be considered in analyses of nucleotide diversity in individual patients.

**REFERENCES**

5. Kao J-H, Chen P-J, Lai M-Y, Chen D-S: Superinfection of
heterologous hepatitis C virus in a patient with chronic type C hepatitis. Gastroenterology 105:583, 1993
20. Schulhof JC, Molko D, Tecule R: The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acid Res 15:397, 1987
NUCLEOTIDE DIVERSITY AND MULTIPLE INFECTIONS


51. Kurosaki M, Enomoto N, Marumo F, Sato C: Rapid sequence variation of the hypervariable region of hepatitis C virus during the course of chronic infection. Hepatology 18:1293, 1993


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