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Abstract

Since detection of hepatitis C virus RNA by the polymerase chain reaction (PCR) showed that there existed anti-C100-3 (anti-HCV) antibody negative patients infected with HCV, we attempted to find out whether there were any clinical or viral genomic differences between the anti-HCV antibody positive and negative groups. One hundred and fifty-nine patients with chronic liver diseases with hepatitis C virus RNA in their sera were selected. Anti-HCV antibody was tested for anti-C100-3 antibody by an enzyme linked immunosorbent assay. The incidence of anti-HCV antibody was 129/159. The concentration of serum gamma-globulin, the titier of ZTT, and the positive rate of the PCR with the primers of the NS3/4 region (NS3/4PCR) were significantly higher in the anti-HCV antibody positive group than in the negative group. However, the other data such as alanine aminotransferase activity or past history were not significantly different. Nucleotide sequence of the cDNA fragments of NS3/4 region amplified by the PCR did not differ significantly between isolates from anti-HCV antibody positive and negative sera. The sequences observed in the present study did not differ significantly from those reported previously. Although there remains the possibility that the variation of viral genomic sequences may cause the absence of anti-HCV antibody, these results suggested that the individual clinical backgrounds or immunoreactivity of the patients might influence the antibody development.

KEYWORDS: hepatitis C virus, polymerase chain reaction, anti-C100-3 antibody, genetic variation

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Anti-C100-3 Antibody Status, Viral Genomic Sequences, and Clinical Features in Chronic Hepatic Patients with Hepatitis C Virus RNA in Sera

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Since detection of hepatitis C virus RNA by the polymerase chain reaction (PCR) showed that there existed anti-C100-3 (anti-HCV) antibody negative patients infected with HCV, we attempted to find out whether there were any clinical or viral genomic differences between the anti-HCV antibody positive and negative groups. One hundred and fifty-nine patients with chronic liver diseases with hepatitis C virus RNA in their sera were selected. Anti-HCV antibody was tested for anti-C100-3 antibody by an enzyme linked immunosorbent assay. The incidence of anti-HCV antibody was 129/159. The concentration of serum gamma-globulin, the titer of ZTT, and the positive rate of the PCR with the primers of the NS3/4 region (NS3/4PCR) were significantly higher in the anti-HCV antibody positive group than in the negative group. However, the other data such as alanine aminotransferase activity or past history were not significantly different. Nucleotide sequence of the cDNA fragments of NS3/4 region amplified by the PCR did not differ significantly between isolates from anti-HCV antibody positive and negative sera. The sequences observed in the present study did not differ significantly from those reported previously. Although there remains the possibility that the variation of viral genomic sequences may cause the absence of anti-HCV antibody, these results suggested that the individual clinical backgrounds or immunoreactivity of the patients might influence the antibody development.

Key words : hepatitis C virus, polymerase chain reaction, anti-C100-3 antibody, genetic variation

Complementary DNA fragments of the hepatitis C virus (HCV) genome have been molecularly cloned (1), and anti-HCV enzyme linked immunosorbent assay (ELISA) using these expressed antigens (C100-3) has been established (2). This anti-HCV antibody (anti-C100-3 antibody) is now routinely used for diagnosis of type C liver diseases. Recently, detection of HCV RNA by the polymerase chain reaction (PCR) has become

available and is a convincing diagnostic procedure (3). Both anti-HCV antibody and detection of HCV RNA by the PCR have revealed HCV as major causative agent of blood-borne non-A, non-B liver diseases (2, 4, 5).

Nucleotide sequences of HCV genomes analyzed in several laboratories are reported to diverge from the prototype sequence cloned by Chiron corporation (HCV-1, (6); HCV-J, (7, 8); HCV-BK, (9); and others (10-13)). According to the nucleotide sequences, HCV can be divided

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into at least 3 basic groups (14). Controversy continues regarding clinical features among the subgroups of HCV (14).

Detection of the HCV genome by the PCR is becoming more widely used and it is well recognized that there exist patients who were diagnosed, via the PCR, as infected with HCV, but did not present anti-HCV antibodies (15). The sequence divergence of HCV genome was thought to be one of the factors that influence the development of anti-HCV antibody. We chose patients whose sera were positive for HCV RNA by the PCR, divided them into anti-HCV antibody positive and negative groups, and attempted to find statistically significant clinical differences between two groups.

Materials and Methods

Materials. All chemicals were reagent-grade and purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) unless otherwise indicated. All manipulations were done under RNase-free conditions, using equipments specified for only RNA experiments.

Patients and sample sera. Sample sera were obtained from 164 (age; 26–79, mean 55.5) patients with chronic non-A non-B liver diseases (Table 1). Patients were diagnosed as non-A, non-B liver diseases if all the other known causes of liver diseases could be excluded by clinical, serological, laboratory, and histological studies. The diagnosis of chronic hepatitis and liver cirrhosis was made from clinical and laboratory data, and confirmed in 64 cases (chronic hepatitis, 46 cases, liver cirrhosis, 18 cases) by histological examination of liver biopsy specimen under laparoscopy. The diagnosis of hepatocellular carcinoma was made from image diagnosis (ultrasonography, computed tomography, magnetic resonance imag-

ing, or angiography) or histological examination of biopsy specimen of the tumors. Sera of the patients who underwent interferon therapy were obtained before the treatment started. Negative control sera were obtained from 34 patients with non-type C chronic liver diseases (chronic hepatitis B, 17; autoimmune hepatitis, 10; primary biliary cirrhosis, 7).

Anti-HCV antibody. Anti-HCV antibody was tested for anti-C100-3 antibody by an ELISA (Ortho Diagnostic Systems, Japan).

RNA extraction. RNA was extracted from sample sera according to the AGPC methods modified from those of Chomczynski (16). In brief, 200 μ l of serum was mixed with 400 μ l of 1.5X solution D (1.5X:6M guanidinium thiocyanate, 37.5mM sodium citrate, pH 7.0, 0.20% sarkosyl, 0.15M 2-mercaptoethanol), 60 μ l of 2M sodium acetate, pH4.0 was added, followed by two extractions of phenol-chloroform (first 5:1; second 1:1). After precipitation with isopropanol, the RNA pellet was suspended in 6 μ l of distilled water.

Reverse transcription and amplification by the PCR. Oligonucleotide primers of C-1, C-2, C-3, N3-1, and N3-2 were synthesized on a DNA synthesizer (Applied Biosystems Japan, Tokyo). NC-1, NC-2, and NC-C were provided from Daichi Pure Chemical Co., Ltd. (Tokyo)(Fig. 1).

N3-1 and N3-2 are deduced from the prototype sequence of HCV (17). C-1, C-2, and C-3 are deduced from the sequence of HC-J1 (7). NC-1, NC-2, and NC-C are deduced from the prototype sequence of HCV. RNA obtained from 100 μ l of serum was subjected to the reverse transcription. Reverse transcription was carried out at 42°C for 30 min in a volume of 10 μ l containing the template, 0.4 units of ribonuclease inhibitor (Promega Biotec, Wisconsin, USA), 1mM of dithiothreitol, 1 μ M of reverse primer (NC-C for 5'-noncoding region; C-3 for core region; N3-2 for NS3/4 region), 2.5 units of M-MuLV reverse transcriptase (New England BioLabs, Massachusetts, USA), 1mM of dNTP (Perkin Elmer Cetus, Connecticut, USA), and the PCR buffer (final concentration: 1mM of Tris-HCl [pH8.3], 5mM of KCl, 0.25mM of MgCl₂).

Serum samples were treated in each experiment in parallel with a negative control, which was subjected to reverse transcription and amplification by the PCR. Stringent care was taken to avoid false positive results due to cross-contamination (18).

The reaction products containing complementary DNA were then amplified by the first stage of the PCR for 35 cycles. Each reaction cycle involves denaturation

Table 1 Clinical diagnosis of selected patients

Diagnosis	Number of cases		
	Male	Female	Total
Chronic hepatitis	48	43	91
Liver cirrhosis	21	17	38
Hepatocellular carcinoma	29	6	35
Total	98	66	164

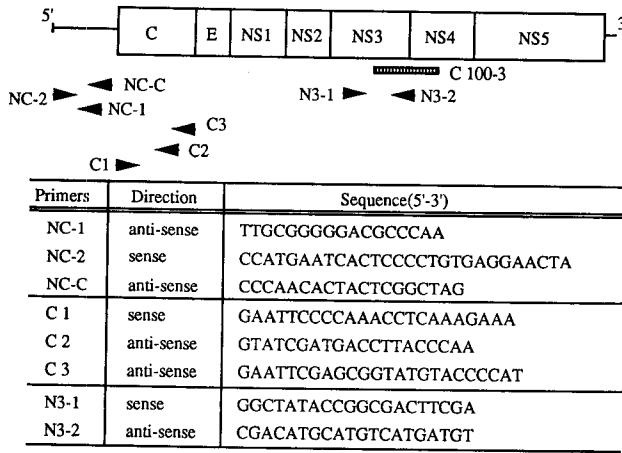


Fig. 1 Schema of primer location on HCV genome deduced from HCV-BK (12). Arrow heads indicate the location and direction of the primers. Dashed box corresponds to the epitope of C100-3. C: core region; E: envelope region; NS1-5: nonstructural regions 1-5.

at 94°C for 1 min, primer annealing at 45°C for 1 min, and primer extension at 72°C for 1 min. The first stage of PCR was carried out in a total volume of 20 μ l containing 0.5 μ M of each primer (NC-2 and NC-C for 5'-noncoding region; C-1 and C-3 for core region; N3-1 and N3-2 for NS3/4 region), 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus), 1 mM of dNTP, and the PCR buffer.

One tenth of the volume of the first PCR products was taken into the re-amplification by the second stage of PCR. The conditions of the second stage of PCR were the same as in the first, except that the primer annealing was performed at 55°C and the amplification was made for 20 cycles. The sets of primers used in the second stage of PCR were NC-1 and NC-2 for 5'-noncoding region, C-1 and C-2 for core region, and N3-1 and N3-2 for NS3/4 region.

Aliquots from the second PCR products were subjected to electrophoresis on a 2.25% NuSieve, 0.75% SeaKem LE agarose gel (FMC Bio-products, Maryland, USA). DNA species were stained with ethidium bromide under ultraviolet light, and analyzed according to the comparison with the migration pattern of the DNA molecular weight marker (pBR322/HaeIII).

Cloning and sequencing. The cDNA fragments amplified by the PCR were cloned with the TA Cloning™ System (Invitrogen, San Diego, USA) following the

manufacturer's instructions.

The nucleotide sequences of cloned cDNA were determined by dideoxy-chain termination methods (19) in use of Sequenase Ver. 2.0 DNA sequencing kits (United States Biochemical Corp.).

The correlation between anti-HCV antibody and clinical backgrounds of the individual patients. Patients whose sera were positive for HCV RNA by the PCR were divided into anti-HCV antibody positive and negative groups. The following data were compared between the two groups; diagnosis of the liver disease, age, sex, incidence of blood transfusion history, family history, alcoholic history, serum level of alanine aminotransferase activity, concentration of gamma-globulin, and titer of ZTT. Statistical analysis was performed by Student's *t* and chi square tests.

Results

The efficiency in detecting of HCV sequences by the PCR. On the HCV genome, 3 different regions were chosen as target for PCR to detect HCV sequences, i.e., the 5'-noncoding region, the putative core region, and the NS3/4 region (the PCRs for above target regions are abbreviat-

ed as follows; 5'-NC PCR, C PCR, NS3/4 PCR, respectively). As given in Fig. 2, cDNA amplified after two stages of PCR showed single bands with expected molecular weight (the 5'-NC PCR products: 214 base pair (bp); the C PCR products: 361 bp; the NS3/4 PCR products: 623 bp). None of the negative controls treated in each experiment showed positive results.

There was a difference in efficiency in detecting HCV sequences among 3 sets of primers. HCV sequences were detected in 159 of 164 patients (97.0 %) with at least one set of primers. The NS3/4 PCR detected HCV sequences in 89 of 164 patients (54.3 %), the 5'-NC PCR in 63 of 66 (95.5 %), and the C PCR in 108 of 132 (81.8 %). Both the 5'-NC PCR and the C PCR were carried out in 42 samples, and the positive rate of each PCR was 85.7 and 47.6 %, respectively. The NS3/4 positive sera were all positive for the 5'-NC PCR and/or the C PCR. HCV sequences were not detected in sera of 5 patients in our study. They were diagnosed as non-A, non-B chronic hepatitis and liver cirrhosis by our criterion, and the diagnosis was confirmed in 2 cases by histological examination of liver biopsy specimen. Thereafter, we used HCV RNA positive 159 patients for further analysis.

Incidence of anti-HCV antibody and differences of the clinical backgrounds between the anti-HCV antibody positive and negative groups. The incidence of anti-HCV antibody was 129/159 (81.1 %). There was a significant difference in the positivity of the NS3/4 PCR between the anti-HCV antibody positive and negative groups. The incidence was 82/129 (63.6 %) in the former and 7/30 (23.3 %) in the latter ($p < 0.01$).

Clinical comparisons between the anti-HCV antibody positive and negative groups were shown in Table 2. There were no significant differences in blood transfusion history, family history, alcoholic history, or severity of the liver diseases between the two groups. The two groups consist of almost the same ratio of patients with chronic hepatitis, liver cirrhosis,

and hepatocellular carcinoma. The laboratory data of the two groups did not provide significant differences in the level of serum alanine aminotransferase activity. The concentration of serum gamma-globulin was higher in the anti-

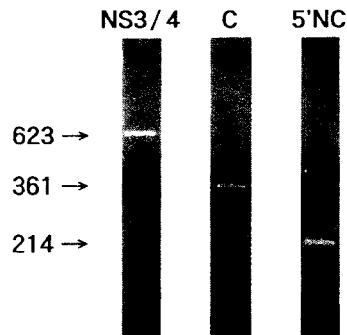


Fig. 2 HCV cDNA amplified by PCR, extracted from serum was reverse-transcribed and the cDNA was amplified by 1st stage of PCR for 35 cycles. After additional 20 cycles of 2nd stage of PCR, cDNA was electrophoresed through an agarose gel, stained with ethidium bromide under ultraviolet light, and the DNA bands were photographed. The figures on the left side indicate size of the cDNA (base pair). N: NS3/4 PCR products; C: C PCR products; 5'NC: 5'-NC PCR products.

Table 2 Clinical differences between anti-hepatitis C virus (HCV) antibody (Ab) positive and negative patients

Clinical findings	HCV RNA (+) ^a (n = 159)	
	Anti-HCV Ab (+) (n = 129)	Anti-HCV Ab (-) (n = 30)
Clinical diagnosis		
Chronic hepatitis	73 (56.6 %)	17 (56.7 %)
Liver cirrhosis	28 (21.7 %)	6 (20 %)
Hepatocellular carcinoma	28 (21.7 %)	7 (23.3 %)
Laboratory tests ^b		
ALT (IU/l)	90.7 ± 7.8	70.0 ± 10.5
ZTT (KU)	14.0 ± 0.33	11.1 ± 0.55**
γ-gl (g/dl)	1.61 ± 0.05	1.27 ± 0.12*
NS3/4 PCR (+) ^c	63.6	23.3**
Blood transfusion ^c	35.5	40.0
Family history ^c	25.8	36.0
Alcoholic history ^c	25.8	36.0

a: Positive for the 5'-NC PCR and/or the C PCR;

b: Mean ± SE;

c: Percent of incidence * $p < 0.05$ ** $p < 0.01$

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4704
[1] GATGCCCACTTCTATCCCAGACAAAGCAGAGTGGGGAGAACCTTCTTACCTGGTA
[2] *****CT*G*****C**A***GCA**A**C*****C**C*****
[3] *****C*****G*****A*****G*****C*****
[4] *****CT*****GCA**A*****C*****
[5] *****C**A***GCA**A*****

4760
GCGTACCAAGCCACCGTGTGCGCTAGGGCTCAAGCCCTCCCCATCGTGGGACCAG
*****A*****C*****G**T**A**T*****A
*****G*****A*****C**G**T**A**T*****T**A
*****A*****C*****A**G**A*****T*****
*****C**C**G**T**A**T*****T**A

4816
ATGTGGAAGTGTGTTGATTCGCCTCAAGCCACCCTCCATGGCCAACACCCTGCTA
*****C*****A*****A**G*****G*****G
*****CA*****A*****T**G*****C*****T**G
*****C**C**G**T**A*****A*****
*****A*****G**T**C*****G*****T**G

4872
TACAGACTGGCGCTGTTTCAGAATGAAATCACCCCTGACGCCACCCAGTACCAAAATC
****G**A**A**C*****A*****GG****T**C**A*****CA**A*****
****G*****A**GG***G***C***G*****GA**A*****G***
**T**G*****T*****A*****T**A*****
*****A**A**C*****A*****C**A*****GA**A*****

4928
ATCATGACATGCATGTCG
*****G*****
*****
*****
*****

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Fig. 3 Sequences of HCV cDNA, encoding a part of C100-3 protein, cloned from HCV infected serum. This region covers 243 nucleotides (nucleotide position of 4704 to 4946 of HCV-1 (6) out of whole 1090 nucleotides encoding a C 100-3 protein. [1]: HCV-1 which was reported by Choo *et al.* (6). [2]: HCV-J which was reported by Kato *et al.* (14). [3][4][5]: isolated from patients' serum by PCR in our study. Serum [3] was positive for anti-HCV antibody, but sera [4] and [5] were negative. Underlined codons indicate the difference from HCV-1 at amino acid level. Wavy line indicates the region corresponding the antisense primer of N3-2.

Table 3 Homology of nucleotide and amino acid sequences

	Nucleotide (amino acid) homology percentage			
	[2]	[3]	[4]	[5]
[1]	84.2 (93.9)	87.9 (91.4)	86.6 (91.5)	87.0 (96.3)
[2]	—	89.5 (93.9)	89.1 (91.5)	93.1 (96.3)
[3]	—	—	87.4 (92.7)	91.9 (92.7)
[4]	—	—	—	89.5 (92.7)

See legend to Fig. 3.

HCV antibody positive group than in the negative group (mean ± SE, 1.61 ± 0.05, 1.27 ± 1.3g/dl, respectively, p < 0.05). Also, the anti-HCV antibody positive group shows a higher titer of ZTT than the negative group (mean ± SE, 14.0 ± 0.33, 11.1 ± 0.13 KU, respectively, p < 0.01).

The nucleotide sequences of the NS3/4 PCR products which overlap the region encoding C100-3 protein. After determining that the inci-

dence of anti-HCV antibody closely relates to that of the NS3/4 PCR, we compared the nucleotide sequences of the part of the NS3/4 PCR products which overlap the region encoding C100-3 protein.

The degrees of nucleotide homology between the anti-HCV antibody positive isolate ([3]) and negative isolates ([4],[5]) were 87.4% ([3],[4]) and 91.9% ([3],[5]), and the degrees of amino acid homology were both 92.7% (Fig. 3 and Table 3). Nucleotide (amino acid) homology among Japanese isolates ranged from 87.4 (91.5)% to 93.1 (96.3)%. There were no significant changes in the nucleotide sequences among these isolates at least in this region. Nucleotide (amino acid) homology between Japanese isolates and the prototype (6) was from 84.2 (91.4)% to 89.5 (96.3)%.

Differences of the clinical backgrounds between the NS3/4 PCR positive and negative groups. The incidence of blood transfusion history, family history, and alcoholic history, the severity of the disease, serum level of alanine aminotransferase activity, serum concentration of gamma-globulin were compared for the NS3/4 PCR positive and negative groups. No significant differences were found (data, not shown).

Discussion

HCV has been cloned as a major cause of non-A, non-B posttransfusion hepatitis. Also, the incidence of anti-HCV antibody is reported to be high in HCC patients in Japan (4, 20), and a close relationship between HCV and hepatocarcinogenesis is suspected. HCV is an RNA virus (1), and displays a high degree of sequence divergence (7, 8, 10, 14, 21). The complete HCV genomes isolated from Japanese patients (HCV-J: (8); HCV-BK: (9)) have been reported to have a divergence of nucleotide sequences of 21 and 23%, respectively, when compared to the prototype sequence cloned by Chiron corporation (HCV-1: (6)). To improve the efficiency in

detecting HCV RNA in the serum, the most appropriate set of primers must be chosen. HCV sequences were detected in the sera of 97.0% of the 164 patients with non-A, non-B chronic liver diseases by the 5'-NCR and /or the C PCR. Samples in which both the 5'-NC PCR and the C PCR were carried out, showed a positive rate for each PCR of 85.7% and 47.6%, respectively. Using the primers at the 5'-noncoding region to detect HCV RNA is obviously advantageous. Okamoto *et al.* also reported a high detectability of HCV RNA by the PCR with primers from the 5'-noncoding region in scoring in 31 (97%) of 32 patients with chronic non-A, non-B hepatitis (22). These results are attributed to the remarkable sequence conservation of the 5'-noncoding region (99%)(11). HCV sequences were not detected in sera of 5 patients in our study. They were diagnosed as having non-A, non-B chronic hepatitis and liver cirrhosis by clinical, serological and laboratory studies. HCV in sera of these 5 patients might be too little to be detected by our methods. HCV sequences might be detected if sample sera were drawn at a different time or of greater volume were used for extraction of RNA.

Infection with HCV is now routinely diagnosed by anti-HCV antibody in serum. Anti-HCV antibody detected by an ELISA kit used in our study is against the recombinant HCV antigen (C100-3) which is located in the non-structural region of the HCV genome. Although a clear association has been shown between the anti-HCV antibody and chronic non-A, non-B liver diseases (2, 4, 5), 30(19.9%) of the 159 patients did not present this antibody in our study and were proved by the PCR to be infected with HCV. These results may suggest that once a person is exposed to the HCV and develops chronic liver diseases, some present anti-HCV antibodies, but others do not. We supposed that the divergence of nucleotide sequences of HCV or the clinical and immunological backgrounds of the individual patients may influence the development of anti-HCV antibody.

We compared the nucleotide sequences

between the isolates from patients who tested positive or negative for the anti-HCV antibody in the NS3/4 region that overlapped the region encoding C100-3 protein, but found no significant differences. We can not completely exclude the possibilities of viral genomic influence in the development of anti-HCV antibodies because of the following 3 facts: 1) the incidence of anti-HCV antibody correlates to that of the NS3/4 PCR; 2) nucleotide sequences we examined were 243 of 1090 nucleotides encoding C100-3 protein; 3) we did not examine nucleotide sequences of the NS3/4 PCR negative isolates. Kato *et al.* reported that sequences of the NS3 region of the HCV-J genome differed approximately 20 % from that of the HCV-1 genome (10, 21). The NS3/4 PCR might fail to detect HCV sequences because of the sequence divergence of the HCV genome (10, 23). Chan *et al.* have reported that three types of HCV were found by the comparison of nucleotide sequences, and that serological diagnosis of infection with HCV types 2 and 3 by the anti-C100-3 antibody had considerable problems for effectiveness (24). They reported that type 1 includes the prototype HCV (HCV-1) and Japanese HCV (HCV-J). Our isolates ([3],[4], and [5] in Fig. 3) are thought to belong to the same group as HCV-J according to the degrees of nucleotide homology. Therefore, it might be difficult to explain anti-HCV antibody development from the point of Chan's subtypes.

When the clinical backgrounds were considered, it was found that anti-HCV antibody positive groups had higher concentrations of serum gamma-globulin than anti-HCV negative groups. In chronic liver diseases, an increase of the concentration of serum gamma-globulin is observed. Two basic mechanisms are considered responsible for the increase of serum gamma-globulin (25). 1) a hyperreactive immune state which may be associated with adjuvant effects of liver cell breakdown products. Depressed cell-mediated immunity may express a compensatory B-cell overaction. 2) an increased antigenic stimu-

lus; The diseased liver may fail to sequester antigens absorbed from the gastrointestinal tract. The increase of serum gamma-globulin in chronic liver diseases reflect liver injuries and a hyperreactive immune state. It may be concluded that anti-HCV antibody may be developed more often in patients in a hyperreactive immune state.

Comparative sequence analysis of all the complete and partial HCV sequences published to date indicates that they can be broadly subdivided into at least three basic groups (6). It is important to determine the clinical consequences of infection with different HCV agents. The pathology of HCV infections differ widely from case to case and can vary from asymptomatic infection to fulminant hepatitis. It is important to clarify the causes of the differences in the clinical courses of HCV infections. In order to determine whether the clinical courses of HCV infection could vary according to the subgroups of infected HCV agents, clinical differences among subgroups must be investigated. Fujio *et al.* found no correlation between clinical features and the variation of nucleotide sequences of the putative core region analyzed by restriction fragment length polymorphism (26). In our study, when patients with HCV infection were divided into the NS3/4 PCR positive and negative groups, no differences were found in diagnosis of the liver diseases, alanine aminotransferase activity, gamma-globulin, ZTT, alcoholic history, family history and blood transfusion history between the two groups.

Little is known about the virology and biology of HCV infections at present, and it is necessary to use information of the molecular biology of the HCV to analyze the pathogenesis of HCV infection. Further study is required to determine the nature of any correlation between the subgroups of HCV and clinical features such as the severity of liver injuries, clinical consequences, response to interferon therapy, and hepatocarcinogenesis (4, 20).

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