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Genetic variation of putative core gene in hepatitis C virus.

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Abstract

Genetic variation of hepatitis C virus was assessed. We prepared RNA fractions from 21 patients' sera which were positive for hepatitis C virus RNA, synthesized their cDNAs, and amplified fragments, 406 base pairs, encoding a putative core protein, by polymerase chain reaction. One of them, N 15, was cloned and sequenced. N 15 showed 92.4% homology at the nucleotide level and 97.0% homology at the amino acid level compared with HC-J 1 which is the first isolated clone in Japan and similar to that isolated in USA. By restriction fragment length polymorphisms analysis, 14 out of 21 patients (66.7%) showed the same pattern as N 15. No patients showed the pattern of HC-J 1. We could not find a correlation between the genetic variation and clinical features of hepatitis C virus infection. These results indicate that the region, which encodes the core protein and is believed to be relatively conserved in hepatitis C virus genome, has several variations at the nucleotide level, and the major part of hepatitis C virus in Okayama district is different from HC-J 1 and the USA clone.

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Genetic variation of hepatitis C virus was assessed. We prepared RNA fractions from 21 patients' sera which were positive for hepatitis C virus RNA, synthesized their cDNAs, and amplified fragments, 406 base pairs, encoding a putative core protein, by polymerase chain reaction. One of them, N 15, was cloned and sequenced. N 15 showed 92.4 % homology at the nucleotide level and 97.0 % homology at the amino acid level compared with HC-J 1 which is the first isolated clone in Japan and similar to that isolated in USA. By restriction fragment length polymorphisms analysis, 14 out of 21 patients (66.7 %) showed the same pattern as N 15. No patients showed the pattern of HC-J 1. We could not find a correlation between the genetic variation and clinical features of hepatitis C virus infection. These results indicate that the region, which encodes the core protein and is believed to be relatively conserved in hepatitis C virus genome, has several variations at the nucleotide level, and the major part of hepatitis C virus in Okayama district is different from HC-J 1 and the USA clone.

Key words : hepatitis C virus, restriction fragment length polymorphisms, polymerase chain reaction, genetic variation

Blood-borne non-A, non-B hepatitis (NANBH) agents have been unidentified for a long time (1). Recently cDNA fragments encoding a specific peptide for NANBH were obtained by immunoscreening of a cDNA library derived from the NANBH infected plasma (2-4). The putative virus from which the cDNAs derived was termed hepatitis C virus (HCV). Its nucleotide sequence was determined to be a positive strand RNA genome.

Epidemiological studies of antibody to the HCV recombinant peptide revealed that HCV was a prime agent responsible for NANBH (5). It has been pointed out that the present antibody assay system has a limitation in its sensitivity and specificity to detect HCV infection (6). HCV genome is scarcely detected with conventional techniques of molecular biology. However, it can be detected specifically and effectively by reverse transcription followed by polymerase chain reaction (PCR)(7). The comparison of obtained nucleotide sequences revealed variation in HCV (8-11). However, sequence analysis of variants is cumbersome and time-consuming (12). Restriction fragment length polymorphisms (RFLP) analysis has been recently developed to determine genomic change easily and rapidly (13). Using the combination of PCR and RFLP, we evaluated variations in the region encoding putative core protein, which is believed to be relatively conser-

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ved in HCV. We also investigated the correlation between the variations of HCV RNA and clinical feature of HCV disease.

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 condition, using equipment specified for only RNA experiments.

Table 1 shows the clinical data of 21 patients whose sera were positive for HCV RNA by PCR. Twenty-two

serum samples were obtained from them. Twenty of them were positive for antibody to HCV by determination using the commercial assay kit (Ortho Diagnostic System, Tokyo, Japan). Case 5 lived in Shiga Prefecture, cases 8 and 19 lived in Hiroshima Prefecture, and the rest lived in Okayama Prefecture.

Reverse transcription and PCR. RNA was extracted by an acid guanidinium thiocyanate-phenol-chloroform method from polyethylen glycol precipitates of sera (14, 15). Briefly, $400\,\mu$l serum was precipitated with $40\,\mu$l of 44 % polyethylen glycol at 4°C for 2 h. After centrifugation at $14000 \times$ g for 20 min at 4°C, the pellet was dissolved in $500\,\mu$l solution (solution D) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % (w/v) sodium lauroylsarcosine, and 0.1 M 2-

Table 1   Clinical data of the patients and the results of RFLP

Case	Age/Sex	Clinical diagnosis	BT/FH	C 100	AST/ALT (IU/ℓ)	RFLP *Kpn* I	result *Msp* I
1	58/M	CH	−/−	+	72/ 90	K 1	M 1
2	58/M	CH	−/−	+	105/125	K 1	M 1
3	62/F	CH	−/−	+	59/ 70	K 1	M 1
4	62/F	CH	−/−	−	73/ 56	K 1	M 1
5	50/F	CH	+/−	+	178/191	K 1	M 1
6	59/F	CH	+/+	+	26/ 24	K 1	M 1
7	49/M	CH	−/−	+	35/ 61	K 1	M 1
8	64/F	LC	−/−	+	44/ 51	K 1	M 1
9	50/M	LC	−/+	+	70/ 92	K 1	M 1
10	52/M	LC	−/−	+	117/ 74	K 1	M 1
11	61/M	LC	+/−	+	59/ 69	K 1	M 1
12	72/M	LC	−/−	+	71/ 37	K 1	M 1
13	73/F	LC[b]	−/−	+	78/140	K 1	M 1
14	71/M	LC[b]	+/+	+	76/ 76	K 1	M 1
15	60/M	AH	+/−	−	67/138	K 1	M 4
16	54/F	CH	−/+	+	20/ 19	K 1	M 4
17[a]	56/M	CH	−/+	+	33/ 36	K 2	M 4
				+	22/ 19	K 2	M 4
18	31/F	CH	−/−	+	35/ 65	K 2	M 4
19	59/F	LC	−/−	+	133/143	K 1	M 4
20	56/M	LC[b]	+/−	+	69/ 46	K 1	M 4
21	75/M	LC[b]	−/−	+	93/ 60	K 2	M 1

BT: Blood transfusion history; Family history of liver disease; C 100: anti-C 100 antibody; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase ; AH: Acute hepatitis; CH: Chronic hepatitis; LC: Liver cirrhosis; K 1, K 2, M 1, M4: see legend to Fig. 3.
a: Paired sera at twenty months' interval.
b: Liver cirrhosis with hepatocollular carcinoma.

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mercaptoethanol. Fifty μl 2 M sodium acetate (pH 4. 0), 500 μl water-saturated phenol, and 100 μl chloroform: isoamyl alcohol (49:1) were mixed sequentially. The solution was incubated at 4°C for 20 min and centrifuged at 14000 × g for 10 min at 4°C. The aqueous phase was removed, re-extracted with phenol-chloroform, and precipitated with equal volume of isopropanol at − 20°C overnight. After centrifugation at 14000 × g for 20 min at 4°C, the pellet was dissolved in 100 μl solution D, and re-precipitated with ethanol. The ethanol precipitation was repeated after dissolving in 50 μl of 10 mM Tris-HCl (pH 8. 0) and 1 mM ethylene diamine tetraacetate (EDTA). Finally RNA was washed in 70 % ethanol, air-dried, and dissolved in 5.3 μl distilled water.

Two microliters of RNA was reverse-transcribed in a total 10 μl PCR buffer including 1. 0 μM anti-sense primer (C3), 4 u RNasin (Promega Biotec, Wisconsin, USA), and 2.5 u Molony murine leukemia virus reverse transcriptase (New England Biolabs, Massachusetts, USA). The PCR was carried out in 20 μl PCR buffer (GeneAmp® PCR reagent kit, Perkin-Elmer Cetus, Connecticut, USA) containing 0.5 μM primers, 0. 1 mM deoxynucleotide triphosphates, and 0. 5 u Taq DNA polymerase on a DNA Thermal Cycler (Perkin-Elmer Cetus) for 35 cycles. Thermal conditions were primer annealing for 1 min at 45°C, extension for 1 min at 72°C, and denaturation for 1 min at 94°C. To obtain enough products for analysis, 2 μl of PCR products were re-amplified in 20 μl PCR buffer for additional 20 cycles. Thermal conditions for annealing, extension, and denaturation were 55°C, 72°C, and 95°C, respectively. The sense primer was C 1 (5′-GAATTCCCCAAACCTCAAAG-AAA-3′) at position 337–353, and the anti-sense primer was C 3 (5′-GAATTCGAGCGGTATGTACCCCAT-3′) at position 724–742. These primers were constructed according to the sequence of HC-J 1 (8), added Eco Rl site at their 5′termini, and synthesized (Applied Biosystems Japan, Tokyo, Japan) following the manufacture's instructions.

Cloning and sequencing of pHSN 15. The cDNA synthesized from case 7 was treated with T 4 polynucleotide kinase (Takara Biochemicals, Kyoto, Japan), purified by electroelution from an agarose gel, and inserted by blunt-end ligation into Sma I site of pGEM-7 Zf (+)(Promega). Two clones with both directions (pHSN 15) were obtained after transformation of E. coli strain with XL 1-Blue. Single strand DNAs obtained by using helper phage, M 13 KO 7 (Takara), were sequenced by dideoxy chain termination method (16).

RFLP analysis. Five microliters of PCR products

were digested with restriction enzymes Kpn I (10 u) or Msp I (8 u)(Takara) for 3 h at 37°C. The digests were electrophoresed through an agarose gel of 3 % NuSieve and 1 % SeaKem® (FMC Bio-Products, Maryland, USA) in 40 mM Tris acetate, and visualized by staining with 1 μg/ml ethidium bromide. To make sure the specificity of the gene amplification and to detect small amounts of cDNA fragment bands, Southern blot analysis was performed. After denaturation in 1.5 M NaCl and 0.5 M NaOH, the DNA were transferred to Biodyne® A membranes (Pall BioSupport, New York, USA) with 1 M ammonium acetate and 20 mM NaOH (17, 18). The membranes were incubated at 60°C in 50 % deionized formamide, 5 X Denhardt's solution, 5 X SSPE (150 mM NaCl, 10 mM NaH$_2$PO$_4$, 1 mM EDTA), 1 % (w/v) sodium dodecyl sulfate (SDS), 0.2 mg/ml salmon sperm DNA, 0.2 mg/ml yeast total RNA, and 1.0×10^7 dpm/ml ^{32}P-labeled anti-sense RNA probe. The membranes were washed 4 times at 60°C for 15 min in 1 × SSPE/0.5 % SDS, once at 60°C for 50 min in 0.1 × SSPE/0.5 % SDS, incubated at 37°C for 15 min in 1 μg/ml RNase A, washed at 60°C for 15 min in 1× SSPE/0.1 % SDS, and exposed to Kodak X-Omat AR film for 2 h at room temperature with intensifying screen. RFLP analysis was performed twice in all samples.

^{32}P-labeled anti-sense RNA probe was produced with T7 RNA polymerase (Takara) and Hind III linearized pHSN 15, and had a specific activity of 8.0×10^8 dpm/μg.

The correlation between RFLP results and clinical feature. We divided patients into two groups. The patients of the same RFLP results with N 15 were in a group and the others were gathered together. We compared the two groups in diagnosis, age, sex, native place, and transaminase levels.

Results

The cDNA products by the PCR method had 417 base pairs (bp)(Figs. 1, 2). It was exactly the same size as predicted from the originally reported sequence. The cDNA synthesized from case 7 was cloned as N 15 and sequenced. Fig. 1 showed N 15 and other published sequences corresponding to N 15 (9, 10). N 15 had 29 base substitutions which lead to only four deduced amino acid differences from HC-J 1. All mutations among N 15, HC-J 1, HC-J 4, and Take-
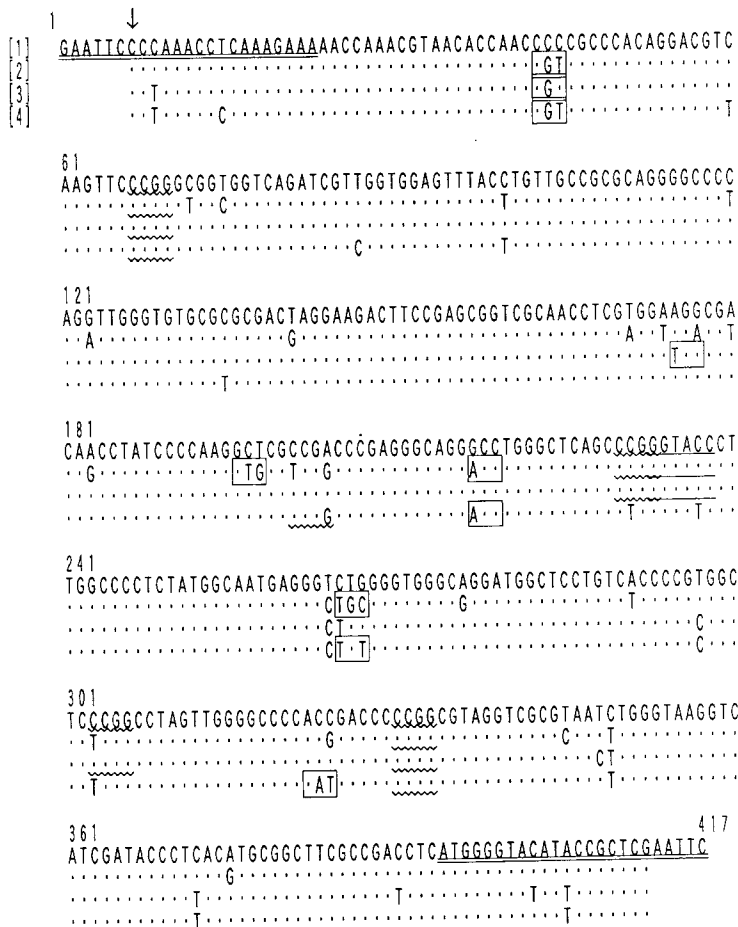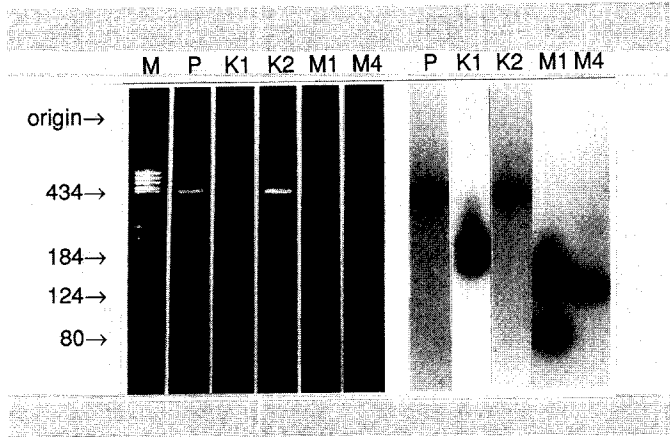
Fig. 1   Sequences of HCVcDNA, encoding a putative core protein, synthesized from RNA extracted from HCV infected serum. [1]: N 15 which is isolated from human serum by PCR in our study; [2] and [3]: HC-J 1 and HC-J 4 which reported by Okamoto *et al.*, respectively (9); [4]: clone which reported by Takeuchi *et al.* (10). Arrow corresponds to nucleotide 337 in HC-J 1. Double underlines indicate PCR primers. Wavy underlines indicate *Msp* I sites, and single underline indicates *Kpn* I site. Boxed codons indicate the difference from N 15 at amino acid level.

uchi's clone were base substitutions, and no insertion, duplication, and deletion were found. Table 2 shows homology of the nucleotide and deduced amino acid sequences among them. Although the primers were derived from HC-J 1, N 15 had the highest homology with HC-J4. N 15, HC-J 4 and Takeuchi's clone were similar each other compared with HC-J1. But amino acid sequences were

Table 2   Homology of nucleotide and amino acid sequences.

	Nucletide (amino acid) homology percentage		
	HC-J 1	HC-J 4	Takeuchi's clone
N 15	92.4 (97.0)	96.8 (98.5)	94.6 (97.0)
HC-J 1	---	92.1 (97.0)	92.4 (97.8)
HC-J 4	---	—	95.6 (97.0)

See legend to Fig. 1.

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Fig. 2 RFLP analysis of HCVcDNA amplified by PCR. RNA extracted from serum was reverse-transcribed and the cDNA was amplified by PCR for 35 cycles. After additional 20 cycles of PCR, cDNA was digested with *Kpn* I or *Msp* I and electrophoresed through an agarose gel of 3% NuSieve and 1% SeaKem. After ethidium bromide staining, the DNA bands were photographed and transferred to filters, hybridized with ^{32}P-labeled anti-sense RNA probe, and autoradiographed for 2 h. The left panels is ethidum bromide staining and the right panel is autoradiogram. M: *Hae* III digested pBR322 as molecular weight marker, their length are shown on the left; P: PCR amplified cDNA; K 1 and K 2: *Kpn* I -digested cDNA from case 7 and case 17, respectively; M 1 and M 4: *Msp* I -digested cDNA from case 7 and case 17, respectively.
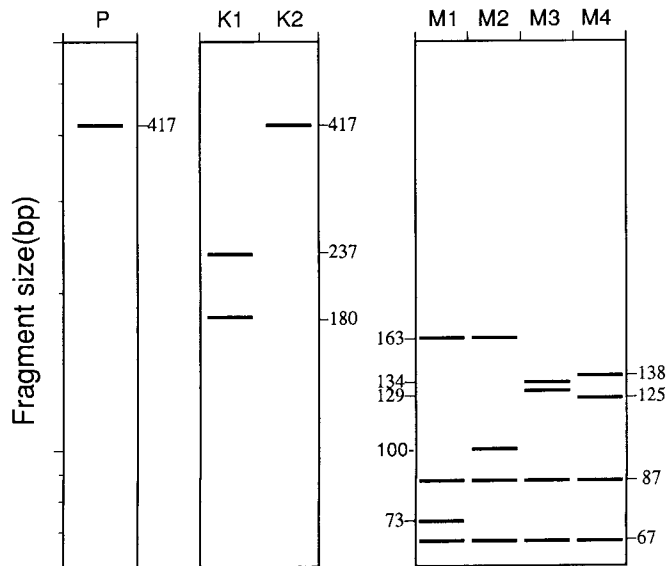
Fig. 3 Predicted schema of restriction fragment length polymorphisms on 4 % agarose gel. N 15 and HC-J 4 show K 1 and M 1. HC-J 1 shows K 1 and M 2. Takeuchi's clone shows K 2 and M 3. Case 17 shows K 2 and M 4. P: PCR product from serum of HCV infected patients; K: *Kpn* I -digested PCR product; M: *Msp* I -digested PCR product.

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conserved in these four clones.

To examine the reproducibility of PCR and RFLP analysis, serum from case 8 was divided into two parts and analyzed separately. The identical results were obtained. Duplicate RFLP analyses brought identical results in all samples (data not shown). Table 1 shows RFLP results of all sera and Fig. 2 represents typical RFLP results. Paired sera at different periods of time from case 17 showed the same results. The DNA bands analyzed by RFLP of most cases could be visualized with ethidium bromide staining, but six cases required Southern blot hybridization analysis. The cDNA fragments less than 60 bp were too small to be detected, as they were interfered by primer dimer on the gel. Fig. 3 shows the predicted RFLP results by *Kpn* I or *Msp* I digestion: N 15 and HC-J 4 are corresponding to K 1 and M 1; HC-J 1, K 1 and M2; Takeuchi's clone, K 2 and M 3. While the cDNAs from 18 out of 21 patients were digested with *Kpn* I into two fragments, 237 and 180 bp as K 1, the rest were not cleaved and categorized as K 2. Those from 15 patients (71.4 %) were digested with *Msp* I into four fragments, 163, 87, 73, and 67 bp long as M 1. There must be, theoretically, another 27 bp band but too small to be observed on this gel. The rest were digested into four fragments as M 4. Samples showing M 2 and M 3 patterns were not observed. Clones belonging to K 1 and M 1 were dominant and recognized in 14 out of 21 patients (66.7 %).

RFLP results had no correlations with clinical data including diagnosis, age, sex, native place, and transaminase levels.

Discussion

The PCR amplified structural region encoding a putative core protein contained 44 point mutations, but there were only six amino acid alterations among N 15, HC-J 1, HCJ 4, and Takeuchi's clone. These results could not be explained by nucleotide misincorporations of Taq DNA polymerase, because most of mutations conserved amino acid sequence. The same phenomenon, *i.e.*, a higher rate of the point mutation without amino acid change, was previously reported in the non-structural region of HCV by Kubo *et al.* (8). As amino acids were substituted with those physicochemically unrelated among their clones, the observed changes may induce the biological behavior of HCV.

Several clones of HCV with different nucleotide sequence may exist in the same patients at the same time. In such cases, sequence analysis could not reflect a dominant HCV clone. RFLP analysis of PCR products will reveal genetic variations of a dominant population of HCV clones in a sample, if mutations occur at the recognition sequence of restriction enzymes. Although RFLP analysis can not decide the whole sequence of HCV, we can theoretically recognize 20 point mutations in N 15 with restriction enzymes *Kpn* I and *Msp* I , and recognize other 36 point mutations occurring in the primers. Altogether 14 % of N 15 can be covered with our analysis. We can thus use RFLP analysis to evaluate a certain genetic variation, and the simplicity of RFLP allows us to analyze many samples as was previously done with dengue type 2 virus (20).

In RFLP analysis, we observed an M 4 pattern which has not been previously reported. One possible explanation for this pattern is that Takeuchi's clone loses a *Msp* I site at 201 nt and gains a *Msp* I site at 205 nt with an alteration from cytidine to guanosine at 207 nt. These mutations are without amino acid replacement. HCV showing M 4 pattern may be thus similar to Takeuchi's clone. The clone genetically similar to HC-J 1, which has M 2 pattern in RFLP analysis, was not found in our study. It was unlikely that HCV like HC-J 1 was eliminated by mismatching during PCR, because the primers used in the present study were derived from HC-J 1. Furthermore, even in the presence of small mismatching of the primers, efficient amplification will be obtained (21).

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If HCV sequences of HC-J 1 and Takeuchi's clone reflected the dominant clones in certain areas, those and our results altogether suggest that there are distinct differences in the HCV genome.   Namely, HCV with the nucleotide sequence like N 15 or HC-J 4 is more common in Okayama district, where most of the patients examined in this study were born, grew up, and suspected to have been infected with HCV. Homology data indicated that HC-J 1 was different from other Japanese clones and rather similar to the original clone isolated by the Chiron group (8). Other researchers suggested that HCV was divided into two subtypes at least, and most HCV isolates in Japan differed from that in USA (8, 11). Our RFLP results also suggested that HCV genetically similar to HC-J1 was rare in Okayama.

The transmission route of HCV infection is chiefly associated with blood (22). And RNA genome, such as HCV, shows millionfold higher mutation frequency than the DNA genome (23). Therefore, HCV may have many geographical variants.   Sera obtained from one patient at different periods of time had the same RFLP results in the present study. HCV genome in this patient might be stable or dominant, even if many variants evolved as suggested in AIDS virus (24, 25). If a certain genomic variant has a tendency to dominate in patients, the analysis on HCV nucleotide sequence and assessment of their homology may be useful for the estimation of transmission route (12).

It is unclear whether mutations influence the biological behavior of HCV. It is possible that mutations in the virus genome influence clinical features. Once hepatitis B virus genome has a point mutation in pre C region, it ceases to produce HBe antigen, but continues to replicate virus particles in patients who have anti-HBe antibody (26, 27). In this study, we could not find the correlation between the clinical features and the genomic variations in the putative core region of HCV. Mutations with amino acid change may influence the clinical course of HCV infection.

Further investigation should reveal such correlations.

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