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DISTRIBUTION OF PLURAL HCV TYPES IN JAPAN*

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SUMMARY: A detection system was developed to distinguish the four different HCV genomes[HCV-J, HCV-US, HCV-K2 and group II HCV(HCV-GII)], involving reverse transcription followed by a nested polymerase chain reaction using specific primers for each HCV type. The putative non-structural(NS) 5 regions of HCV-J, HCV-US and HCV-K2 and the putative NS3 region of HCV-GII were amplified. Of 95 specimens from patients with acute hepatitis, chronic hepatitis, liver cirrhosis or hepatocellular carcinoma, 67 specimens were positive for HCV-J, 2 for HCV-US, 23 for HCV-K2 and 11 for HCV-GII. About half the specimens that were positive for HCV-K2 or HCV-GII were coinfected with HCV-J and all those that were positive for HCV-GII were also positive for HCV-K2. Nucleotide sequence analysis of several amplified cDNA products revealed that HCV-K2 and HCV-GII could each be classified into two groups, and the pattern of classification of HCV-K2 was identical with that of HCV-GII. Therefore, our results strongly suggest that HCV-K2 is the same as HCV-GII.

Hepatitis C virus(HCV) is the major causative agent of post-transfusional non-A, non-B hepatitis(NANBH) throughout the world(1). Recently, almost the entire HCV genome has been cloned in Japan(2,3) and in the United States(US)(4). The HCV genome is a positive-stranded RNA molecule of about 9.4 kilobases and encodes a large

* The nucleotide sequences of clones #4, #8 to #11 and #13 have been submitted to the DDBJ/EMBL/GenBank DNA databases with accession numbers D90513 to D90524.

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polyprotein precursor of about 3000 amino acids. This structural analysis revealed that HCV belongs to a new genus distinct from the flaviviruses and pestiviruses of the *Flaviviridae*, although some parts of the genome show slight homologies with those of flaviviruses and pestiviruses(2,3,5,6).

Comparison of the sequences of the genomes cloned in Japan and the US suggests that these genomes are different subtypes(HCV-J in Japan and HCV-US in the US), because two Japanese isolates showed about 23% difference in nucleotide sequence and about 15% difference in amino acid sequence from a US isolate(2-4). HCV-J and HCV-US are considered to be representative types in Japan and the US, respectively(7,8), although some sequence diversity has been observed in both types(7-9). On the other hand, recently, two Japanese groups independently reported the partial sequences of HCV genomes [the non-structural(NS) 5 region of HCV-K2 and NS3 region of group II HCV(HCV-GII)], which were highly divergent from both HCV-J and HCV-US(10,11). HCV-K2 and HCV-GII showed more than 30% difference in nucleotide sequence and 20-30% difference in amino acid sequence from that of HCV-J or HCV-US. Furthermore, both HCV-K2 and HCV-GII could be divided into two groups: groups HCV-K2a and HCV-K2b of HCV-K2, and tentative groups HCV-GIIa and HCV-GIIb of HCV-GII, which showed about 20% inter-group nucleotide divergence and about 5% intra-group nucleotide divergence(10,11). However, the distribution of these HCV variants in Japan is not clear. It is also not clear whether HCV-K2 and HCV-GII are the same type of HCV, because of lack of information on the nucleotide sequences of the same regions of HCV genome, and because HCV-K2 and HCV-GII were detected using probes of different regions of the HCV genome. HCV-K2 and HCV-GII may be different because one third of the cases of HCV-GII were coinfected with HCV-J, but cases of HCV-K2 were not infected with HCV-J(10,11). However, the difference in findings may have been due to the different primers used for the detection by the polymerase chain reaction.

To clarify these problems, we examined 95 HCV specimens by reverse transcription followed by a nested polymerase chain reaction(RT-nested PCR) using specific primers for each type of HCV genome. Our results suggest that HCV-K2 includes HCV-GII.

MATERIALS AND METHODS

Samples. Plasma were obtained from 8 patients with acute NANBH(AH), 66 patients with chronic NANBH(CH), 5 patients with liver cirrhosis(LC) and 16 patients with hepatocellular carcinoma(HCC).

Synthetic oligonucleotides. Oligonucleotide primers for PCR were synthesized in an Applied Biosystems model 380A(CA, U.S.A.). The sequences of primers in the first PCRs for HCV-J, HCV-US and HCV-K2 were 166, 5'-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3' as a sense primer[corresponding to positions 8230 to 8260 of the HCV-J(2)] and 167R, 5'-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3' as an antisense primer[corresponding to positions 8601 to 8630 of the HCV-J(2)]. The sequences of primers in the second PCRs for HCV-J, HCV-US and HCV-K2 are 192, 5'-TGACATCCGTGTTGAGGAGT-3', 194, 5'-CGACATCCGTACGGAGGAGG-3' and 151, 5'-ACTGAGAGAGACATCAGAAC-3', respectively, as sense primers, and 193R, 5'-CAGGCCGCAGAGGCCTTCAA-3', 195R, 5'-CAGGCTGCCCGGGCCTTGAT-3' and 153R, 5'-GTGATGGTGTTCCCCATGCT-3', respectively, as antisense primers. The sequences of these primers were designed from the data of Enomoto et al.(10). The sequences of primers in the first PCR for HCV-GII were 164, 5'-GCCACGTGCATGCAAGTGAA-3' as a sense primer [corresponding to positions 5265 to 5284 of HCV-J(2)] and 165R, 5'-GACTTGAGCATCTCCGCCAT-3' as an antisense primer[corresponding to positions 5499 to 5518 of HCV-J(2)]. The sequences of primers in the second PCR for HCV-GII were 175, 5'-AGATCATGACGAGCACCTGG-3' as a sense primer and 178R, 5'-CCATCTCATCAAAGGCCTCA-3' as an antisense primer. These sequences for HCV-GII were designed from the data of Tsukiyama-Kohara et al.(11).

RT-nested PCR. RNA was extracted from 0.2 ml of plasma as described(12) and the final product was dissolved in 20 μl of distilled water. An aliquot of RNA(1 μl) was used for cDNA synthesis and following nested PCR as described previously(7,13) with the specific primers described above. For the 1st PCR reaction was carried out for 35 cycles of 1 min at 94 °C, 1 min at 40 °C and 1 min at 72 °C, and for the 2nd PCR reaction was carried out for 35 cycles of 1 min at 94 °C, 45 s at 55 °C and 1 min at 72 °C. The amplified products were separated by electrophoresis on 3 % agarose gel and located by ethidium bromide staining.

Cloning and sequencing of PCR products. Several PCR products that were positive for the HCV-K2 or HCV-GII genome were cloned into the pTZ19R plasmid vector as described(7,12). To reduce the possibility of misreading by *Thermus aquaticus* polymerase, we isolated three clones and determined their nucleotide sequences by the dideoxy-nucleotide chain-termination method(DNA sequencing kit, USB Corp., OH. USA).

RESULTS AND DISCUSSION

The HCV genome in plasma RNAs from 95 patients with no hepatitis B viral surface antigen with hepatic diseases(8 AH, 66 CH, 5 LC and 16 HCC) was examined. cDNA was synthesized using RNA extracted from the plasma as template with a synthetic oligonucleotide primer 167R for detection of the NS5 region of HCV-J, HCV-US or HCV-K2 genome,

Table I. Summary of typing analysis of HCV genomes and analysis of antibody against the viral protein, C100

HCV Type	Status of hepatic disease					Antibody C100 + [1]
	AH	CH	LC	HCC	Total	
J	3	43	1	8	55	41
J + K2	0	5	0	1	6	3
J + K2 + GII	0	5	0	1	6	3
K2 + GII	0	3	1	1	5	3
K2	0	4	1	1	6	1
US	1	1	0	0	2	2
GII	0	0	0	0	0	0
ND [2]	4	5	2	4	15	9

[1] measured by ELISA with a kit from Ortho Diagnostic System.
[2] no HCV genome detected.

or primer 165R for detection of the NS3 region of the HCV-GII genome. The cDNA was then amplified by the nested-PCR as described in "materials and methods". After the second PCR, the products of 216 base-pairs(bp)(HCV-J), 216bp(HCV-US), 197bp(HCV-K2) and 165bp (HCV-GII) were detected by staining with ethidium bromide after agarose gel electrophoresis. The results of RT-nested PCR are summarized in Table I. The HCV-J, HCV-US, HCV-K2 and HCV-GII genomes were detected in 67, 2, 23 and 11, respectively, of 95 specimens. Six cases were positive for both HCV-J and HCV-K2 and 5 were positive for both HCV-K2 and HCV-GII. Furthermore, 6 cases were positive for HCV-J, HCV-K2 and HCV-GII.

To check the specificity of the primers used in this study, we cloned several amplified PCR products into plasmid vector and determined their nucleotide sequences. We confirmed the justification for this typing of HCV genomes, by showing that each sequenced clone was well matched with either consensus sequence or the expected representative nucleotide sequence of HCV-J or HCV-US (data not shown) and with that of HCV-K2 or HCV-GII(Fig.1).

There was no correlation between typing of HCV and the status of hepatic diseases, as shown in Table I. These data showed that HCV-J

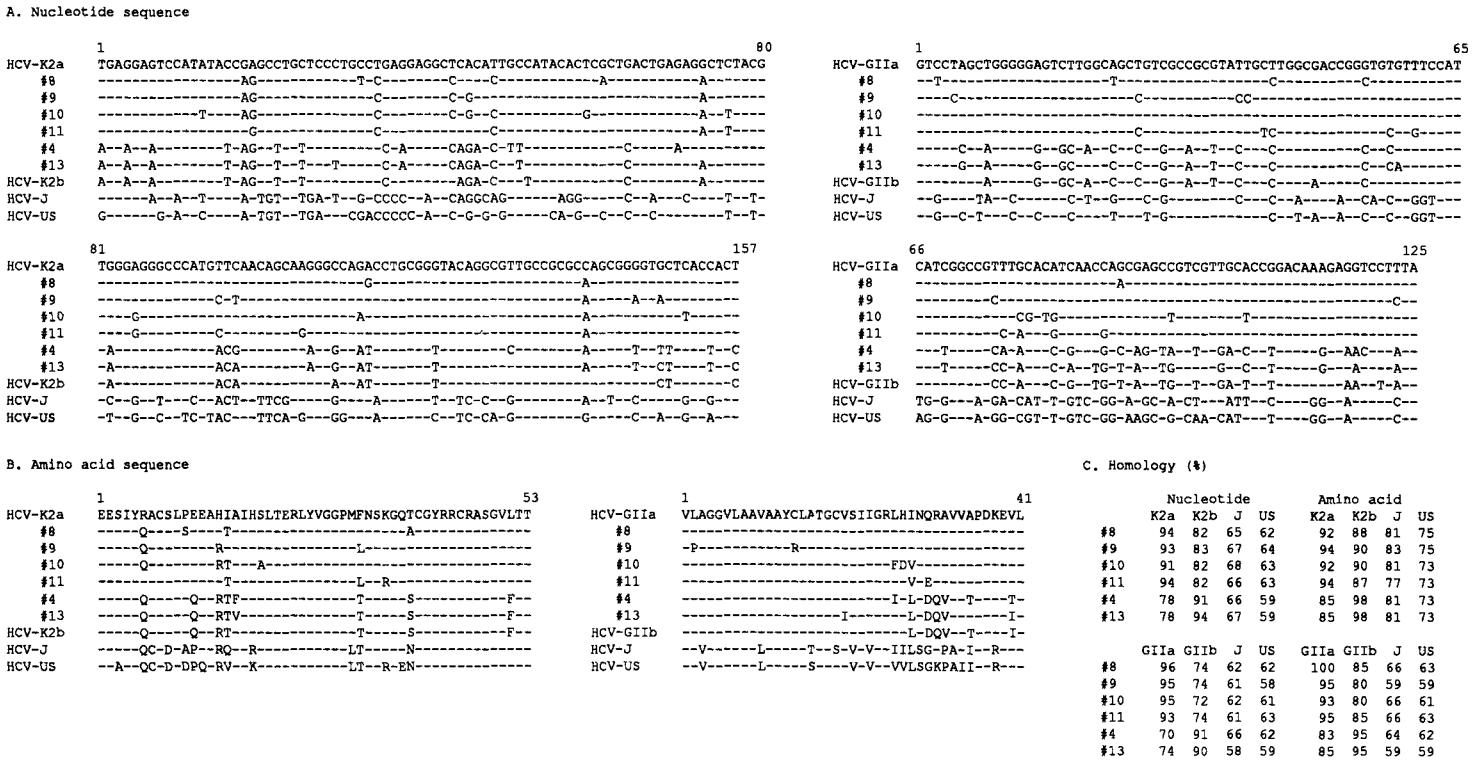

Figure 1. Nucleotide sequences of HCV cDNAs (A) and the deduced amino acid sequences (B) in the amplified region. Sequences of HCV-K2a and HCV-K2b are cited from Enomoto et al. (10) and the first nucleotide of HCV-K2a or HCV-K2b corresponds to nucleotide position 31 in the sequence of Enomoto et al. (10). Sequences of HCV-GIIb are cited from Tsukiyama-Kohara et al. (11) and the first nucleotide of HCV-GIIb corresponds to nucleotide position 1165 in the sequence of Tsukiyama-Kohara et al. (11). Consensus sequences of HCV-GIIa were obtained from those of #8, #9, #10, and #11. Sequences of HCV-J and HCV-US are cited from Kato et al. (2) and Choo et al. (4), respectively. Amino acid sequences are indicated by the single letter code. Nucleotide and amino acid sequence homologies (C) are summarized at the right side of bottom.

was the major type in Japan and about half the specimens with HCV-K2 were coinfected with HCV-J, in contrast with the result of Enomoto et al.(10).

It is noteworthy that all specimens that were positive for HCV-GII were included in the HCV-K2 positive group and 6 specimens were positive for HCV-J, HCV-K2 and HCV-GII. From these findings, we considered that the HCV-GII type might be genetically the same as HCV-K2 type. This is also suggested from the evidence that HCV-K2 and HCV-GII are classified genetically into two groups(HCV-K2a and HCV-K2b for HCV-K2; tentatively named HCV-GIIa and HCV-GIIb for HCV-GII)(10,11). To clarify these groups of HCV-K2 and HCV-GII, the amplified DNAs with K2 or GII specific primers in 6 specimens (specimens #4, 8, 9, 10, 11, 13), which were positive for HCV-K2 and HCV-GII, were examined further by nucleotide sequence analysis. As shown in Figure 1, RNAs from specimens #8, 9, 10 and 11 contained the typical sequence for HCV-K2a and HCV-GIIa groups at both nucleotide sequence and deduced amino acid sequence levels, and those from specimens #4 and 13 belonged to the HCV-K2b and HCV-GIIb groups. These results strongly suggest that HCV-K2a is identical to HCV-GIIa and HCV-K2b is identical to HCV-GIIb. It is unlikely that three different types of HCV(HCV-J, HCV-K2a or -K2b and HCV-GIIa or GIIb) were coinfected in 6 patients.

We failed to detect the HCV-GII genome in about half the HCV-K2 positive specimens. This low level of detection of HCV-GII might be due to the low specificity of primers for HCV-GII in this work, because there is only one report of the sequence of HCV-GII (11). In fact, as shown in Figure 1, we found sequence diversity(8 to 11%) among the isolates of the HCV-GII group as well as the HCV-J type (7). By using the primers designed from the consensus sequences of HCV-GII as used in the case of HCV-K2, it might be possible to detect HCV-GII in specimens containing HCV-K2 more efficiently.

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