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Molecular Epidemiology of Hepatitis C Virus Infection in an Area Endemic for Community-Acquired Acute Hepatitis C

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Yoshii, E., Shinzawa, H., Saito, T., Shao, L., Kuboki, M., Saito, K., Togashi, H., Takahashi, T. and Mizokami, M. *Molecular Epidemiology of Hepatitis C Virus Infection in an Area Endemic for Community-Acquired Acute Hepatitis C.* Tohoku J. Exp. Med., 1999, **188** (4), 311-316 —— The southern district of N city (U area), Yamagata Prefecture, is highly endemic for hepatitic C virus (HCV) infection. Around 20% of the general population are positive for antibodies to HCV (anti-HCV). Community-acquired, acute non-A, non-B hepatitis was epidemic from 1967 to 1972 in this area. Our previous study revealed that these people are actually infected with HCV, but a relationship between this outbreak and the high positivity rate of anti-HCV in the U area has not been shown. We followed up 15 anti-HCV-positive individuals who developed hepatitis during the epidemic and used the serum collected to conduct molecular evolutional analysis to reveal the characteristics of the HCV epidemic in the U area. HCV genotypes in the U area were also analyzed. Phylogenetic analysis of the HCV core gene sequences showed that the subjects' HCV sequences were closely related and derived from the same cluster. All subjects were infected with HCV genotype 1b, which was frequently detected with a high positivity of over 80% of HCV-infected individuals in the U area. These results confirm that the community-acquired hepatitis C epidemic occurred around three decades ago through an unidentified route, and suggest that this episode may result in a continuing increase in the number of HCV-1b positive patients in this small area. —— hepatitis C; phylogenetic tree; epidemiology; HCV RNA

The southern district of N city (U area), Yamagata Prefecture, has been reported to be highly endemic for hepatitis C virus (HCV) infection (Kuboki et al. 1999). A community-acquired, acute non-A, non-B hepatitis epidemic occur-

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red from 1967 to 1972 in this area. One hundred eighteen patients were treated for acute hepatitis from 1967 to 1972 in N City Hospital (Kuboki et al. 1991). The discovery of HCV in 1989 (Choo et al. 1989) enabled us to reveal retrospectively that this epidemic of hepatitis was caused by HCV infection (Kuboki et al. 1991). Despite intensive investigation to determine the possible route of infection, it has not been identified (Kuboki et al. 1991). In this study, we carried out a phylogenetic, molecular evolutional analysis, using serum samples taken from 15 individuals who were involved in the outbreak of acute hepatitis from 1967 to 1972, to reveal the genetic relatedness of individual nucleotide sequences. We also investigated the HCV genotypes in the U area. This approach enabled us to reveal the relationship of the acute outbreak in the past with the high endemic status at present in this HCV-endemic small community.

Subjects and Methods

Subjects

There were 3814 registered inhabitants aged 6 years and older on file in the southern part of N city (U area), Yamagata Prefecture, as of April 1, 1991. We conducted a mass health survey for HCV infection in 1991 and 1992 among 2382 inhabitants from whom informed consent was obtained. Of these, 528 (22.2%) were positive for anti-HCV in repeated testing with second-generation enzyme-linked immunosorbent assay, as described previously (Kuboki et al. 1999). We then tested the relatedness of HCV RNA in 528 anti-HCV positive samples by nested reverse-transcription polymerase chain reaction (PCR) using specific primers encoding the 5' noncoding region of HCV, which is highly conserved in the HCV genome (Widell et al. 1991). Using a genotyping assay, we tested 334 of these samples that were positive for both anti-HCV and HCV RNA. For molecular evolutional analysis of the outbreak of community-acquired hepatitis in this area, 15 of 334 cases could be chosen according to the following criteria: a) the subjects were living in the U area before 1967, b) their medical records confirmed that they had a history of hospitalization in the N City hospital for acute hepatitis during 1967–1972, and c) the subjects had no known history of parenteral exposure, including blood transfusion, surgery, oriental needlestick therapy or any folk remedies. The serum samples used in the molecular evolutional analysis were collected in 1994. All subjects had prolonged abnormal liver function with an elevation of alanine-aminotransferase (ALT) level. Liver biopsy from 10 of the subjects showed that all had chronic liver diseases.

Genotyping assay. HCV RNA was extracted from 100 μl of serum using the Sepa Gene-RV kit (Sanko Chemical Co., Tokyo). The complementary DNA (cDNA) was synthesized using 200-units Moloney murine leukemia virus reverse transcriptase (Promega Co., Madison, WI, USA) with 100 pmol random primer (Takara, Tokyo). The HCV genotype was determined after amplifying cDNA by PCR with genotype-specific primers derived from the core region using the method

of Okamoto et al. (1992) and then classified according to the system proposed by Simmonds et al. (1993).

Sequencing and molecular evolutional analysis. The HCV cDNA was amplified by nested PCR using primers derived from the core region. Primers were designated for the 365-bp amplification product of the HCV core region. The outer primers for the first-stage PCR were 5′ TAG GAT CCG GGA GGT CTC GTA GAC CGT GCA CCA TG 3′ and 5′ CAG AAT TCG AGV GGK ATR TAC CCC ATG AGR TCG GC 3′. The inner primers for the second-stage PCR were 5′ TAG GAT CCG GGA GGT CTC GTA GAC CGT GCA CCA TG 3′ and 5′ TTT GAA TTC CTA CGC CCC CCC TCW GTR GGG CCC CA 3′. The PCR-amplified DNA was sequenced with the 5′ sequencing primer 5′ TAG GAT CCG GGA GGT CTC GTA GAC CGT GCA CCA TG 3′ and the 3′ sequencing primer 5′ TTT GAA TTC CTA CGC CCC CCC TCW GTR GGG CCC CA 3′ using the fluorescent dye terminator cycle method in an ABI 373 automated sequencer (Applied Biosystems Japan, Chiba). The nucleotide sequences of these 15 samples were labeled from N1 to N15, and then aligned with other reported sequences of HCV. The evolutionary relationship between these samples and previously reported isolates was elucidated by the six-parameter method (Gojobori et al. 1982). A phylogenetic tree was constructed by the neighbor-joining method using the ODEN computer software program (Ina 1994). The molecular distance calculated between two isolates of HCV-1a, H77 and H90, that were derived from the same HCV-1a infected patient with a posttransfusion hepatitis in 1977 and 1990, respectively, was used to estimate the time that each isolate of the 15 samples diverged from its precursor.

Results

HCV genotype. It was possible to determine the HCV genotype in 332 samples. According to the system proposed by Simmonds et al. (1993), HCV genotype 1b (HCV-1b) was detected in 263/332 (79.2%), HCV-2a in 30/332 (9.0%), HCV-2b in 26/332 (7.8%), HCV-1b plus HCV-2a in 3/332 (0.9%), HCV-1b plus HCV-2b in 1/332 (0.3%), HCV-1b, HCV-2a plus HCV-2b in 1/332 (0.3%), HCV-2a plus HCV-2b in 1/332 (0.3%). HCV-1b was detected totally in 268/332 (80.7%), which is a higher than average incidence for HCV-1b in Japan (Yamada et al. 1995).

Phylogenetic analysis. The HCV core region from each of the 15 selected samples was sequenced to construct a phylogenetic tree, as shown in the Fig. 1. All 15 subjects were infected with HCV-1b. The core regions showed 96.3% homology in nucleotide sequence and 96.0% homology in deduced amino acid sequence. A phylogenetic tree showed that the nucleotide sequences of these 15 samples were closely related and derived from the same cluster. The core region is known to be conserved among HCV genotypes (Major and Feinstone 1997). The molecular distance of 13 years between H77 and H90 suggested that the 15

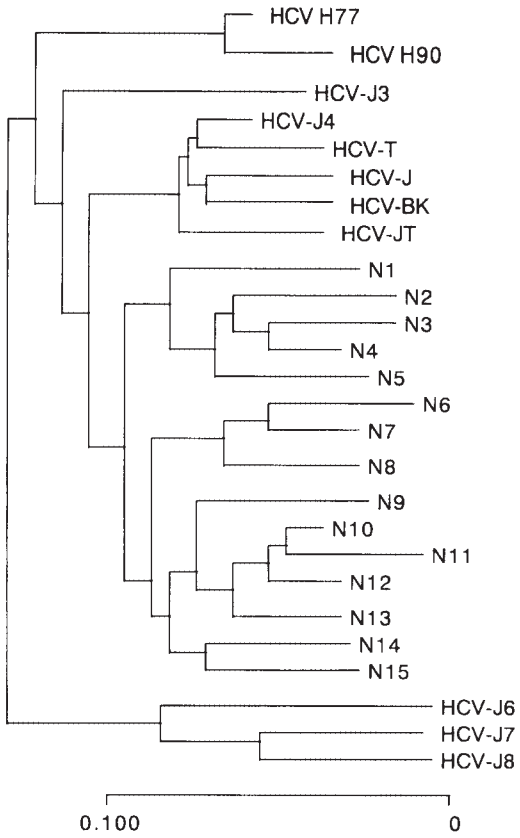

Fig. 1. A phylogenetic tree of isolates from 15 individuals of the U area which are labeled N from 1 to 15, and previously reported HCV isolates, showing the genetic relatedness of each nucleotide sequence. The Gen Bank accession numbers of the reported HCV isolates are: HCV H77, M62381; HCV H90, M62382; HCV-J, D90208; HCV-BK, M58335; HCV-T, M84754; HCV-J4, D10750; HCV-JT, D01171; HCV-JK3, X61592; HCV-J6, D00944; HCV-J7, D10077; HCV-J8, D10988. Our nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank databases with the accession numbers AB030159-AB030173.

sequences should have around three decades (30.7 ± 4.6 years, mean $\pm$ S.D.) molecular divergence, consistent with the known interval between the original outbreak of acute hepatitis and the high endemic status of HCV at present.

Discussion

In this study, we showed that the outbreak of the community-acquired acute hepatitis around three decades ago was caused by an HCV-1b cluster with close genetic relatedness, and that this episode could have resulted in the increase of HCV-1b positive patients in the small area.

It has been reported that the HCV genotype distribution in Japan comprises around 70% and 30% for HCV-1b, and HCV-2a and 2b, respectively (Yamada et al. 1995). The deviation of genotype distribution in the U area, where HCV-1b is predominant (over 80% of HCV-infected individuals), suggests that the infection by HCV-1b occurred as a mass outbreak at some time in the past. After the discovery of HCV and subsequent development of hepatitis C diagnosis, our

epidemiological study revealed that all the patients we followed up and who were hospitalized because of acute hepatitis at that time were infected with HCV (Kuboki et al. 1991). The results of the phylogenetic analysis of the HCV epidemic in the U area supported the evidence from genotype distribution that the current endemic status of chronic HCV infection may have been caused by an outbreak of the virus in the past. However, we had not been able to find direct evidence that this outbreak was associated with the acute HCV infection epidemic around three decades ago. When it was possible to identify that all 15 individuals analyzed were infected with the same HCV variant, HCV-1b, we were able to estimate when HCV infection occurred in these patients using the phylogenetic analysis. In this study, we chose to sequence the relatively conserved region of HCV, the core region, because its genomic stability makes it the most suitable region for estimating the time of divergence using the known molecular distance. The results of the phylogenetic tree analysis confirmed that these samples were separated by a molecular distance of around three decades, consistent with the time interval from the outbreak until the present.

It is well known that there are several areas in Japan highly endemic for HCV infection (Tajima et al. 1991; Ishibashi et al. 1996). Some folk remedies are reported to be closely related to the spread of HCV among populations through parenteral routes (Kiyosawa et al. 1994), but in most areas the reason why HCV is spread and accumulates in small communities has not been clarified. Despite intensive investigation of how HCV contaminated this area three decades ago, the infection route remains unknown (Kuboki et al. 1999). Further studies are needed to draw more definitive conclusions about how HCV is spread in such areas where the virus is endemic. Molecular evolutional approaches including genotyping assays and phylogenetic analyses of HCV using various appropriate regions of the virus (Mizokami et al. 1994; Smith and Simmonds 1997) would be useful for investigating the epidemiology of hepatitis C further.

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