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Subtype Analysis of Hepatitis C Virus in Indonesia on the Basis of NS5b Region Sequences

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A recently identified subtype of hepatitis C virus, subtype 1d, was found to be common in Indonesia, being isolated from 4 (20%) of 20 and 11 (34%) of 32 patients with chronic hepatitis and liver cirrhosis, respectively. A new group of sequence variants was also identified, although its prevalence ratio was not as high in the area surveyed.

Hepatitis C virus (HCV), a member of the family *Flaviviridae*, is a major etiologic agent of posttransfusion and sporadic non-A, non-B viral hepatitis worldwide (2, 8). The viral genome encodes a polyprotein precursor, which is cleaved by cellular or virus-encoded proteases to generate at least nine viral proteins; the core and two envelope proteins (E1 and E2) and six nonstructural proteins (NS2, NS3, NS4a, NS4b, NS5a, and NS5b) (4, 5). Among different isolates the nucleotide sequences of the HCV genome show considerable degrees of variation. Simmonds et al. (17) have recently proposed that HCV be classified into at least six major types, each of which may be further divided into a few subtypes, e.g., HCV-1a, HCV-1b, and HCV-1c. Recently, we and another research group have independently identified the presence of a novel subtype of HCV in Indonesia (6, 7, 13), which we refer to as HCV-1d in this paper. The prevalence of HCV subtypes differs among different geographical regions. For example, HCV-1b is the most prevalent subtype in Japan (14), whereas HCV-1a is the most prevalent subtype in the United States (8). Likewise, HCV-3a is commonly found in Thailand and the United Kingdom (12, 17), but not in Japan (3). Besides the geographical distribution, clinical features of HCV infection as well as responsiveness to interferon treatment appear to vary with different subtypes (9, 15, 16). It is thus important to determine HCV subtypes for epidemiological and clinical purposes. In the present study, we performed subtype analysis of HCV isolates from Indonesia to look into a possible association between HCV subtypes and the clinical features of chronic liver disease.

Sera were obtained from a total of 61 Indonesian patients at Dr. Soetomo Hospital, Airlangga University, Surabaya; 20 patients with chronic hepatitis (mean age, 56 years), 32 patients with liver cirrhosis (mean age, 59 years), and 9 individuals of a miscellaneous group (mean age, 49 years). Most of the patients were Javanese, and approximately half of them had experienced a blood transfusion, a surgical operation, or both. The sera were tested for antibodies against HCV by using a second-generation enzyme-linked immunosorbent assay (ELISA) (Ortho HCV Ab ELISA Test II). The HCV RNAs extracted from the sera were reverse transcribed into cDNA, which was then amplified by PCR as described previously, with slight modifications (3, 6, 7). The nucleotide sequences of the amplified fragments were determined by the direct sequencing method by using the *Taq* Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc.) and an ABI 373A DNA Sequencer (Applied Biosystems, Inc.), and the sequences were compared with each of the sequences of known types or subtypes (13, 17, 18) by using GENETYX MAC version 6.0.2 software (Software Development Inc., Tokyo, Japan). Phylogenetic trees were constructed by the unweighed pairwise grouping method (10).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the GSDB/DDBJ/EMBL/NCBI DNA databases with the following accession numbers, D26383 to D26391, D30046, D30047, and D37897 to D37899.

When primers HC23 and HC24 (3) were used in the second-round PCR for the NS5b region, positive amplification was observed with 32 (52.5%) of the 61 serum specimens (Table 1). Of the 29 negative serum specimens, 14 became positive with primers HC23 and HC26 (3). Two serum specimens were identified as positive with primers HC15 and HC16 (3), and four serum specimens were identified as positive with primers HC23 and HC28 (antisense; 5′-CACGAGCATGGT GCAGTCCCGGAGC-3′). Nine serum specimens (14.8%) were negative for NS5b by PCR with the above sets of primers.

Following the criteria suggested by Simmonds et al. (17), the subtypes of the HCV isolates described above were determined. Isolates showing sequence homology at the nucleotide level of more than 88% with any of the reported subtypes were assigned to the corresponding subtype. A substantial number of isolates, including Td-34/92 and Td-47/92, showed high degrees of homology with HC-G9 (13), and those isolates were assigned to subtype 1d (Fig. 1). Also, phylogenetic analysis on the basis of the E1 region demonstrated that the previously reported variant Td-6 (6, 7), as well as isolates Td-34/92 and Td-47/92, shares a high degree of sequence similarity with HC-G9 (Fig. 2). The same phylogenetic trees identified sequence variants Td-3/93 and Td-35/93 on another branch. These variants are distantly related to subtypes of type 3 on the basis of sequence homology in the NS5b and E1 regions (Fig. 1 and 2). Percent homologies in core region sequences between Td-3/93 and each of the six subtypes of type 3 subtypes

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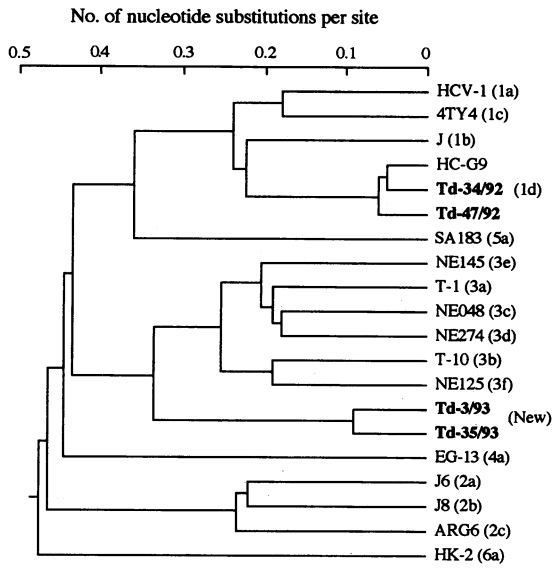

FIG. 1. Phylogenetic analysis of NS5b region of HCV genome on the basis of nucleotide sequences. The clones described in this report are set in boldface type. Subtypes are given in parentheses. No subtype has yet been assigned to the new sequence variants Td-3/93 and Td-35/93. GSDB/DDBJ/EMBL/NCBI accession numbers of the clones in the phylogenetic tree are as follows: HCV-1, M62321; 4TY4, L23447; J, D90208; HC-G9, D14853; Td-34/92, D26389; Td-47/92, D26390; SA183, L23472; NE145, D16619; T-1, D10078; NE048, D16613; NE274, D16621; T-10, D10081; NE125, D16615; Td-3/93, D26387; Td-35/93, D37898; EG-13, L23469; J6, D00944; J8, D01221; ARG6, L23457; HK-2, L23475.

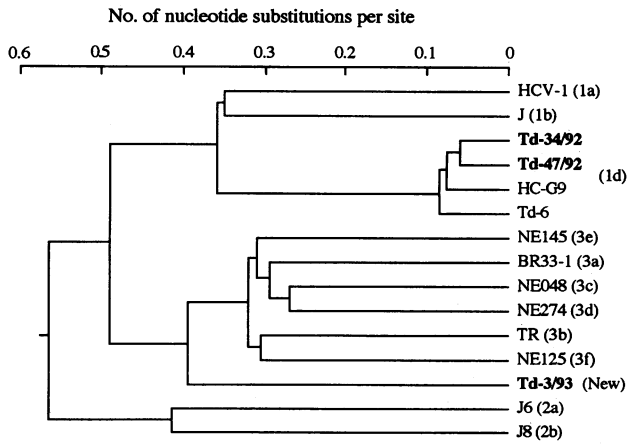

FIG. 2. Phylogenetic analysis of E1 region of HCV genome on the basis of nucleotide sequences. The clones described in this report are set in boldface type. Subtypes are given in parentheses. No subtype has yet been assigned to the new sequence variant Td-3/93. GSDB/DDBJ/EMBL/NCBI accession numbers of the clones in the phylogenetic tree are as follows: HCV-1, M62321; J, D90208; Td-34/92, D26385; Td-47/92, D26386; HC-G9, D14853; Td-6, D13732; NE145, D16618; BR33-1, D14596; NE048, D16612; NE274, D16620; TR, D11443; NE125, D16614; Td-3/93, D30046; J6, D00944; J8, D01221.

3a to 3f (1, 18) were 84.3, 86.3, 85.2, 87.7, 82.1, and 86.3%, respectively. On the other hand, the nucleotide sequence of the 5′-untranslated regions (5′-UTRs) of Td-3/93 and Td-35/93 did not match any of the particular sequence motifs for types 1 to 4 (11) or subtypes 3a to 3f (1, 18) (data not shown). At present, we are unable to draw a conclusion as to whether Td-3/93 and Td-35/93 represent a new subtype of type 3 or a new type. Sequence analysis of other regions of the viral genome will help to determine the phylogenetic positions of these variants.

Among patients with chronic hepatitis, HCV-1b was the most common subtype (45%); this was followed by HCV-1d (20%) (Table 2). Interestingly, HCV-1d was found more frequently among patients with liver cirrhosis (34%) than among those with chronic hepatitis, although the difference was not statistically significant. HCV-1a was isolated from 2 of 3 hemodialysis patients, but not from 52 patients with chronic hepatitis or liver cirrhosis. Nine samples that were negative for NS5b by PCR were further subjected to PCR for the 5′-UTR. Six samples became positive by this analysis, while three samples were still negative. Sequence analysis of the 5′-UTR (11) classified four isolates into type 1 and 2 isolates into type 2 (data not shown).

Clinical features of HCV infection appear to vary with different subtypes; HCV-2a and HCV-2b are more susceptible than HCV-1b to interferon treatment (9, 16). Also, it has been reported that HCV-1b might be associated more closely with serious liver disease than the other subtypes (15, 16). In the present study there was a tendency for HCV-1d to be more common in patients with liver cirrhosis than in patients with chronic hepatitis, suggesting the more pathogenic features of HCV-1d, as is the case with HCV-1b. This point should be addressed in detail in a larger-scale study.

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TABLE 1. Efficacy of each primer set in second-round PCR to amplify NS5b region of HCV genome from Indonesian serum samples

Primer set	No. positive/ no. examined (%)
HC23, HC24	32/61 (52.5)
HC23, HC26	14/29[a]
HC15, HC16	2/15[a]
HC23, HC28	4/13[a]
Total	52/61 (85.2)

[a] Negative samples in the previous PCR experiment were examined.

TABLE 2. Prevalence of HCV subtypes in different groups of patients in Indonesia

Patient group	Sample no.	No. (%) of HCV isolates with subtype:					
		1a	1b	1d	2a	New[a]	UC[b]
Chronic hepatitis	20	0 (0)	9 (45)	4 (20)	3 (15)	0 (0)	4 (20)
Liver cirrhosis	32	0 (0)	11 (34)	11 (34)	4 (13)	2 (6)	4 (13)
Miscellaneous[c]	9	2[d]	2	0	4	0	1
Total	61	2[d] (3)	22 (36)	15 (25)	11 (18)	2 (3)	9 (15)

[a] New sequence variants.
[b] Unclassifiable because of unsuccessful amplification of the NS5b region.
[c] One patient with hepatocellular carcinoma, five asymptomatic carriers, and three hemodialysis patients.
[d] Isolated from hemodialysis patients.

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