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Hepatitis C Virus (HCV) Subtype Prevalence in Chiang Mai, Thailand, and Identification of Novel Subtypes of HCV Major Type 6

HISAYA DOI,[1] CHATCHAWANN APICHARTPIYAKUL,[2] KEN-ICHI OHBA,[3] MASASHI MIZOKAMI,[3] AND HAK HOTTA[1]*

Department of Microbiology, Kobe University School of Medicine, Chuo-ku, Kobe, Hyogo 650,[1] and Second Department of Internal Medicine, Nagoya City University Medical School, 1-1 Kawasumi, Mizuho-ku, Nagoya, Aichi 467,[3] Japan and Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50002, Thailand[2]

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Subtype analysis of hepatitis C viruses (HCVs) obtained from patients with chronic liver disease in Chiang Mai, Thailand, was performed. Of 46 HCV isolates, 13 (28%) were shown to belong to HCV subtype 3a (HCV-3a), 10 (22%) to belong to HCV-1a, 7 (15%) to belong to HCV-1b, 1 (2%) to belong to HCV-3b, and 1 (2%) to belong to a variant group, as determined from partial nucleotide sequences of the NS5B region of the viral genome. Analysis of 5′ untranslated region sequences identified five other isolates (11%) of HCV type 1 and two other isolates (4%) of type 3. Detailed phylogenetic positions for the variant described above and those previously obtained from blood donors and drug addicts in Chiang Mai were determined by a six-parameter neighbor-joining method on the basis of core, E1, and NS5B region sequences. The results revealed that those sequence variants represent novel subtypes of HCV type 6. The HCV type 6 isolates appear to be antigenically different from isolates of HCV types 1 and 2, as determined by a serotyping method that utilizes recombinant peptides corresponding to a portion of the NS4 protein. The significance of subtype analysis around this area is discussed.

Hepatitis C virus (HCV) has been known to be a major etiologic agent of posttransfusion as well as sporadic non-A, non-B hepatitis worldwide (14). The genome of HCV is a single-stranded RNA with positive polarity of about 9,400 bases, which has a long open reading frame encoding four structural proteins (core, E1, E2 type A, and E2 type B) and at least six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) with untranslated regions at both the 5′ and 3′ ends (5′UTR and 3′UTR, respectively) (8, 10, 18). The viral genome exhibits considerable degrees of sequence diversity among different isolates. On the basis of this diversity, HCV has been classified into a number of distinct types or groups, each of which can be further divided into several subtypes (4, 24–26). Viral pathogenicity and susceptibility to interferon treatment appear to vary among different subtypes (15, 21, 22). Also, the prevalence of each subtype among HCV isolates varies among geographical areas (5, 9, 25). Moreover, the identification of sequence variants that might represent novel types or subtypes has been increasing as surveillance is extended to previously overlooked areas (1, 4, 11–13, 20, 28, 29).

We previously determined the prevalence of each subtype among HCV isolates from anti-HCV-positive blood donors and drug addicts in Chiang Mai, Thailand, and identified a number of unique sequence variants (1). However, the exact phylogenetic positions of the variants could not be determined at that time. In the present study we have determined the HCV subtype prevalence among anti-HCV-positive patients with chronic liver disease. We also performed a detailed phylogenetic analysis that shows that the sequence variants obtained in this area represent novel subtypes of HCV type 6. We discuss how the variants are likely to possess an antigenic epitope(s) that is distinct from those of HCV types 1 and 2.

* Corresponding author. Mailing address: Department of Microbiology, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650, Japan. Phone: 81-78-341-7451, ext. 3300. Fax: 81-78-351-6347. Electronic mail address: hotta@icluna.kobe-u.ac.jp.

MATERIALS AND METHODS

Serum samples. Sera were collected from patients with chronic liver disease at Chiang Mai University Hospital, Chiang Mai, Thailand. The sera were tested for anti-HCV antibodies by second-generation enzyme-linked immunosorbent assays (ELISA) (Abbott Diagnostics, Inc.; Ortho Diagnostics Systems, Inc.). Positive sera were subjected to further analysis.

Reverse transcription and PCR. HCV RNA extracted from anti-HCV-positive sera was reverse transcribed into cDNA by using an HCV-specific primer, and the resultant cDNA was amplified by nested PCR as described previously (1, 6, 11–13). We first aimed to amplify NS5B region sequences. In the case of negative amplification for the NS5B region, 5′UTR sequences were subjected to amplification by PCR. In this analysis, RNA was reverse transcribed by using a primer UTR2 (antisense; 5′-AGTACCACAAGGCCTTTCGC-3′), and the resultant cDNA was amplified by nested PCR with UTR1 (sense; 5′-CCGGGAGAGC CATAGTGGTC-3′) and UTR2 as an outer primer set and UTR3 (sense; 5′-TGGTCTGCGGAACCGGTGAG-3′) and UTR4 (antisense; 5′-ACCCAA CACTACTCGGCTAG-3′) as an inner primer set. In some experiments, core and E1 region sequences were also amplified as described previously (11, 12) with some modifications. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and visualized by UV illumination.

Sequence analysis and subtyping. Amplified DNA fragments were sequenced by a direct sequencing method with a *Taq* Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) and an ABI 373A Autosequencer (Applied Biosystems, Inc.). Each sequence obtained was compared with those of reported types and subtypes (4, 25, 28), and on the basis of percent homologies, each isolate was assigned a subtype (1, 13, 25).

Phylogenetic analysis. For HCV isolates that could not be assigned to any known subtypes, phylogenetic analysis was performed as described previously (12, 19). Briefly, the nucleotide sequences obtained in this study and those available from the international DNA data banks (DDBJ, NCBI, and EMBL) were maximally aligned by using HOMOGAPN, GENETYX version 8.0 (Software Development Co., Ltd., Tokyo, Japan), and the number of nucleotide substitutions per site (genetic distance) at all positions between each possible

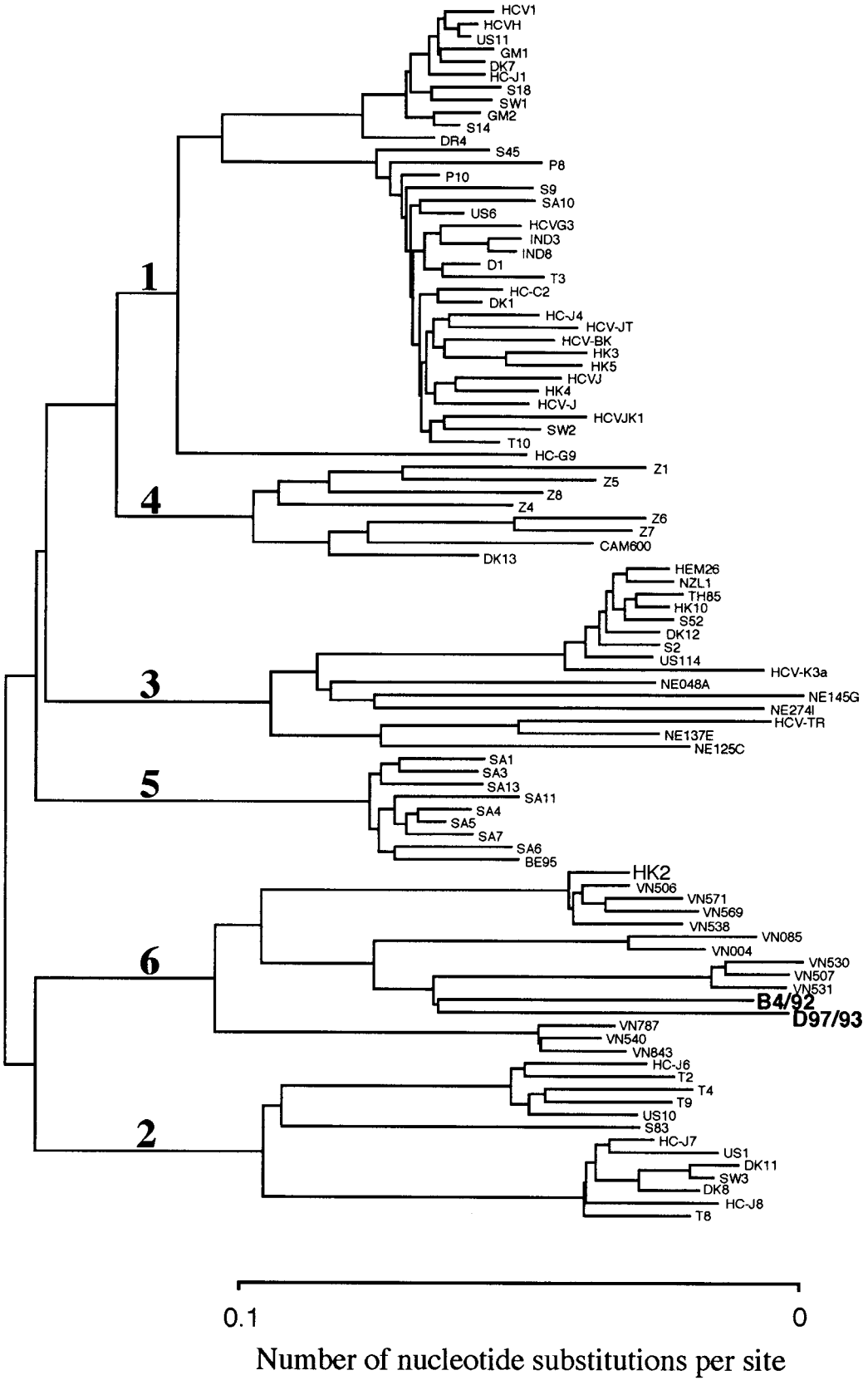

FIG. 1—*Continued*.

FIG. 1. Phylogenetic analysis of the entire core region sequences of the HCV genome. HCV types are indicated by numerals, and Thai variants are shown in boldface. The GSDB/DDBJ/EMBL/NCBI accession numbers for the sequence data for the clones in the phylogenetic tree are as follows: HCV1, M62321; HCVH, M67463; US11, U10232; GM1, M61718; DK7, U10194; HC-J1, D10749; S18, U10207; SW1, U10222; GM2, M61719; S14, U10206; DR4, U10196; S45, U10209; P8, U10205; P10, U10204; S9, U10212; SA10, U10213; US6, U10234; HCVG3, M86779; IND3, U10202; IND8, U10203; D1, U10189; T3, U10227; HC-C2, D10934; DK1, U10193; HC-J4, D00832; HCV-JT, D11168; HCV-BK, M58335; HK3, U10199; HK5, U10201; HCVJ, D00574; HK4, U10200; HCV-J, D90208; HCVJK1, X61596; SW2, U10223; T10, U10225; HC-G9, D14853; Z1, U10235; Z5, U10237; Z8, U10240; Z4, U10236; Z6, U10238; Z7, U10239; CAM600, L29587; DK13, U10192; HEM26, D14311; NZL1, D17763; TH85, D14307; HK10, U10197; S52, U10210; DK12, U10191; S2, U10208; US114, D14309; HCV-K3a, D28917; NE048A, D16612; NE145G, D16618; NE274I, D16620; HCV-TR, D11443; NE137E, D16616; NE125C, D16614; SA1, U10216; SA3, U10217; SA13, U10215; SA11, U10214; SA4, U10218; SA5, U10219; SA7, U10221; SA6, U10220; BE95, L29577; HK2, U10198; VN506, D17500; VN571, D17507; VN569, D17506; VN538, D17504; VN085, D17497; VN004, D17496; VN530, D17502; VN507, D17501; VN531, D17503; B4/92, D63943; D97/93, D63946; VN787, D17508; VN540, D17505; VN843, D17509; HC-J6, D00944; T2, U10226; T4, U10228; T9, U10230; US10, U10231; S83, U10211; HC-J7, D10077; US1, U10233; DK11, U10190; SW3, U10224; DK8, U10195; HC-J8, D10988; and T8, U10229.

pair of isolates was estimated by the six-parameter method (7). The analyses were carried out with the supercomputer at DDBJ, the National Institute of Genetics, Mishima, Japan, using the computer program ODEN version 1.1. On the basis of these reliable estimates (genetic distance matrices), phylogenetic trees were constructed by using the neighbor-joining method (23).

Serotype analysis. Serotypes of HCV isolates were judged on the basis of serum antibody responses of the patients against the C14-1 and C14-2 recombinant peptides, which carry antigenic determinants specific for serotype 1 (corresponding to HCV type 1) and serotype 2 (corresponding to HCV type 2), respectively (27). ELISA kits to detect antibodies against those peptides were a generous gift from M. Kohara, The Tokyo Metropolitan Institute of Medical Sciences.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB/DDBJ/EMBL/NCBI DNA databases under accession numbers D63943 to D63947.

RESULTS

Prevalence of each subtype among HCV isolates obtained from liver disease patients in Chiang Mai. A total of 50 anti-HCV-positive sera obtained from patients with chronic liver disease (29 with chronic hepatitis [22 male and 7 female; mean age, 40.1 years], 16 with liver cirrhosis [9 male and 7 female; mean age, 51.7 years], and 5 with hepatocellular carcinoma [4 male and 1 female; mean age, 62.5 years) were examined for the presence of HCV RNA by using reverse transcription and PCR for NS5B and 5′UTR sequences. Only four sera, all of which were from patients with chronic hepatitis, were negative for HCV RNA by this method (data not shown). Of the 25 HCV isolates amplified by PCR from sera of patients with chronic hepatitis, 5 (20%), 5 (20%), and 4 (16%) were shown to be HCV subtype 1a (HCV-1a), HCV-3a, and HCV-1b, respectively, on the basis of NS5B sequences (Table 1). A sequence variant (LD47/93) was isolated from this patient group; this variant represents a novel subtype of HCV type 6, as described below. It should be noted that four other isolates (16%) were determined to belong to HCV type 1 and two others (8%) were determined to belong to HCV type 3 on the basis of 5′UTR sequences (16), although further classification into subtypes could not be done because of unsuccessful amplification of NS5B sequences. Four isolates (16%) identified by PCR for the 5′UTR sequences were unclassifiable because of some mismatches with the consensus sequences for each of HCV types 1 to 6. Of the 16 HCV isolates obtained from liver cirrhosis patients, 7 (44%) were HCV-3a and 3 (19%) each

TABLE 1. Prevalence of each subtype among HCV isolates obtained from liver disease patients in Thailand

Disease	No. of patients	No. (%) of HCV isolates in subtype or type:							
		1a	1b	3a	3b	New[a]	1[b]	3[b]	UC[c]
Chronic hepatitis	25	5 (20)	4 (16)	5 (20)	0 (0)	1 (4)	4 (16)	2 (8)	4 (16)
Liver cirrhosis	16	3 (19)	3 (19)	7 (44)	1 (6)	0 (0)	1 (6)	0 (0)	1 (6)
Hepatoma	5	2 (40)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	2 (40)
Total	46	10 (22)	7 (15)	13 (28)	1 (2)	1 (2)	5 (11)	2 (4)	7 (15)

[a] Referred to as HCV-6(new).
[b] Based on 5′UTR sequences.
[c] UC, Unclassifiable.

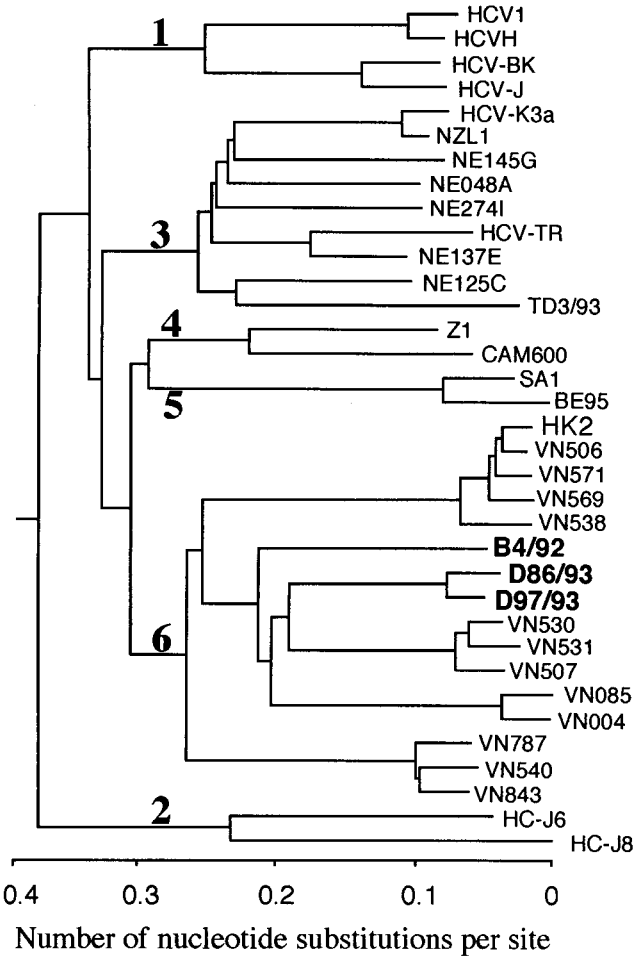

FIG. 2. Phylogenetic analysis of partial E1 sequences of the HCV genome. HCV types are indicated by numerals, and Thai variants are shown in boldface. The GSDB/DDBJ/EMBL/NCBI accession numbers for sequence data for the clones in the phylogenetic tree are as follows: HCV1, M62321; HCVH, M67463; HCV-BK, M58335; HCV-J, D90208; HCV-K3a, D28917; NZL1, D14305; NE145G, D16618; NE048A, D16612; NE274I, D16620; HCV-TR, D11443; NE137E, D16616; NE125C, D16614; Td-3/93, D30046; Z1, D16677; CAM600, L29587; SA1, L16642; BE95, L29578; HK2, L16634; VN506, D17500; VN571, D17507; VN569, D17506; VN538, D17504; B4/92, D63944; D86/93, D63945; D97/93, D63947; VN530, D17502; VN531, D17503; VN507, D17501; VN085, D17497; VN004, D17496; VN787, D17508; VN540, D17505; VN843, D17509; HC-J6, D00944; and HC-J8, D10988.

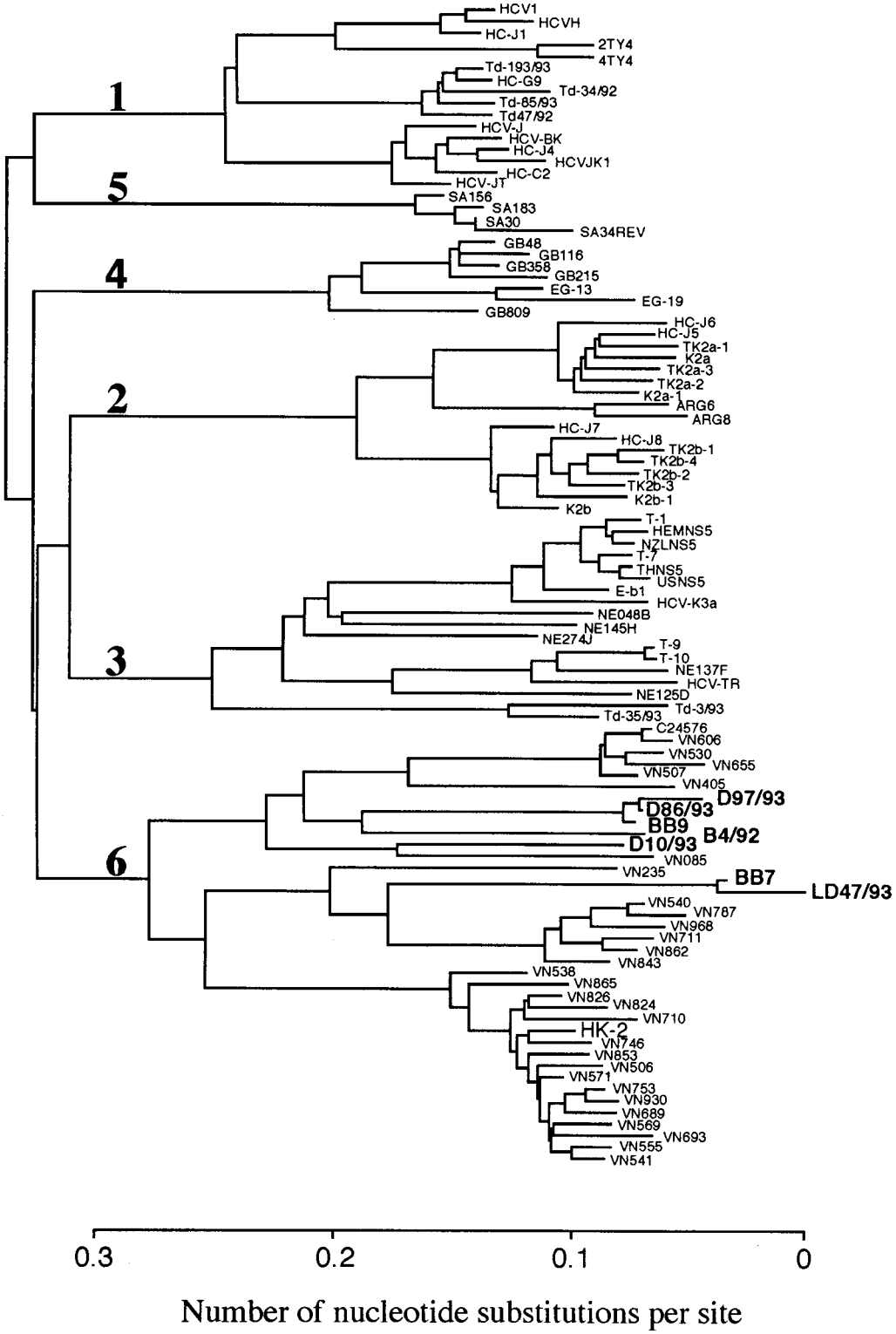

FIG. 3. Phylogenetic analysis of partial NS5B sequences of the HCV genome. HCV types are indicated by numerals, and Thai variants are shown in boldface. The GSDB/DDBJ/EMBL/NCBI accession numbers (or reference) for the sequence data for the clones in the phylogenetic tree are as follows: HCV1, M62321; HCVH, M67463; HC-J1, D00825; 2TY4, L23446; 4TY4, L23447; Td-193/93, D26388; HC-G9, D14853; Td-34/92, D26389; Td-85/93, D26391; Td-47/92, D26390; HCV-J, D90208; HCV-BK, M58335; HC-J4, D00826; HCVJK1, X61596; HC-C2, D10934; HCV-JT, D11168; SA156, L23471; SA183, L23472; SA30, L23473; SA34REV, L23474; GB48, L29614; GB116, L29602; GB358, L29607; GB215, L29605; EG-13, L23469; EG-19, L23470; GB809, L29626; HC-J6, D00944; HC-J5, D10075; TK2a-1, 4a; K2a, D10647; TK2a-3, 4a; TK2a-2, 4a; K2a-1, D10648; ARG6, L23457; ARG8, L23458; HC-J7, D00828; HC-J8, D10988; TK2b-1, 4a; TK2b-4, 4a; TK2b-2, 4a; TK2b-3, 4a, K2b-1, D10650; K2b, D10649; T-1, D10078; HEMNS5, D14312; NZLNS5, D14306; T-7, D10079; THNS5, D14308; USNS5, D14310; E-b1, D10130; HCV-K3a, D28917; NE048B, D16613; NE145H, D16619; NE274J, D16621; T-9, D10080; T-10, D10081; NE137F, D16617; HCV-TR, D26556; NE125D, D16615; Td-3/93, D26387; Td-35/93, D37898; C24576, D14208; VN606, D17478; VN530, D21321; VN655, D17479; VN507, D21320; VN405, D21318; D97/93, D28546; D86/93, D28545; BB9, D28542; B4/92, D28543; D10/93, D28544; VN085, D21316; VN235, D21317; BB7, D28541; LD47/93, D28547; VN540, D21324; VN787, D21327; VN968, D17494; VN711, D17483; VN862, D17491; VN843, D17489; VN538, D21323; VN865, D17492; VN826, D17488; VN824, D17487; VN710, D17482; HK-2, L23475; VN746, D17484; VN853, D17490; VN506, D17473; VN571, D17477; VN753, D17485; VN930, D17493; VN689, D17480; VN569, D17476; VN693, D17481; VN555, D17475; and VN541, D17474.

were HCV-1a and -1b. In total, 13 (28%) of the 46 HCV isolates were shown to be HCV-3a on the basis of NS5B sequences, while 10 (22%), 7 (15%), and 1 (2%) were classified as HCV-1a, -1b, and -3b, respectively. Five other isolates (11%) were determined to belong to HCV type 1, and two others (4%) were determined to belong to HCV type 3, on the basis of 5′UTR sequences. Seven HCV isolates (15%) were unclassifiable.

Phylogenetic analysis of the HCV variants. We previously reported the presence of sequence variants in blood donors and drug addicts in Chiang Mai (1). At that time, however, we could not determine whether they represented novel HCV types or novel subtypes of a known HCV type(s). To clarify this matter, we performed a detailed phylogenetic analysis of those variants. Figure 1 illustrates a phylogenetic tree based on the entire core region sequences of the viral genome (573 bases), including those of Vietnamese variants that had tentatively been classified as HCV types 7, 8, and 9 (28). In this phylogenetic tree, Thai (B4/92 and D97/93) and Vietnamese (VN085 to VN843) variants and HK-2, a representative strain of HCV-6a (25), formed a diverse single phylogenetic group. The result indicates that the Thai and Vietnamese variants are members of HCV type 6, representing two and three independent novel subtypes, respectively. Similarly, phylogenetic analysis of partial E1 region sequences (381 bases) classified the Thai (B4/92, D86/93, and D97/93) and Vietnamese (VN530 to VN843) variants in a diverse single phylogenetic group (type 6) that consisted of six subtypes (Fig. 2); this result was consistent with that obtained with the entire core region sequences. Moreover, phylogenetic analysis based on partial sequences of the NS5B region (225 bases) identified four other novel subtypes of HCV type 6 (Fig. 3). Included among them were Thai variants D10/93, BB7, and LD47/93 and Vietnamese variants VN405 and VN235.

Serotype analysis of HCV isolates. A serotyping method to differentiate individuals infected with HCV type 1 from those infected with type 2 has recently been developed (27). We carried out experiments to determine whether this serotyping method could be applicable to HCV type 6 infection. Patients infected with HCV type 6 isolates (D22/93 [subtype 6a] and D97/93, D10/93, and LD47/93 [new subtypes]) did not show detectable levels of antibody responses to either the C14-1 or C14-2 recombinant peptide, despite their strong antibody responses to more conserved antigenic determinants used in the commercially available second-generation ELISA (data not shown).

DISCUSSION

In Chiang Mai, Thailand, HCV-3a, HCV-1a, and HCV-1b were commonly found among anti-HCV-positive patients with chronic liver disease (chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma), with the prevalences being 28% (13 of 46), 22% (10 of 46), and 15% (7 of 46), respectively, on the basis of partial nucleotide sequences of the NS5B region of the viral genome (Table 1). Five (11%) of the remaining isolates, which had shown unsuccessful amplification for NS5B sequences, were determined to belong to HCV type 1, and two other isolates (4%) were determined to belong to type 3, on the basis of 5′UTR sequences. In total, 22 isolates (48%) were shown to belong to HCV type 1 (subtypes 1a and 1b), and 16 isolates (35%) were shown to belong to type 3 (subtypes 3a and 3b). In this connection, it was previously reported that HCV-3a was the most common subtype in liver disease patients in Thailand, followed by HCV-1b and -1a in that order, on the basis of subtype-specific amplification of core region sequences (17). Significant inconsistency between the previous results and ours was not observed.

A sequence variant (LD47/93) was found in the serum of a patient with chronic hepatitis (Table 1). We previously reported the presence of other sequence variants among blood donors and drug addicts (1). At that time, however, we could not draw a conclusion as to whether those variants represented novel HCV types or novel subtypes of a known type(s). In the present study, we performed a more detailed phylogenetic analysis with a six-parameter neighbor-joining method. The results based on the entire core region sequences and partial sequences of the E1 and NS5B regions have demonstrated that the Thai variants represent novel subtypes of HCV type 6 (Fig. 1, 2, and 3). In this connection, Tokita et al. (28) reported the presence of sequence variants in Vietnam, which they classified into HCV types 7, 8, and 9. However, our present analysis has revealed that the Thai and Vietnamese variants as well as HCV-6a isolates form a diverse single phylogenetic group. This result indicates that the Vietnamese variants also belong to HCV type 6, representing new subtypes. A detailed analysis like ours would be needed to determine the exact phylogenetic positions of HCV variants obtained in these areas. We also found similar HCV type 6 variants in southern China (17a). It seems likely that HCV type 6 exists rather commonly in southeastern parts of the Asian continent. In our previous study, HCV type 6 isolates (including the new subtypes) were found in 7 (15%) of a total of 47 blood donors and drug addicts (1). On the other hand, only a single isolate of HCV type 6 (LD47/93) was identified among 46 patients with chronic liver disease (Table 1). The difference between the two prevalence ratios is statistically significant ($P < 0.05$ by Fisher's exact test). A determination of whether HCV type 6 is less pathogenic than other types awaits further investigation.

The HCV type 6 isolates appear to be antigenically different from isolates of HCV types 1 and 2, as determined by a serotyping method that utilizes recombinant peptide C14-1 and C14-2 antigens. We previously observed that patients infected with HCV-3a did not show detectable antibody responses to either the C14-1 or C14-2 antigen (2). It is of great importance to establish another serotyping system(s) that is applicable to HCV types 3 and 6 in order to facilitate molecular epidemiological analysis of HCV in Southeast Asian countries, where those HCV types are very common (1, 2, 5, 28, 29). A serotyping system recently reported by Bhattacherjee et al. (3) would be useful for that purpose.

We found seven HCV isolates with apparently unique 5′UTR sequences that were different from any of the consensus sequences for known HCV types (16). Amplification of coding-region sequences such as the core, E1, and NS5B regions has been unsuccessful so far. These results suggest the possible presence of another novel type(s) or subtype(s) of HCV. Further study is needed to clarify the matter.

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