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Short Communication

Three Type 6 Hepatitis C Virus Subgroups among Blood Donors in the Yangon Area of Myanmar Are Identified as Subtypes 6m and 6n, and a Novel Subtype by Sequence Analysis of the Core Region

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Previously, using phylogenetic analysis of NS5b sequences, we found that three type 6 variant subgroups (M6–1, M6–2 and M6–3) exist in Myanmar. According to the new nomenclature of hepatitis C, M6–1 and M6–2 belong to subtypes 6m and 6n, respectively, but M6–3 is unassigned. In this study, we sequenced and phylogenetically analyzed the core region of these type 6 variant subgroups. Serum samples assigned as 6m or 6n by NS5b sequence were also identified as 6 m or 6n by core region analysis. The M6–3 (sample name MYAN-3E-3) remained unassigned to a subgroup based on its core region analysis. The findings of this study suggest that either the core region or the NS5b region can be analyzed for HCV subtype classification.

Key words: HCV genotype, type 6 subgroup, Myanmar, HCV core, phylogenetic analysis

A higher prevalence of HCV infection has been found in Southeast Asia than in other geographic locations. Myanmar is located in Southeast Asia and geographically bordered by other Southeast Asian countries, namely China, Thailand, Laos, and South Asian countries such as India or Bangladesh. The HCV genotype distributions of these countries differ [1–5]. Therefore, information on HCV genotypes in Myanmar is epidemiologically significant; yet such information is scarce.

We previously reported HCV genotypes of the Yangon city area of Myanmar [6]. Genotypes 3 and 1 and genotype 6 variants are the main genotypes. Using phylogenetic analysis of NS5b sequences, we found that three type 6 variant subgroups exist in Myanmar. According to the newly proposed nomenclature of hepatitis C, the M6–1 and M6–2 groups belong to subtypes 6m and 6n, respectively [7]. But M6–3 is still unassigned [8].

In this study, we sequenced the core region of these type 6 variant subgroup sera samples, compared the sequences with other reported sequences by phylogenetic analysis, and examined whether these samples are classified the same way by core region as by NS5b sequence analysis.

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Materials and Methods

Serum samples. Four HCV antibody positive serum samples representing three type 6 subgroups found for a previous study were used in this study. MYAN-8H-1 and MYAN-9H-1 represent the M6–1 group. MYAN-2I-2 and MYAN-3E-3 represent M6–2 and M6–3, respectively.

RNA extraction. Amplicor HCV 2.0 positive sera were HCV-RNA extracted for further experimentation. RNA was extracted from 0.125 ml of serum by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction [9] using Isogen-LS reagent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Extracted samples were precipitated with isopropanol and washed with 70% ethanol. The resulting pellet was resuspended in 20 μl of RNase-free water.

RT-PCR and nested PCR. RT-PCR and nested PCR were performed according to the method of Ohno *et al.* [10] with minor modifications. Briefly, extracted RNA was amplified by reverse transcriptase (RT)-PCR using sense primer Sc2 and antisense primer Ac2 at a portion of the core region (Table 1). RT-PCR was performed by the following protocol. We first prepared 20 μl of reaction mixture, containing 4 μl of 5 x reaction buffer, 0.8 μl of RNase inhibitor, 0.8 μl of rTTH DNA polymerase (Toyobo, Osaka, Japan), 0.25 μM of each primer and 0.3 mM of deoxynucleotide. The thermal cycler was programmed at 60 °C for 30 min and 95 °C for 1 min for the RT step; 95 °C 15 sec, 60 °C 20 sec for 2 cycles; and 90 °C 15 sec, 60 °C 20 sec for 38 cycles, followed by extension at 60 °C for 4 min.

The second PCR was performed using sense primer S7 (Table 1) and antisense primer A5 at 95 °C for 10 min and at 95 °C for 15 sec; and 60 °C for 20 sec for 30 cycles followed by at 60 °C for 4 min.

The resultant PCR product was electrophoresed on 3% agarose gel in 1x TAE, stained in ethidium bromide solution, and evaluated under UV light. To avoid the risk of false-positive results, PCR assays were done with strict precautions against cross-contamination.

Direct sequencing. Direct sequencing was performed as follows. Five microliters of crude PCR product was purified by MonoFas DNA kit I (GL Science, Tokyo Japan). 2.5 or 5.0 μl of purified PCR product was used as a template, and cycle-sequencing reactions were completed with the addition of 2 μl (3.2 pmol) internal sense or antisense sequencing primer using S7 or A5, plus 8 μl of the dye terminator included in the Big Dye Terminator 1.1 (Applied Biosystems, Foster City, CA, USA) cycle sequencing reaction kit and RNase-free water (total reaction amount 20 μl). Thermal cycling was performed on a Gene Amp 9600 thermal cycler at 96 °C for 10 sec, at 50 °C for 5 sec and at 60 °C 4 min, for a total of 25 cycles. After heat shock (95 °C for 2 min, at −80 °C for 5 min, sequencing was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis by core region sequences of Myanmar HCV type 6 subgroup samples. We analyzed a phylogenetic tree with core sequences of the four Myanmar HCV type 6 subgroup samples of this study and with other known genotype sequences obtained from the GenBank database.

ODEN (version 1.1.1) [11] using the six-parameter method [12] was used to determine the number

Table 1 Oligonucleotide primers used for PCR sequencing of the core region

Primer	Nucleotide Sequence	Nucleotide Position
Sc2	5'-GGGAGGTCTCGTAGACCGTGCACCATG-3'	nt.318–344
Ac2	5'-GAGMGGKATRTACCCCATGAGRTCGGC-3'	nt.758–732
S7	5'-AGACCGTGCACCATGAGCAC-3'	nt.330–349
A5	5'-TACGCCGGGGTCAKTRGGGCCCA-3'	nt.684–660

R, A.G; M, A.C; K, G.T;
S represents the sense primer; A represents the antisense primer.
Nucleotide numbering is from the 5'end of the HCV genome of HC-J4/83 (accession no. D13558).

of nucleotide substitutions per site (genetic distance) between the isolates. Based on these values, a phylogenetic tree was constructed by the neighbor-joining (N-J) method [13]. The tree was plotted by a program from DDBJ with the mid-point rooting option. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1,000 times [14].

Nucleotide sequence accession numbers. The sequences obtained in this study have been submitted to the DDBJ, GeneBank and EMBL nucleotide sequence databases and have been assigned accession numbers AB254860 to AB254863.

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MYAN-8H-1 1:CAACACCAACCGCCGCCCACAGGACGTTAAGTTCCCGGGCGGCGGCCAGATCGTCGGTGG 60
MYAN-9H-1 1:TAACACCAACCGCCGCCCACAGGACGTTAAGTTCCCGGGCGGTGGCCAGATCGTCGGTGG 60
MYAN-2I-2 1:AAACACCAACCGTCGTCCAATGGATGTTAAGTTCCCGGGCGGCGGTCAGATCGTTGGTGG 60
MYAN-3E-3 1:AAACACCAACCGTCGCCCACAGAACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGG 60
VN506-6a  1:AAACACCAACCGTCGCCCAATGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGCGG 60
VN843-7a  1:AAACACCAACCGCCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGCCAGATCGTTGGTGG 60
VN530-8a  1:AAACACCAACCGTCGCCCAATGGACGTAAAGTTCCCGGGTGGCGGTCAGATCGTCGGTGG 60
VN004-9a  1:AAACACTAACCGTCGCCCTATGGACGTCAAGTTCCCGGGCGGCGGCCAGATCGTTGGTGG 60
             ***** ***** ** **   * * ** ********** ** ** ******** ** **

MYAN-8H-1 61:AGTCTACTTGTTGCCGCGCAGGGGCCCACGTTTGGGTGTGCGCGCAGTGAGGAAGACCTC 120
MYAN-9H-1 61:AGTCTACTTGTTGCCGCGCAGGGGCCCACGTTTGGGTGTGCGCGCAGTGAGGAAGACCTC 120
MYAN-2I-2 61:AGTCTACTTATTGCCGCGCAGGGGCCCACGTTTGGGTGTGCGCGCGACGAGGAAGACTTC 120
MYAN-3E-3 61:AGTTTACTTGCTGCCGCGCAGGGGCCCTAGATTGGGTGTGCGCGCAGTTAGGAAGACTTC 120
VN506-6a  61:AGTTTACTTGTTGCCGCGCAGGGGCCCCCGGTTGGGTGTGCGCGCAACGAGAAAGACTTC 120
VN843-7a  61:AGTTTACTTGTTGCCGCGCAGGGGCCCTCGTTTGGGTGTGCGCGCGACGAGGAAAACTTC 120
VN530-8a  61:AGTCTACTTATTGCCGCGCAGGGGCCCTAGATTGGGTGTGCGCGCAACGAGGAAGACCTC 120
VN004-9a  61:AGTTTACTTGTTGCCGCGCAGGGGCCCTCGTTTGGGTGTGCGCGCGACGAGGAAGACTTC 120
             *** *****  ****************  * ***************    ** ** ** **

MYAN-8H-1 121:TGAACGATCCCAGCCTAGAGGCAGGCGCCAGCCCATACCAAAGGCGCGCCAGCCGACGGG 180
MYAN-9H-1 121:TGAACGATCCCAGCCTAGAGGCAGGCGTCAGCCTATACCAAGGGCGCGCCAGCCGACGGG 180
MYAN-2I-2 121:TGAACGATCCCAGCCCAGAGGCAGGCGTCAGCCCATACCAAAGGCGCGCCAGCCAACGGG 180
MYAN-3E-3 121:TGAACGATCCCAGCCCCGAGGGAGACGCCAGCCTATACCAAAGCAGCGTCCTCCGACGGG 180
VN506-6a  121:CGAGCGATCCCAGCCCAGAGGCAGGCGCCAACCTATACCAAAGGCGCGCCAGTCCCAGGG 180
VN843-7a  121:TGAACGGTCCCAGCCCAGGGGTAGACGCCAACCTATACCGAAAGCGCGTCACCAAACGGG 180
VN530-8a  121:CGAGCGGTCCCAGCCCAGAGGCAGGCGTCAGCCCATACCAAAGGCACGCCAGCCAACGGG 180
VN004-9a  121:CGAACGGTCCCAGCCTAGAGGCAGGCGCCAGCCCATACCAAAGGCGCGCCAGCCGATAGG 180
               ** ** ********  * ** ** ** ** ** ***** *     ** *        **

MYAN-8H-1 181:CCGGCACTGGGCTCAGCCTGGTTACCCTTGGCCCCTCTATGGGAATGAGGGCTGCGGTTG 240
MYAN-9H-1 181:CCGGCACTGGGCTCAGCCTGGTTACCCCTGGCCCCTTTATGGCAACGAGGGCTGCGGTTG 240
MYAN-2I-2 181:CCGGCACTGGGCTCAGCCTGGTTACCCTTGGCCCCTTTATGGGAATGAGGGCTGCGGATG 240
MYAN-3E-3 181:CCGGCACTGGGCTCAGCCCGGTTACCCCTGGCCCCTTTACGGTAACGAGGGCTGCGGCTG 240
VN506-6a  181:CCGGCACTGGGCTCAGCCCGGATACCCTTGGCCTCTTTATGGAAACGAGGGCTGCGGGTG 240
VN843-7a  181:CCGTACCTGGGCACAGCCTGGGTACCCCTGGCCTCTTTATGGGAATGAGGGTTGTGGCTG 240
VN530-8a  181:CCGGCACTGGGCGCAGCCTGGTTACCCCTGGCCCCTGTATGGTAACGAGGGCTGCGGATG 240
VN004-9a  181:CCGTAGCTGGGGTCAACCCGGCTACCCTTGGCCCCTTTATGGCAACGAGGGCTGCGGATG 240
             ***   *****  ** ** ** ***** ***** ** ** ** ** ***** ** ** **

MYAN-8H-1 241:GGCAGGGTGGCTCCTGTCCCCTCGCGGTTCTCGCCCTAAT                   280
MYAN-9H-1 241:GGCAGGGTGGCTCCTGTCCCCCCGCGGTTCTCGCCCTCAT                   280
MYAN-2I-2 241:GGCGGGATGGCTCCTGTCTCCCCGCGGTTCCCGCCCTAAT                   280
MYAN-3E-3 241:GGCAGGGTGGCTCCTGTCCCCCCGTGGTTCTCGTCCGCAT                   280
VN506-6a  241:GGCAGGTTGGCTCCTGTCCCCCCGCGGCTCCCGGCCACAT                   280
VN843-7a  241:GGCAGGGTGGCTCCTGTCCCCCCGCGGCTCTCGCCCTAAT                   280
VN530-8a  241:GGCAGGGTGGCTCCTGTCCCCCCGCGGCTCTCGTCCTCAC                   280
VN004-9a  241:GGCGGGATGGCTCCTGTCCCCCCGCGGATCTCGGCCTAAT                   280
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Fig. 1 Comparison of sequences of the core region (nucleotides 380–659) of type 6 subgroups. VN506 represents type 6a. VN843, VN530 and VN004 represent types 7a, 8a and 9a respectively. MYAN-8H-1 and MYAN-9H-1 represent the M6-1 subgroup. MYAN-2I-2 represents the M6-2 subgroup. MYAN-3E-3 represents M6-3. Asterisks indicate identical nucleotides.

Results

Sequence results and phylogenetic tree of HCV core region including Myanmar type 6 subgroup samples. A comparison of sequences of the core region nucleotides 380–659 of type 6 subgroups including representatives of M6–1 to M6–3 is shown in Fig. 1.

As with the NS5b analysis, phylogenetic analysis of the core region classified the type 6 variants into 3 subgroups (Fig. 2). The first 2 of these subgroups, which we had formerly named M6–1, M6–2 and M6–3, have been assigned according to the new nomenclature, to 6m and 6n [7], but M6–3 (sample name

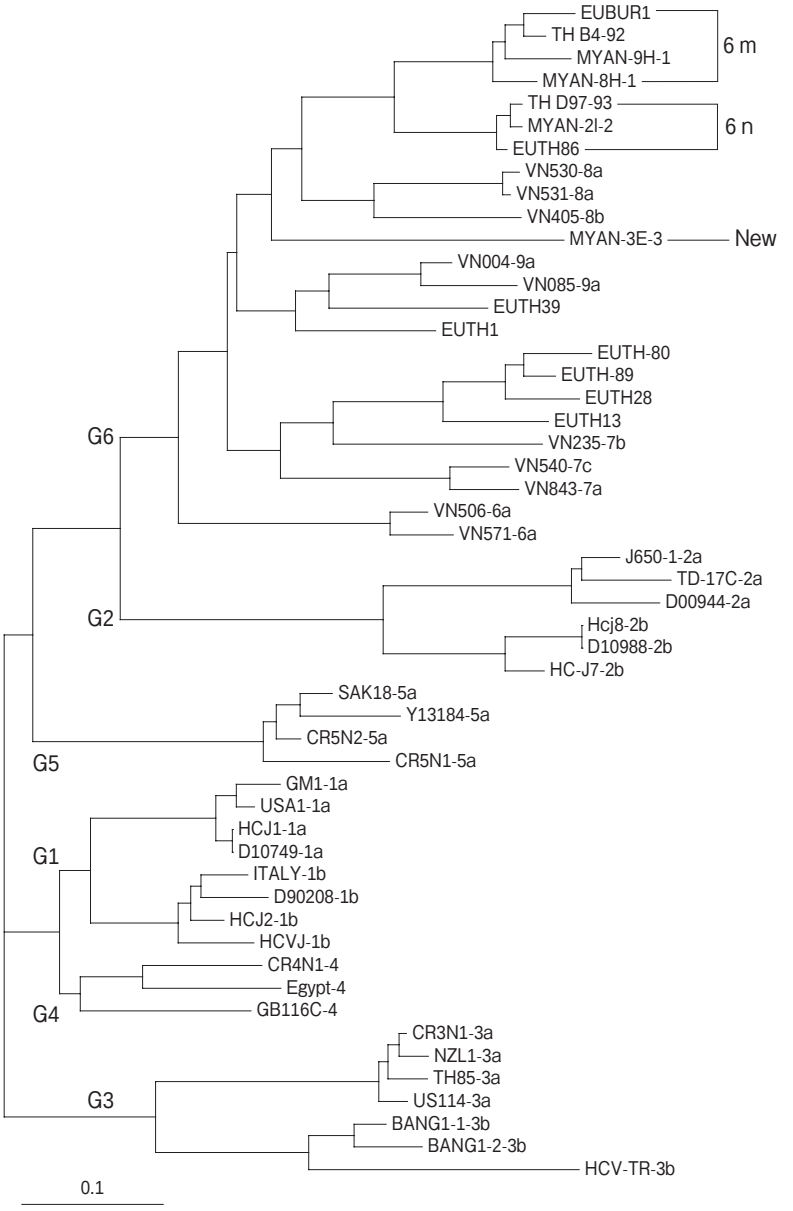

Fig. 2 Phylogenetic tree constructed from the core region of 4 HCV type 6 variants in Myanmar and previously reported type 6 group variants, along with representatives of the other 5 genotypes. Bootstrap values are shown along each main branch. The length of the horizontal bar indicates the number of nucleotide substitutions per site. Isolates labeled "MYAN-" were sequenced in the present study.

MYAN-3E-3) has not yet been assigned.

Discussion

HCV genotype 6 variants are common in the Southeast Asian countries, and also are restricted to this region. They have been classified into many subgroups from 6a to 6q [7]. 5'UTR PCR is often preferred for sensitive diagnostic assays, but sequence analysis of the region is not appropriate to differentiate genotypes 1 and 6 [3, 7]. To propose a new subtype, 3 or more examples of infection and sequences from both the core/ E1 region and the NS5b region are required [7]. Previously we reported our analysis of the NS5b region of type 6 subgroups in Myanmar 6. In this study, we analyzed the core regions of the same samples.

By the new nomenclature system [7], the M6-1 group (MYAN-8H-1, MYAN-9H-1) is classified as subtype 6m, and the M6-2 group (MYAN-2I-2) is classified as subtype 6n. At this point, subtype 6m has been reported only in Myanmar and northern Thailand. On the other hand, subtype 6n has been reported in Myanmar, Thailand and Kunming city in southwestern China [8]. Subtype 6n may more widely spread in this area than 6m.

The M6-3 group (MYAN-3E-3) was an unassigned type 6 subtype by NS5b analysis [6, 8], and remained as an unassigned type 6 subtype by the core sequence analysis in this study. Phylogenetic analysis of both the core region and NS5b region showed the same results. These findings suggest that, for HCV subtype classification, either the core region or the NS5b region can be analyzed.

Currently, we are analyzing sequences of HCV samples from the Myanmar - China border area and from the Myanmar - India border area. As a preliminary result, we found several samples that form a group of MYAN-3E-3. This group could be provisionally classified as a new subtype of HCV. We are performing further study for the assignment of new subtype of HCV from Myanmar.

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