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Severity-Related Molecular Differences among Nineteen Strains of Dengue Type 2 Viruses

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Abstract: **Comparative nucleotide sequencing was carried out on dengue type 2 virus (DEN-2) strains isolated from patients in Northeast Thailand during the epidemic season in 1993. The patients exhibited different clinical manifestations ranging from dengue fever (DF) to dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS). The results classified 19 DEN-2 strains into 3 subtypes according to nonsynonymous amino acid replacements. The strain isolated from a DSS patient eliciting secondary serological response belonged to subtype I, whereas 13 strains isolated from DHF patients with secondary response and 2 strains from DF patients with primary response belonged to subtype II. On the other hand, 3 strains isolated from DF cases evoking either primary or secondary response belonged to subtype III. These results suggest that subtype III virus infection could result in clinically milder manifestation irrespective of the serological response compared with subtype I or II viruses. The RNA secondary structure predicted for the 3′ noncoding region showed 4 different structures (A, B, C, and D). The result also indicates that different subtypes of DEN-2 serotypes are circulating in a single epidemic in Thailand.**

Key words: **Dengue-2 virus, Thailand, Sequencing, Subtype**

Dengue (DEN) viruses with 4 different serotypes, causing dengue fever (DF) and dengue haemorrhagic fever (DHF), are medically the most important arthropod-borne viruses affecting humans in terms of morbidity (30). DF/DHF are reemerging diseases and a major health concern in tropical and subtropical regions because of the increasing number of patients, expanding epidemic areas and increased occurrence of severe clinical manifestations: DHF/dengue shock syndrome (DSS). It is estimated that 100 million cases of DF occur annually and that fatality due to DHF/DSS is about 30,000 infected individuals per year (9, 22, 29). DHF has become the leading cause of death and hospitalization among children in Southeast Asian countries during the last 20 years (29).

Dengue viruses belong to the genus *flavivirus*, family *Flaviviridae*, possessing a positive-sense, single-stranded RNA genome which is approximately 10,700 bases long and contains a single open reading frame. A polyprotein, which is encoded by the open reading frame, is cleaved into 3 structural and 7 nonstructural proteins. The gene order is 5′-C-PrM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′, similar to all sequenced members of the genus *flavivirus* (35). Representative strains of all 4 serotypes of dengue virus have been sequenced (4, 7, 11, 18, 33, 44).

Infection of dengue virus may classically result in uncomplicated DF or severe manifestation characterized by capillary leakage and thrombocytopenia known as DHF/DSS. The case fatality rate of DHF/DSS comprises about 5% among children and young adults (38). The pathogenesis of DHF/DSS has not been fully elucidated although several hypotheses have been proposed. It is still uncertain what kind of host and virus-specified factors determine why a certain individual develops life-threatening infection while others recover without consequences or experience only mild DF. It has been proposed that secondary infection with a dengue virus serotype which is different from the primary infection is a risk factor for development into DHF/DSS, through hypersensitivity reaction (40) or antibody dependent enhancement (13). However, the secondary infection theory cannot explain the infection of cells without Fc

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Abbreviations: cDNA, complementary DNA; DEN-2, dengue-2; DF, dengue fever; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; FFU, focus forming unit; PrM, precursor of membrane associated protein; NGC, New Guinea C; NS, nonstructural protein; PBMC, peripheral blood mononuclear cells; RT-PCR, reverse transcription polymerase chain reaction; UTR, untranslated region.

receptors nor does it explain the occurrence of DHF in primarily infected patients lacking dengue antibodies. Molecular epidemiological studies on DEN-2 virus strains indicated that the Southeast Asian genotype is associated with severe clinical manifestations, while classical Caribbean genotype is associated with milder diseases in the Western Hemisphere (37). Information obtained so far indicates that both host-related factors and virus virulence factors (39) can contribute to the pathogeneses of DHF/DSS (8, 24, 37). Although host factors responsible for DHF/DSS have not yet been clearly identified, it has been suggested that cytokines and some chemical mediators, such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-2, IL-6, platelet activating factor (PAF), complement activation products like C3a and C5a, and histamine could be responsible for increased vascular permeability leading directly to plasma leakage in DHF/DSS (22, 25).

Since there is no animal model for DHF/DSS, we have been comparing nucleotide and deduced amino acid sequences of DEN-2 virus strains isolated from patients exhibiting different clinical manifestations in the same epidemic area during the same epidemic season. Results obtained so far have shown certain strain-specific secondary structures in the 3′ untranslated region and amino acid replacements in viral proteins (26, 27, 42). However, such molecular changes have not yet been able to be related to the clinical manifestations and serological response of the patients.

Molecular markers of virulence and attenuation have most frequently been shown to occur among flaviviruses (5, 11, 14, 16, 19, 31, 41). Recent studies (24) on 11 strains of DEN-2 virus showed that several structural differences in viral genome could be responsible for the pathogenesis of severe disease in Southeast Asia.

In this study, the nucleotide sequence of the whole genome was analyzed for several additional strains of DEN-2 virus isolated from patients exhibiting different disease severity during the same epidemic season in the same epidemic area in Nakhon Phanom, Northeast Thailand. The results for a total of 19 strains were compared with the clinical diagnosis and serological responses of the patients from whom each strain was isolated.

Materials and Methods

Viruses. The DEN-2 virus strains used in this study were isolated from patients in Nakhon Phanom Provincial Hospital, Northeastern Thailand, during the dengue outbreak in 1993. The samples from the DF cases were collected from the outpatient department, while other samples were taken from patients hospitalized with DHF/DSS. The clinical diagnosis and the disease severity grading were classified according to the World Health Organization (WHO) (43). All strains were determined as DEN-2 serotype by type-specific reverse transcription polymerase chain reaction (RT-PCR). Each strain was inoculated once into a monolayer culture of *Aedes albopictus* clone C6/36 cell line and incubated at 28 C for 7 days in Eagle's medium containing 2% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acids at final concentrations. The infected culture fluid was harvested 7 days after inoculation, aliquoted, and stored at −80 C as the seed virus. The seed virus was passaged in C6/36 cells 1–3 times before sequencing.

Sample preparation for sequencing. RNA was extracted from the infected C6/36 cell culture fluid by using Trizol LS (GIBCO BRL, U.S.A.). cDNA products were prepared from the RNA using superscript II, RNase H-Reverse Transcriptase (GIBCO BRL) as described in the manufacturer's instruction manual. The PCR amplification was performed using the cDNA products according to the manufacturer's instructions. Primer sequences for cDNA synthesis were obtained from the published data of DEN-2 candidate vaccine strain PR 159/S1, Jamaica 1409 strain, and prototype New Guinea C (NGC) strain (4, 11, 18). The primer sequences for the 5′ and 3′ UTR were obtained from a previous publication (2). The 3′ terminal sequences were determined using the 3′ RACE Kit (GIBCO BRL) by tailing the genomic RNA with poly (A) using *E. coli* poly (A) polymerase as recommended by the manufacturer. The cDNA nucleotide regions 1–276, 36–1830, 1735–3559, 3405–5193, 4768–6118, 6054–7595, 7164–9429, 8836–10342 and 10210–10723 were amplified according to the manufacturer's instructions (Boehringer Mannheim, Germany).

The PCR products of 3′ UTR and C-PrM and NS1 regions were electrophoresed in agarose gel. The band with expected size was excised from the gel or purified using the QIAGEN, QIAEX II, Gel Extraction Kit (150). The newly synthesized RT-PCR product was ligated directly into a TA cloning kit, pCR® vector, and transfected to *E. coli* XL blue cells as described in the instruction manual. Transformants were replated, and plasmid was extracted by using QIAGEN and digested with *Eco*RI restriction enzyme. Five clones for sequencing analysis were chosen from transformants that produced restriction digest products with lengths equivalent to the original RT-PCR products. The PCR product of the rest of the genome starting from NS2A-NS2B-NS3-NS4A-NS4B-NS5 was purified using Microcon microconcentrators (Amicon, U.S.A.) according to the instruction manual. The purified PCR product was used for direct sequencing.

Sequencing strategy. The nucleotide sequencing was determined by the primer extension dideoxy chain termination method and was performed as recommended in the Taq and Big Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems, U.S.A.). For each sequencing reaction, approximately 30–90 ng of purified DNA from direct PCR product or 250 to 500 ng of purified dsDNA was combined with 3.2 pmol of primer and dRhodamine Terminator Cycle Sequencing ready reaction mixture containing the four dye-labeled deoxynucleotide terminators. The cycle sequencing parameters used were as described in the manufacturer's protocol (25 cycles of 96 C for 30 sec, 50 C for 60 sec, and 60 C for 4 min). The reaction mixture was column purified (CentriSeps, U.S.A.), and the DNA was vacuum dried for 30 min. The pellet was resuspended in 15 μl of template suppression reagent, heated at 92 C for 2 min, and kept on ice until loaded on the sequencer (Applied Biosystems Prism 310, U.S.A.) using a capillary (47 cm with the inside diameter of 50 μm) and Performance Optimized Polymer 6 (Perkin-Elmer/Applied Biosystems).

Nucleotide and amino acid sequence analysis. The DNASIS-Mac Version 3.6 Software System (Hitachi, 1995) was used for primer selection, homology search, comparison of all obtained sequences, and secondary structure prediction. The protein analysis software was based on the Chou and Fasman (3) criteria for secondary structure analysis from protein primary structure. Construction and analysis of the genomic dengue DNA were done using sequence Navigator and Factura software (Perkin-Elmer/Applied Biosystems).

Phylogenetic analysis. The whole genome was used to draw a phylogenetic tree for comparison among the 19 DEN-2 strains, prototype NGC strain 16681 and the American Mara 4 strain. Sequences of DEN-1, 3 and 4 viruses were included as an outgroup (data not shown).

The PHYLIP package of software programs, distributed by Felsenstein (1989, 1993) SeqBoot, DNADIST, Neighbor and CONSENSE, that utilize the UPGMA method using DNA distance relationships were used to calculate nucleotide evolutionary distances and to prepare a dendrogram for the sequences, which were viewed using the TREEVIEW program as described previously. Bootstrap reassembly of the data set 1,000 times was done to ascertain support for major branches of the tree.

Assay of virus infectivity. The infectivity assay of the seed virus, prepared in C6/36 cells cultures, was performed using a microfocus method described previously (32) with certain modifications. Approximately 1×10^4 cells/ml of BHK-21 cells were cultured on 96-well microplates. An overlay medium containing 0.5% methyl cellulose 4000 and 1% FCS in Eagle's medium was used after infecting diluted virus specimens onto the cell sheet. Infectivity titer was expressed as focus forming units (FFU/ml).

Nucleotide sequence accession number. The nucleotide sequences reported in this study have been deposited in the GenBank database under accession nos. AF169678 (ThNH29/93), AF169679 (ThNH36/93), AF169680 (ThNH45/93), AF169682 (ThNH54/93), AF169681 (ThNH55/93), AF169683 (ThNH62/93), AF169684 (ThNH63/93), AF169685 (ThNH69/93), AF169686 (ThNH73/93), AF169687 (ThNH76/93), and AF169688 (ThNH81/93).

Results

Sequence Analysis of the DEN-2 Isolates

We determined the entire genome sequence for the 19 strains of DEN-2 virus isolated from patients exhibiting different disease severity. The strain code, infectivity and subtyping of the virus based on the amino acid sequence are shown together with the patient's history such as age, gender, clinical diagnosis, serological response, and dates of onset and sampling (Table 1). The infectivity titers in the culture fluids of the C6/36 cells infected with the DEN-2 isolates, as determined by the focus forming method, did not correlate with the severity of the disease of the patient from whom each strain was isolated. Three strains isolated from the DSS, DHF and DF representative of subtypes I, II and III were comparatively inoculated into the primary culture of peripheral blood mononuclear cells (PBMC). The percentage of DEN-2 antigen-positive cells and the amount of TNF-α in the infected PBMC culture fluid were highest for the subtype I strain followed by subtype II and lowest for subtype III strain (data not shown).

Table 2 summarizes nonsynonymous amino acid replacements found in these DEN-2 strains. The results classified the 19 strains into 3 subtypes in terms of amino acid replacements (Table 2). Amino acid replacement at the PrM 16 position (R↔I) could alter the electric charge of the protein, while replacement at PrM 81 (T↔A) could bring about a change in the secondary structure on the C-terminus of the PrM protein. We could not find any amino acid replacements in the E protein except for a single synonymous amino acid replacement (K↔R). In contrast, many nonsynonymous amino acid replacements were scattered along the nonstructural proteins of the virus. The amino acid replacements which could bring about changes in the characteristics of the nonstructural proteins are as follows: (D↔G) at 278 of NS1; (M↔V) at 41, (M↔I) at 136 and (N↔D) at 139 of NS2A; (M↔I) at 13 and (A↔T) at 118 of NS3; and (M↔T) at 337 of NS5. In addition,

Table 1. Clinical information of the patients, serological response and amino acid (AA) subtyping of the DEN-2 virus

Name of Isolates	Diagnosis	Serological response	Sex	Age (years)	Relevant dates		FFU/ml[c] BHK-21	AA sub-type
					Onset	Sampling		
ThNH7/93[a]	DSS	Secondary	F	12	06-17	06-18	1.75×10^4	I
ThNH28/93[a]	DHF(2)	Seconday	M	10	06-19	06-21	7.5×10^4	II
ThNH52/93[a]	DHF(1)	Secondary	M	7	06-22	06-23	1.5×10^6	II
ThNHp11/93[a]	DF	Primary	M	14	I.U[b]	06-30	1.75×10^5	III
ThNHp12/93[a]	DF	Secondary	F	11	I.U[b]	06-30	2.0×10^5	III
ThNHp14/93[a]	DF	Secondary	M	11	06-26	06-29	6.0×10^5	III
ThNHp16/93[a]	DF	Primary	F	12	I.U	06-30	10^4	II
ThNHp36/93[a]	DF	Primary	F	9	I.U	06-30	10^4	II
ThNH29/93	DHF(2)	Secondary	M	11	06-20	06-21	1.86×10^5	II
ThNH36/93	DHF(2)	Secondary	M	13	06-20	06-21	8.0×10^5	II
ThNH45/93	DHF(2)	Secondary	F	9	06-21	06-23	7.35×10^5	II
ThNH54/93	DHF(1)	Secondary	M	13	06-21	06-23	2.88×10^3	II
ThNH55/93	DHF(1)	Secondary	M	7	06-20	06-23	2.36×10^4	II
ThNH62/93	DHF(2)	Secondary	M	13	06-23	06-24	5×10^3	II
ThNH63/93	DHF(1)	Secondary	F	11	06-19	06-24	7.42×10^4	II
ThNH69/93	DHF(1)	Secondary	M	9	06-23	06-24	2.46×10^4	II
ThNH73/93	DHF(1)	Secondary	F	8	06-22	06-30	2.24×10^6	II
ThNH76/93	DHF(1)	Secondary	F	9	06-24	06-30	2.6×10^3	II
ThNH81/93	DHF(1)	Secondary	F	8	06-24	07-01	1.1×10^4	II

[a] Mangada and Igarashi, 1998, [b] information not available, [c] infectivity of the isolated virus.

Table 2. Summary of nonsynonymous amino acid (AA) changes among DEN-2 viruses

Isolates	Clinical severity	Position of amino acid replacement in the dengue-2 virus											AA sub-type
		PrM		NS1		NS2A				NS3		NS5	
		16	81	278	281	41	136	139	215	13	118	337	
ThNH7/93	DSS	I	T	G	D	V	I	D	N	I	T	T	I
ThNH28/93	DHF II	-	-	D	-	-	-	N	-	M	-	-	II
ThNH52/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNHp11/93	DF	R	A	D	E	M	M	N	S	M	A	M	III
ThNHp12/93	DF	R	A	D	E	M	M	N	S	M	A	M	III
ThNHp14/93	DF	R	A	D	E	M	M	N	S	M	A	M	III
ThNHp16/93	DF	-	-	D	-	-	-	N	-	M	-	-	II
ThNHp36/93	DF	-	-	D	-	-	-	N	-	M	-	-	II
ThNH29/93	DHF II	-	-	D	-	-	-	N	-	M	-	-	II
ThNH36/93	DHF II	-	-	D	-	-	-	N	-	M	-	-	II
ThNH45/93	DHF II	-	-	D	-	-	-	N	-	M	-	-	II
ThNH54/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH55/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH62/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH63/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH69/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH73/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH76/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
ThNH81/93	DHF I	-	-	D	-	-	-	N	-	M	-	-	II
*Mara 4	DHF	R	-	D	E	-	M	N	S	V	-	M	III
**IQT1797	DF	R	-	D	E	L	M	N	S	V	-	M	III

The amino acid residue number corresponds to the position in respective proteins. GenBank accession no. of *Mara 4 (Venezuela) and **IQT1797 (Peru) isolates are AF100466 and AF100467, respectively.

ThNH7/93 is shown on the top as a reference, and the residues of the other isolates with identical AA to ThNH7/93 are shown by a hyphen (-).

subtype III viruses were found to possess E at 281 in NS1 instead of D in subtype I and II viruses, although this replacement is synonymous.

The amino acid sequence subtype of the DEN-2 strain was compared with the clinical severity and serological response of the patient from whom each strain was isolated, and the results are summarized in Tables 1 and 2. A single strain of subtype I virus was isolated from a

DSS patient showing secondary type antibody response. Thirteen strains of subtype II virus were isolated from DHF patients with secondary type antibody response, whereas 2 strains of this subtype were isolated from mild DF cases with primary antibody response. On the other hand, all 3 strains of subtype III virus were isolated from mild DF cases, whose antibody responses were secondary for one case and primary for the remaining 2 cases.

Nucleotide sequence variation was scattered throughout the entire genome except for the 5′ UTR, where all the isolates possessed sequences identical to that of the prototype NGC strain (18) and 16681 strain (21). Additionally, amino acid sequences of the C and M proteins were perfectly conserved among all of the isolates. No nucleotide deletions or insertions were detected within the coding region; however, subtype I virus (ThNH7/93) possessed one nucleotide insertion at position 335 of the 3′ UTR, bringing it a total genome length of 10,724 nucleotides compared with 10,723 nucleotides for the other 18 isolates. Hydrophobicity profiles of the viral polyprotein were similar for all isolates, and mostly hydrophobic. We also confirmed our previous data on 8 strains of DEN-2 virus (26) where potential N-glycosylation sites and RGD cell membrane binding sequences found at position 538 of the NS3 protein were perfectly conserved among all the newly sequenced isolates.

Nucleotide and Amino Acid Sequence Homology

The nucleotide and amino acid sequence similarities were compared among 19 Thai strains, DEN-2 NGC and 16681 (data not shown). An analysis of 18 strains from DHF and DF cases revealed nucleotide and amino acid sequence similarities of 97.33–99.96% and 99.0–99.97%, respectively. On the other hand, nucleotide and amino acid sequence similarities between the subtype I virus (ThNH7/93) and other isolates were 95.5–96.3% and 99.2–99.6%, respectively. The homologies among the 19 isolates and the prototype NGC strain, 16681 and other Thai isolates from the 1994 outbreak in Kamphaeng Phet, Thailand, were compared. The percentage of nucleotide sequence similarities between the prototype NGC strain and all of the isolates varies from 93.19 to 95.76%. Thai strain K0008, isolated from a DHF case, possessed higher amino acid homology, ranging from 99.41 to 99.61%, with the 19 isolates. On the other hand, the amino acid homology of the 16681 strain with the 19 Thai isolates varied from 98.61 to 98.99%, indicating a low mutation rate among the Thai isolates since 1964.

Phylogenetic Analysis of Full Genome Sequence of All Isolates

To determine the genetic relationship among DEN-2 strains, a phylogenetic analysis was done using the whole genome of the 19 Thai strains, the prototype NGC strain, 16681 strain and American strain Mara 4. The 18 isolates from DHF and DF cases showed a closer relationship with the 16681 strain and belonged to South Asian genotypes (36). UPGMA analysis of the

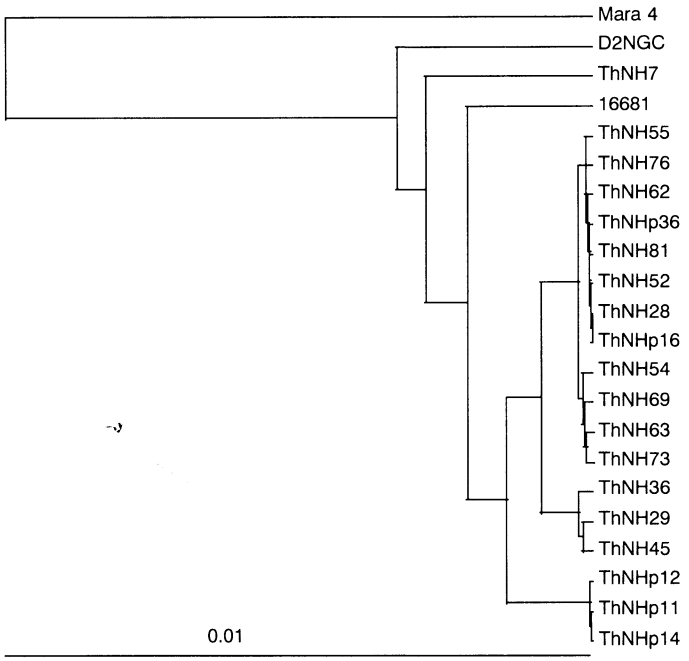

Fig. 1. Phylogenetic tree generated using the entire genome sequences of 19 DEN-2 strains, the 16681 strain, the prototype NGC strain and American isolate Mara 4. The tree was constructed by the UPGMA method. The branches are drawn with proportional distances.

Table 3. Comparison of the nucleotide evolutionary distances among DEN-2 virus isolates

	ThNH7	NGC	Th 28	29	36	45	52	54	55	62	63	69	73	76	81	p11	p12	p14	p16	p36	16681
ThNH7																					
D2NGC	0.0684																				
ThNH28	0.0347	0.0449																			
ThNH29	0.0433	0.0430	0.0110																		
ThNH36	0.0447	0.0424	0.0133	0.0030																	
ThNH45	0.0444	0.0438	0.0114	0.0024	0.0040																
ThNH52	0.0351	0.0453	0.0004	0.0114	0.0137	0.0118															
ThNH54	0.0376	0.0444	0.0035	0.0085	0.0108	0.0089	0.0038														
ThNH55	0.0359	0.0457	0.0011	0.0118	0.0141	0.0125	0.0015	0.0046													
ThNH62	0.0356	0.0456	0.0008	0.0119	0.0142	0.0123	0.0012	0.0043	0.0020												
ThNH63	0.0376	0.0452	0.0027	0.0100	0.0123	0.0108	0.0027	0.0028	0.0038	0.0034											
ThNH69	0.0373	0.0449	0.0024	0.0101	0.0128	0.0108	0.0028	0.0025	0.0032	0.0031	0.0022										
ThNH73	0.0374	0.0448	0.0027	0.0096	0.0119	0.0102	0.0027	0.0022	0.0038	0.0034	0.0013	0.0022									
ThNH76	0.0360	0.0461	0.0012	0.0123	0.0146	0.0126	0.0016	0.0047	0.0024	0.0017	0.0038	0.0035	0.0038								
ThNH81	0.0353	0.0453	0.0006	0.0116	0.0139	0.0120	0.0009	0.0040	0.0017	0.0010	0.0031	0.0028	0.0031	0.0014							
ThNHp11	0.0396	0.0452	0.0168	0.0254	0.0275	0.0265	0.0172	0.0194	0.0180	0.0177	0.0194	0.0191	0.0192	0.0181	0.0174						
ThNHp12	0.0397	0.0455	0.0171	0.0257	0.0278	0.0268	0.0175	0.0197	0.0183	0.0180	0.0197	0.0194	0.0195	0.0184	0.0177	0.0008					
ThNHp14	0.0394	0.0453	0.0169	0.0255	0.0276	0.0266	0.0173	0.0195	0.0181	0.0178	0.0195	0.0192	0.0193	0.0182	0.0175	0.0005	0.0009				
ThNHp16	0.0348	0.0452	0.0003	0.0113	0.0136	0.0117	0.0007	0.0038	0.0014	0.0011	0.0030	0.0027	0.0030	0.0015	0.0008	0.0171	0.0174	0.0172			
ThNHp36	0.0352	0.0450	0.0007	0.0117	0.0140	0.0121	0.0007	0.0041	0.0018	0.0015	0.0030	0.0031	0.0030	0.0019	0.0012	0.0175	0.0178	0.0176	0.0009		
16681	0.0515	0.0308	0.0273	0.0300	0.0319	0.0310	0.0277	0.0282	0.0285	0.0282	0.0293	0.0289	0.0291	0.0285	0.0277	0.0302	0.0305	0.0303	0.0276	0.0280	
Mara 4	0.1358	0.1202	0.1374	0.1358	0.1352	0.1376	0.1374	0.1379	0.1377	0.1378	0.1381	0.1378	0.1378	0.1387	0.1378	0.1356	0.1356	0.1353	0.1376	0.1375	0.1279

genomic sequences showed that DEN-2 NGC, ThNH7/93 and 16681 strain showed a closer relationship with each other compared to the other 18 isolates (Fig. 1). The American isolate, Mara 4, was distantly related to all of Thai isolates. Three subtype III viruses isolated from mild DF cases formed one group and subtype II virus strains isolated from 13 DHF cases and 2 DF cases made up a cluster separate from the subtype III strains. A single subtype I virus isolated from a DSS patient formed a branch distinct from the branch clusters composed of subtype II or III virus strains. There is no substantial difference between the phylogenetic trees constructed using the partial sequences of E/NS1 gene junction and the entire genome sequences.

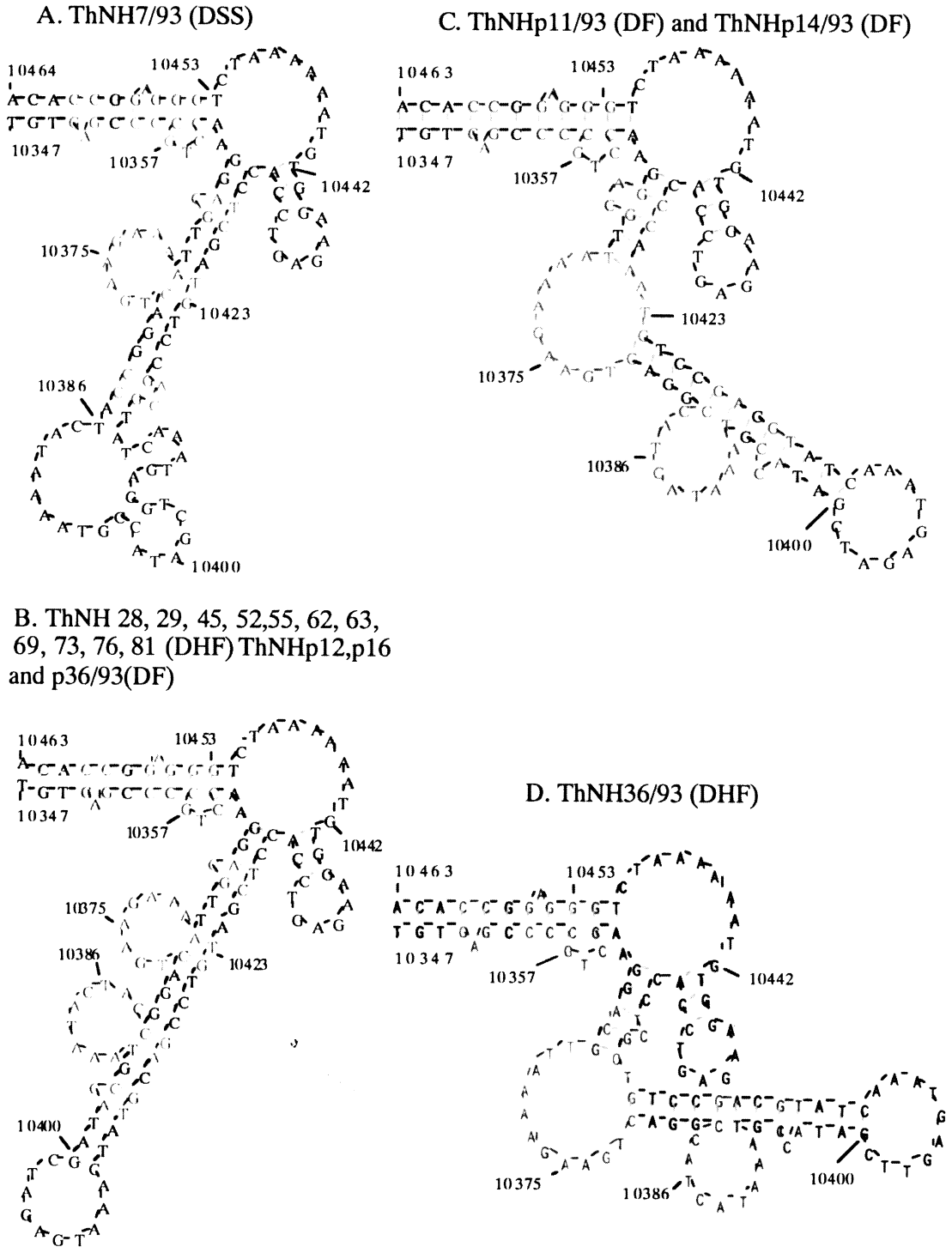

Fig. 2. Comparison of predicted secondary structures of nucleotides 10347–10463 (10464) in the 3′ UTR among the 19 DEN-2 strains. Four different secondary structures (A, B, C, and D) were predicted.

The nucleotide evolutionary distances within the 19 Thai strains, 16681, DEN-2 NGC and American isolate Mara 4 were analyzed. The result showed that the 19 isolates from DHF and DF were more closely related to 16681 than DEN-2 NGC, with the evolutionary distance ranging from 0.0273 to 0.0515 (Table 3). The Thai isolates were distantly related to Mara 4, with the evolutionary distance ranging between 0.1202 and 0.1387.

Secondary Structure Analysis of the 3′ UTR

A RNA secondary structure was predicted for the 3′ UTR of all 19 DEN-2 strains based on their nucleotide sequences. The result confirmed our previous report (26) that four kinds of secondary structure could be predicted. The first structure (A) was predicted for a DSS strain (ThNH7/93), the second structure (B) for 12 DHF strains represented by ThNH29/93 as well as 3 DF strains (ThNHp12/93, ThNHp16/93, and ThNHp36/93), the third structure (C) for 2 DF strains (ThNHp11/93 and ThNHp14/93), and the fourth structure (D) for another DHF strain (ThNH36/93) similar to the third structure (C) but possessing a slightly smaller angle between its stems (Fig. 2). Strains 16681, DEN-2 NGC and Mara 4 were also examined for the secondary structure of 3′ UTR. The result suggested that the 16681 had structure B, whereas DEN-2 NGC and Mara 4 had a structure different from the four structures listed above (data not shown).

Discussion

Dengue virus infection represents a worldwide health problem of increasing magnitude: increasing number of cases, expanding epidemic areas, as well as severity of the disease. The pathogenesis of DHF has been studied intensively for the past 30 years. Previous studies (2, 6, 20, 21, 24, 26–28, 34) have been tried to determine the molecular markers for attenuation and virulence of dengue viruses. To clarify the role of genetic variation in the occurrence of severe disease, a useful step is to obtain the complete nucleotide sequence for comparison on several strains of the same serotype isolated from DSS, DHF and DF cases in a particular outbreak area. The sequence data on the virus strains and clinical as well as serological characteristics of the patient from whom each strain is isolated will be the first step towards understanding the molecular determinants for virus virulence. Our results indicated that the clinical severity of DEN-2 virus infection depends both on the molecular structure of the infecting virus, particularly amino acid sequence of viral proteins, and the serological response of the patients (Table 1). Although the number of analyzed virus strains is still limited, the comparison of amino acid subtypes of infecting virus with clinical manifestation and serological response of the patients suggested the following possibilities: (1) secondary infection of subtype I virus could be very severe (DSS); (2) secondary infection of subtype II virus could be quite severe (DHF), but its primary infection could be mild (DF); and (3) infection of subtype III virus could be mild (DF) irrespective of serological response, either primary or secondary. These possibilities should be confirmed by analyzing an increased number of virus strains obtained in different geographical areas during different epidemic seasons.

The translation of DEN-2 virus polyprotein is initiated by ribosome binding to the 5′ terminus of the positive strand RNA, whereas replication of the positive strand RNA is initiated by replicase binding to the 3′ terminus of the minus strand. The 3′ UTR region of the viral genome is essential for viral replication, serving as a signal for the initiation of minus-strand synthesis (11, 28). Therefore, mutation in the 3′ UTR would bring about changes in its secondary structure of viral RNA and could potentially affect the virus virulence (17, 21). Among the 4 kinds of secondary structure predicted for the 3′ UTR, the first one (A) was unique for the strain isolated from a DSS case (ThNH7/93). Therefore, there remains some possibility that this unique secondary structure in the 3′ UTR has something to do with the biological characteristics or virulence of this particular virus strain. However, a comparison of the predicted secondary structure of each virus strain with clinical or serological data of the patients could not bring about a certain conclusion as in the case of amino acid sequence subtypes.

The comparative study among the Thai and American strains (23, 27) showed certain amino acid replacements in the whole genome of DEN-2 viruses which might correlate with the severity of disease. The present study, which is a continuation of a previous study (27), found a possible correlation between the amino acid sequence subtype of virus strain and clinical severity and serological response of the patient from whom each virus strain was isolated. A majority of the amino acid substitutions were found within the nonstructural proteins although 2 changes were observed in a structural protein PrM. Three amino acid replacements were characteristic to a single subtype I virus strain isolated from a DSS case: D↔G change at position 278 in NS1; N↔D change at 139 in NS2A; and M↔I change at 13 in NS3 (Table 2). Five nonsynonymous amino acid replacements were characteristic to subtype III virus strains, all isolated from DF cases: I↔R change at 16 and T↔A at 81 in PrM; I↔M at 136 in NS2A; A↔T at 118 in NS3; and T↔M at 337 in NS5. Some of these replace-

ments have been described in previous reports (2, 6, 21). At this moment, it is difficult to assess the significance of each of the observed amino acid substitutions. *In vitro* site-directed mutagenesis using the whole length of the cDNA clone and biological characterization of the progeny virus could address the question as to which one of these amino acid substitutions in the dengue virus genome is related to virulence and attenuation of the virus. Our phylogenetic analysis also supported the finding by amino acid sequence subtyping. The phylogram constructed using either E/NS1 gene junction as proposed by Rico-Hesse (36) or using whole genome gave an almost identical result (Fig. 1).

Parallel experiments in our laboratory have shown that representative strains from each of the 3 different amino acid sequence subtypes of the virus showed different infection rates to a primary culture of human PBMC. The infection rate correlated with the disease severity of the patients from whom each strain was isolated. The levels of some cytokines, such as TNF-α or IL-6, released into the infected culture supernatant roughly correlated with the infection rate. The present study also indicates that multiple subtypes of DEN-2 virus are circulating in a single epidemic in Thailand. Particularly, subtype I virus seems to be more closely associated with severe clinical manifestations, suggesting that some pathogenic virus factors may directly correlate with the occurrence of DHF.

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References

1) Barrett, A.D.T., Monath, T.P., Crop, C.B., Adkins, J.A., Ledger, T.N., Gould, E.A., Schlesinger, J.J., Kinney, R.M., and Trent, D.W. 1990. Attenuation of wild-type yellow fever virus by passage in HeLa cells. J. Gen. Virol. **71**: 2301–2306.
2) Blok, J., Mc William, S.M., Butler, H.C., Gibbs, A.J., Weiller, G., Herring, B.L., Hemsley, A.C., Aaskov, J.G., Yoksan, S., and Bhamarapravati, N. 1991. Comparison of a dengue-2 virus and its candidate vaccine derivative: sequence relationships with the flaviviruses and other viruses. Virology **187**: 573–590.
3) Chou, P.Y., and Fasman, G.D. 1974. Prediction of protein conformation. Biochemistry **13**: 222–244.
4) Deubel, V., Kinney, R.M., and Trent, D.W. 1988. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology **165**: 234–244.
5) Evans, D.M.A., Dunn, G., Minor, P.D., Schild, G.C., Cann, A.J., Stanway, G., Almond, J.W., Currey, K., and Maizel, J.V., Jr. 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature **314**: 548–550.
6) Fong, M.Y., Koh, C.L. Samuel, S., Pang, T., and Lam, S.K. 1990. Nucleotide sequences of the nonstructural protein NS1 gene of three dengue-2 viruses, M1, M2, and M3, isolated in Malaysia from patients with dengue haemorrhagic fever, dengue shock syndrome and dengue fever, respectively. Nucleic Acids Res. **18**: 1642.
7) Fu, L., Tan, B.H., Yap, E.H., Chan, Y.C., and Tan, Y.H. 1992. Full length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). Virology **188**: 953–958.
8) Gubler, D.J., and Trent, D.W. 1994. Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. Infect. Agent. Dis. **2**: 383–393.
9) Gubler, D.J., and Clark, G.G. 1995. Dengue/dengue haemorrhagic fever, the emergence of a global health problem. Emerg. Infect. Dis. **1**: 55–57.
10) Hack, C.E., Degroot, E.R., Feltbersma, R.J.F., Nuijens, J.H., Vanschijndel, R.J.M.S., Eerenberberbeimer, A.J.M., Thijs, L.G., and Aarden, L.A. 1989. Increased plasma levels of interleukin-6 in sepsis. Blood **74**: 1704–1710.
11) Hahn, Y.S., Galler, R., Hunkapillor, T., Dalrymple, J.M., Strauss, J.H., and Strauss, E.G. 1988. Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology **162**: 167–180.
12) Halstead, S.B. 1980. Dengue haemorrhagic fever. A public health problem and a field for research. Bull. WHO **58**: 1–21.
13) Halstead, S.B. 1988. Pathogenesis of dengue: challenges to molecular biology. Science **288**: 476–481.
14) Hasegawa, H., Yoshida, M., Shiosaka, T., Fujita, S., and Kobayashi, Y. 1992. Mutations in the envelope protein of Japanese encephalitis virus affect entry into cultured cells and virulence in mice. Virology **191**: 158–165.
15) Hober, D., Poli, L., Roblin, B., Gestes, P., Chungue, E., Granic, G., Imbert, P., Pecarere, J.L., Pascal, R.V., Wattre, P., and Montreull, M.M. 1993. Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in dengue infected patients. Am. J. Trop. Med. Hyg. **48**: 324–331.
16) Holzman, H., Heinz, F.X., Mandl, C.W., Guirakhoo, F., and Kunz, C. 1990. A single amino acid substitution in envelope protein E of tick borne encephalitis virus leads to attenuation in the mouse model. J. Virol. **64**: 5156–5159.
17) Iizuka, N., Kohara, M., Hagino-Yamagishi, Abe, S., Komatsu, T., Tago, K., Arita, M., and Nomoto, A. 1989. Construction of less neurovirulent polioviruses by introducing deletions into the 5′ noncoding sequence of the genome. J. Virol. **63**: 5354–5363.
18) Irie, K., Mohan, P.M., Sasaguri, Y., Putnak, R., and Padmanabhan, R. 1989. Sequence analysis of cloned dengue virus type 2 genome (New Guinea-C strain). Gene **75**: 197–211.

19) Jennings, A.D., Gibson, C.A., Miller, B.R., Mathes, J.H., Mitchell, C.J., Roehrig, J.T., Wood, D.J., Taffs, F., Sil, B.K., Whitby, S.N., Whitby, J.E., Monath, T.P., Minor, P.D., Sanders, P.G., and Barrett, A.D.T. 1994. Analysis of a yellow fever virus isolated from a fatal case of vaccine associated human encephalitis. J. Infect. Dis. **169**: 512–518.
20) Kawano, H., Rostapshov, V., Rosen, L., and Lai, C.J. 1993. Genetic determinants of dengue type 4 virus neurovirulence for mice. J. Virol. **67**: 6567–6575.
21) Kinney, R.M., Butrapet, S., Chang, G.J.J., Tsuchiya, K.R., Roehrig, J.T., Bhamarapravati, N., and Gubler, D.J. 1997. Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. Virology **230**: 300–308.
22) Knudsen, A.B. 1997. Global strategy for the prevention and control of dengue and dengue haemorrhagic fever, p. 129–140. *In* Saluzzo, J.F., and Doded, B. (eds), Emerging diseases: factors in the emergence of arbovirus diseases, Elsevier, Paris.
23) Kurane, I., and Ennis, F.A. 1997. Immunopathogenesis of dengue virus infections, p. 273–290. *In* Gubler, D.J., and Kuno, G. (eds), Dengue and dengue haemorrhagic fever. CAB International, London.
24) Leitmeyer, K.C., Vaughn, D.W., Watts, D.M., Salas, R., Chacon, I.V., Ramos, S., and Rico-Hesse, R. 1999. Dengue virus structural differences that correlate with pathogenesis. J. Virol. **73**: 4738–4747.
25) Malasit, P. 1987. Complement and dengue haemorrhagic fever/shock syndrome. Southeast Asian J. Trop. Med. Public Health **18**: 316–320.
26) Mangada, M.N.M., and Igarashi, A. 1997. Sequences of terminal non-coding regions from four dengue-2 viruses isolated from patients exhibiting different disease severities. Virus Genes **14**: 5–12.
27) Mangada, M.N.M., and Igarashi, A. 1998. Molecular and *in vitro* analysis of eight dengue type 2 viruses isolated from patients exhibiting different disease severities. Virology **244**: 458–466.
28) Men, R., Bray, M., Clark, D., Chanock, R.M., and Lai, C.J. 1996. Dengue type 4 virus mutant containing deletions in the 3′ noncoding region of the RNA genome: analysis of the growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. J. Virol. **70**: 3930–3937.
29) Monath, T.P. 1994. Yellow fever and dengue—the interactions of virus, vector and host in the reemergence of epidemic disease. Semin. Virol. **5**: 133–145.
30) Monath, T.P., and Heinz, F.X. 1996. Flaviviruses, p. 961–1034. *In* Fields, B.N., Knipe, D.M., Howeley, P.M., et al (eds), Fields virology, 3rd ed, Lippincott-Raven, Philadelphia.
31) Ni, H., and Barrett, A.D.T. 1996. Molecular differences between wild-type Japanese encephalitis virus strains of high and low mouse neuroinvasiveness. J. Gen. Virol. **77**: 1449–1455.
32) Okuno, Y., Fukunaga, T., Tadano, M., Okamoto, Y., Ohnishi, T., and Tagaki, M. 1985. Rapid focus reduction neutralization test of Japanese encephalitis virus in microtiter system. Arch. Virol. **86**: 129–135.
33) Osatomi, K., and Sumiyoshi, H. 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176**: 643–647.
34) Pletnev, A.G., Bray, M., and Lai, C.J. 1993. Chimeric tick-borne encephalitis and dengue type 4 viruses: effects of mutations on neurovirulence in mice. J. Virol. **67**: 4956–4963.
35) Rice, C.M. 1996. Flaviviruses: the viruses and their replication, p. 931–959. *In* Fields, B.N., Knipe, D.M., Howley, P.M., et al (eds), Fields virology, 3rd ed, Lippincott-Raven Publishers, Philadelphia.
36) Rico-Hesse, R. 1990. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology **17**: 479–493.
37) Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramous, C., Boshell, J., de Mesa, M.T.R., Nogueria, R.M.R., and da Rosa, A.T. 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology **230**: 244–251.
38) Rico-Hesse, R., Harrison, L.M., Nisalak, A., Vaughn, W.D., Kalayanarooj, S., Green, S., Rothman, A.L., and Ennis, A.F. 1998. Molecular evolution of dengue type 2 virus in Thailand. Am. J. Trop. Med. Hyg. **58**: 96–101.
39) Rosen, L. 1977. The emperor's new clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever: Presidential address. Am. J. Trop. Med. Hyg. **26**: 337–343.
40) Russell, P.K. 1971. Immunopathological mechanisms in the dengue shock syndrome, p. 831–838. *In* Amos, D.B. (ed), Progress in immunology, Academic Press, New York.
41) Sumiyoshi, H., Tignor, G.H., and Shope, R.E. 1995. Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA. J. Infect. Dis. **171**: 1144–1151.
42) Thant, K.Z., Morita, K., and Igarashi, A. 1996. Detection of the disease severity-related molecular differences among the new Thai dengue-2 isolates in 1993, based on their structural proteins and major nonstructural protein NS1 sequences. Microbiol. Immunol. **40**: 581–590.
43) World Health Organization. 1986. Dengue haemorrhagic fever: diagnosis, treatment and control. WHO, Geneva.
44) Zhao, B., Machow, E., Buckler-White, A., Markoff, L., Chanock, R.M., Lai, C.J., and Makino, Y. 1986. Cloning full length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology **155**: 77–88.