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Molecular epidemiology of enterovirus 71 in Taiwan

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Summary. Taiwan suffered a severe and widespread outbreak of enterovirus infection in 1998. More than 400 children were hospitalized, with seventy-eight fatalities due to central nerve system (CNS) involvement and cardiopulmonary collapse. Enterovirus 71 (EV71) was incriminated as the causative agent for the fatal cases. To understand the viral molecular epidemiology in this outbreak, fragments of 207-bp length of the VP4 region in 23 Taiwanese EV 71 isolates were sequenced. Pair-wise comparison revealed a 17.5–24.4% difference between the isolates and the prototype BrCr. However, all the changes in the VP4 region of the isolated strains were synonymous substitutions. Phylogenetic analysis was performed on these 23 isolates and 21 others deposited in GenBank. In this study, forty-four EV71 isolates from the world were separated into three distinct genotypes: A, B and C. The EV71 prototype strain, BrCr/70, is the only strain of genotype A. Group B included strains from the United States, Japan and Taiwan. Most strains in genotype B were isolated prior to 1990. Group C consisted of strains from Japan and Taiwan. Most strains of genotype C were isolated after 1990, they were further divided into 3 clusters: i.e. C-1, C-2 and C-3. In Taiwan, two genotypes, B and C-3, were co-circulating during the outbreak in 1998, although a minor group of genotype B may have appeared in Taiwan before 1986. The majority of the isolates clustered in genotype

C-3. Genotype C showed a higher evolutionary rate than genotype B (3.9×10^{-3} vs. 1.4×10^{-3}) in the VP4 region. There seems to be a worldwide trend with strains of genotype B appearing earlier than strains of genotype C which took over later in the dominance.

Introduction

Since the first record of EV71 infection occurred in California in 1969 [1], worldwide reports of outbreaks have followed. In the earlier outbreaks during 1969–1974, serious CNS complication was uncommon [2, 3]. However, subsequent reports revealed that the disease spectrum ranged from febrile disease, hand-foot-and-mouth disease (HFMD), herpangina, aseptic meningitis, poliomyelitis-like paralysis, encephalitis and even death [4]. Although mild diseases were the predominant clinical features of EV71 infection, neurologic involvement was the most serious complication, and would accounted for the remarkable fatality rate [5, 6]. Rapid clinical deterioration and death occurred in four outbreaks: 44 fatal cases in Bulgaria in 1975 [7], 47 fatal cases in Hungary in 1978 [8], at least 34 fatal cases in Malaysia in 1997 [9, 10], and 78 fatal cases in Taiwan in 1998 [11, 12]. Therefore, several papers have warned that enterovirus outbreaks caused by EV71 should not be taken lightly [9–12].

There were two epidemics of minor EV71 infection in Taiwan in 1980 and in 1986. Although both caused at least three cases of poliomyelitis-like flaccid paralysis, no children died during these two epidemics [12, 13]. We isolated and serum-confirmed 14 strains of EV71 from 30 cases in the 1986 HFMD outbreak. In 1998, the virus reappeared. At this time, brain stem involvement and pulmonary edema or hemorrhage were the signatures for the fatal cases [11, 12, 14, 15]. To understand the molecular diversity of this outbreak and investigate the molecular epidemiological linkage, we analyzed the nucleotide sequence of the most conserved VP4 region among EV71 isolates from both non-fatal and fatal cases. The property of conservation of the amino acid sequence of the VP4 region suggests that its functions are fundamentally important [16–18]. In addition, most of the data of the East Asian strains deposited in the GenBank were from the VP4 region. Hence, analysis of the 207 bp of nucleotide sequence in VP4 would offer more epidemiological information. In this study, isolates from both the 1986 and 1998 outbreaks in Taiwan and previous isolates worldwide were compared. Furthermore, a phylogenetic analysis was performed and the epidemiological trend was analyzed.

Materials and methods

Virus isolation and viral stock

Viruses were isolated from throat swabs, stool, and cerebrospinal fluid specimens taken from patients. The specimens were inoculated into Vero, RD, or HEL cells. The culture cells, which exhibited an EV-like cytopathic effect, were identified by immunofluorescent assay (IFA) with monoclonal antibody (catalog numbers 3323 and 3324, Chemicon, International Inc., CA). In addition, serum typing was confirmed by microneutralization assay (NT) [19] using in house polyclonal rabbit antiserum against the EV71 prototype BrCr/70 (American

Type Culture Collection, Rockville, MD) and 236/86 strains (an isolate from the 1986 EV71 Taiwanese outbreak) [13]. Virus stocks were prepared in Vero cells with Eagle's minimal essential medium containing 2% fetal bovine serum, and were harvested when the cytopathic effect was almost 100%. Virus fluid was centrifuged at 14000 **g** for 10 min, then after three freeze-thaw cycles, the supernatant was aliquoted and stored at −70 °C.

Viral RNA extraction, RT-PCR, and sequencing

The VP4 region of a total of 23 strains of clinical EV71 isolates from Taiwan was sequenced (Table 1). These strains consisted of three strains from the 1986 outbreak and 20 strains from the 1998 outbreak. The 20 strains included nine isolated from nonfatal cases and eleven from fatal cases. The eleven isolates taken from fatal cases consisted of five isolates from Taipei (two of these were different specimens from the same patient), five from Tainan and one from Kaohsiung. Viral RNA was extracted according to the manufacturer's instructions (QIAamp viral RNA purification kit, Qiagen Co.). The primers OL68-1, EVP2, and EVP4 were used for the amplification of the VP4 region. Reverse transcription (RT) and nested polymerase chain reaction (PCR) were performed as previously described by Kitamura [13].

Table 1. Isolates used in molecular analysis of EV71

Strains/ Year of isolation	Origin	Outcome	GenBank accession no.		
			VP1	VP4	Full length
236/86	Kaohsiung/TW	Paralysis		E11643	
240/86	Kaohsiung/TW	HFMD		AB037267	
244/86	Kaohsiung/TW	HFMD		AB037268	
5033/98	Tainan/TW	Fatality		AB037250	
5142/98	Tainan/TW	Fatality		AB037251	
H0106/98	Tainan/TW	Fatality		AB037252	
5929/98	Tainan/TW	Fatality		Pending	
7008/98	Tainan/TW	Fatality		Pending	
1657/98	Taipei/TW	Fatality	AF116812[a]	AB037253	
1658/98	Taipei/TW	Fatality	AF177911[a]	AB037254	
1569/98	Taipei/TW	Fatality	AF116811[a]	AB037255	
1499/98	Taipei/TW	Fatality	AF116808[a]	AB037256	
2272/98	Taipei/TW	Fatality			AF119795[a]
480/98	Kaohsiung/TW	HFMD		AB037257	
874/98	Kaohsiung/TW	Herpangina		AB037258	
737/98	Kaohsiung/TW	Meningitis		AB037259	
607/98	Kaohsiung/TW	Meningitis		AB037260	
602/98	Kaohsiung/TW	HFMD		AB037261	
588/98	Kaohsiung/TW	HFMD		AB037261	
1226/98	Kaohsiung/TW	Fatality		AB037263	
1288/98	Kaohsiung/TW	Meningitis		AB037264	
1386/98	Kaohsiung/TW	HFMD		AB037266	
693/98	Kaohsiung/TW	Herpangina		AB037266	
5929/98	Tainan/TW	Fatality		AB046379	
7008/98	Tainan/TW	Fatality		AB046740	

[a] Strains sequenced by Chang Gung University

Briefly, 5 μl of the RNA mixed with 1 μl of 50 μM of each primer (EVP-2, 5′-CCT CCG GCC CCT GAA TGC GGC TAA-3′ and OL68-1, 5′GGT AAQ TTC CAC CAC CAY AA-3′, Y is either C or T) was heated for 5 min at 95 °C and immediately put on ice. Then 43 μl of reactive cocktail containing 5 μl of 10X DNA polymerase reaction buffer, and 1 μl of each of the following was added, 40 U of RNase inhibitor (Promega), 200 U MMLV (BRL), 2.5 U of cloned PfuTurbo DNA polymerase (Stratagene) and 15 mM of dNTP (Amershan). The one-step RT-PCR program consisted of incubation for 1 h at 37 °C, for 5 min at 94 °C, 40 cycles of incubation for 1 min at 95 °C, 30 sec at 55 °C, 1 min at 72 °C, and followed by 5 min at 72 °C. The second PCR was performed at a final volume of 50 μl including 2.5 U of cloned PfuTurbo DNA polymerase, 1 μl of 15 mM dNTP, 4.5 μl 10X Pfu reaction buffer, and 1 μl of 50 μM of each primer (EVP-4, 5′-CTA CTT TGG GTG TCC GTG TT-3′ and OL68-1) in the same PCR program. A fragment of approximately 650 base pairs spanning the 5′-noncoding region to one third of VP2 including the entire region of VP4 was amplified. Cycle sequencing was performed using the purified PCR products with the Prism Ready Reaction Dideoxy Terminator cycle sequencing kit (Perkin-Elmer Corporation-Applied Biosystems, Forster City, CA, USA) and the 207 bp nucleotide sequence was determined with an automated sequencer (ABI Model 373A).

Phylogenetic analysis

Nucleotide sequences of the VP4 region of these 23 EV71 strains were analyzed together with the 21 strains in GenBank, including the prototype strain BrCr/70 [20] and those strains isolated in the United States [20], Japan [13] and Taiwan [21]. The coxsackievirus A16 (CA16) prototype strain, G-10 [22], was included as an out-group in the phylogenetic analysis. The nucleotide sequences were aligned using the Genetics Computer Group Sequence Analysis Package, Madison, Wisconsin (GCG). Distances were measured by Kimura Two-Parameter method. A phylogenetic tree was constructed by the modified neighbor-joining (NJ) method [23, 24]. One thousand times bootstrap [25, 26] was used for confirmation of statistical significance of phylogenetic analysis by Mega [27] and Phylip [25] software. The dendrogram was displayed using TreeView [29]. To estimate the evolutionary rate, pairwise comparison distances matrix was calculated according to the Kimura two-parameter method by the Distances program (GCG). The oldest strain in each set as a reference, two separate analyses were performed. The evolutionary rate was calculated by linear regression of the genetic distance from the oldest isolate versus year of isolation.

Accession numbers of the nucleotide sequences

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: 5033/98, AB037250; 5142/98, AB037251; H0106/98, AB037252; 1657/98, AB037253; 1658/98, AB037254; 1569/98, AB037255; 1499/98, AB037256; 480/98, AB037257; 874/98, AB037258; 737/98, AB037259; 609/98, AB037260; 602/98, AB037261; 588/98, AB037261; 1226/98, AB037263; 1288/98, AB037264; 1386/98, AB037266; 693/98, AB037266; 240/86, AB037267; 244/86, AB037268; 5929/98, AB046739; 7008/98, AB046740.

Results

Nucleotide sequences alignment

Nucleotide sequences of the VP4 region among the 23 strains of EV71 from Taiwan and 21, including bother Taiwanese strains of EV71 deposited in GenBank were aligned using the pileup program of GCG package (Fig. 1). Compare of the

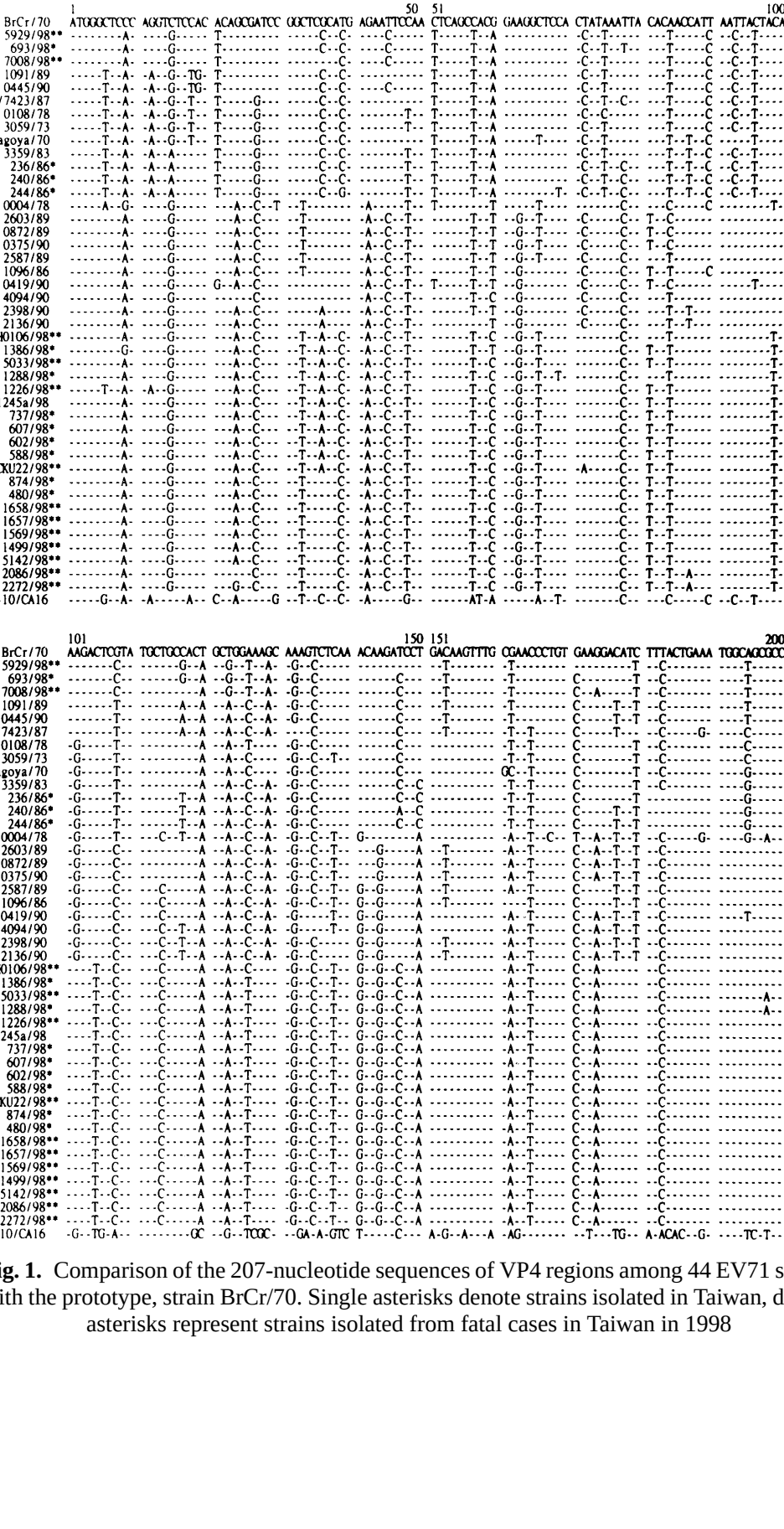

Fig. 1. Comparison of the 207-nucleotide sequences of VP4 regions among 44 EV71 strains with the prototype, strain BrCr/70. Single asterisks denote strains isolated in Taiwan, double asterisks represent strains isolated from fatal cases in Taiwan in 1998

207 bp nucleotide sequence of VP4 region among forty-three worldwide EV71 strains with BrCr/70, a total of sixty-five sites had been changed. And 94.2% of all substitutions occurred in the third codon position (data not shown). Fifty-five nucleotide substitutions were found between all of the 1998 Taiwanese isolates and BrCr/70. No discernible variations were found between fatal and non-fatal cases. Eleven of the 55 substitutions were found in strains from all the strains from Taiwanese 1998 outbreak. Moreover, twenty-one plus those eleven of the 55 substitutions (23 transitions and 9 transversions) were specific to the isolates from Taiwan in 1998, except for strain 693/98, 5929/98, and 7008/98 (Fig. 1). Two strains isolated from the 1986 outbreak showed single amino acid substitution; strain 240/86 showed D→E in residue 49 (nucleotide position 147), strain 244/86 showed H→Q in residue 13 (nucleotide position 39).

Pairwise comparisons

Pairwise comparison among the forty-four EV71 strains in the VP4 region showed nucleotide differences from 17.5% to 24.4% between EV71 isolates worldwide and the prototype BrCr/70 (data not shown). Interestingly, though all 1998 Taiwanese isolates exhibited a significant divergence from BrCr/70 with nucleotide differences of 17.5 to 23.1%, all of them were synonymous mutations. All of the isolates from the 1998 outbreak showed an identical amino acid sequence with prototype BrCr/70. Furthermore, two bunches of identical nucleotide sequences in the VP4 region were shown in the 20 Taiwanese strains isolated in 1998. One, composed of eight strains, was isolated from Taipei, Tainan and Kaohsiung; six of them were isolated from fatal cases. The remainder consisted of five strains, which were isolated from mild cases in Taipei and Kaohsiung; except for 607/98, which was isolated from severe case (Figs. 1, 2).

The variation was less than 3% among most strains of the 1998 outbreak. Except for strains 693/98, 5929/98 and 7008/98, the variation of the latter were less than 1% with each other but with a variation of 20.4–23.4% with the former group. Even with a difference of 9.5 to 12.4%, the three strains were still closer to the strains from Taiwan collected in 1986. Three strains isolated from Taiwan in 1986 showed greater nucleotide differences (22.3–24.1%) with BrCr/70 than with 1998 isolates (17.5–23.1%). These strains from 1986 showed 2.0–9.5% nucleotide differences from Japanese isolates from 1970 to 1989. However, they were distinctly different from Japanese isolates from 1989 to 1990 (15.9% to 24.4%).

Phylogenetic analysis

After conducting phylogenetic analysis of the 44 EV71 strains worldwide, the genetic relationships were inferred by Neighbor-joining method, and three genotypes A, B and C, were discerned in the tree (Fig. 2). Genotype C was further divided into three clusters C-1, C-2 and C-3. To check the reliability of the branches defined by the tree, one thousand replicates of bootstrap were performed by Phylip and Mega packages. The bootstrap value was 97% in genotype B, 90%

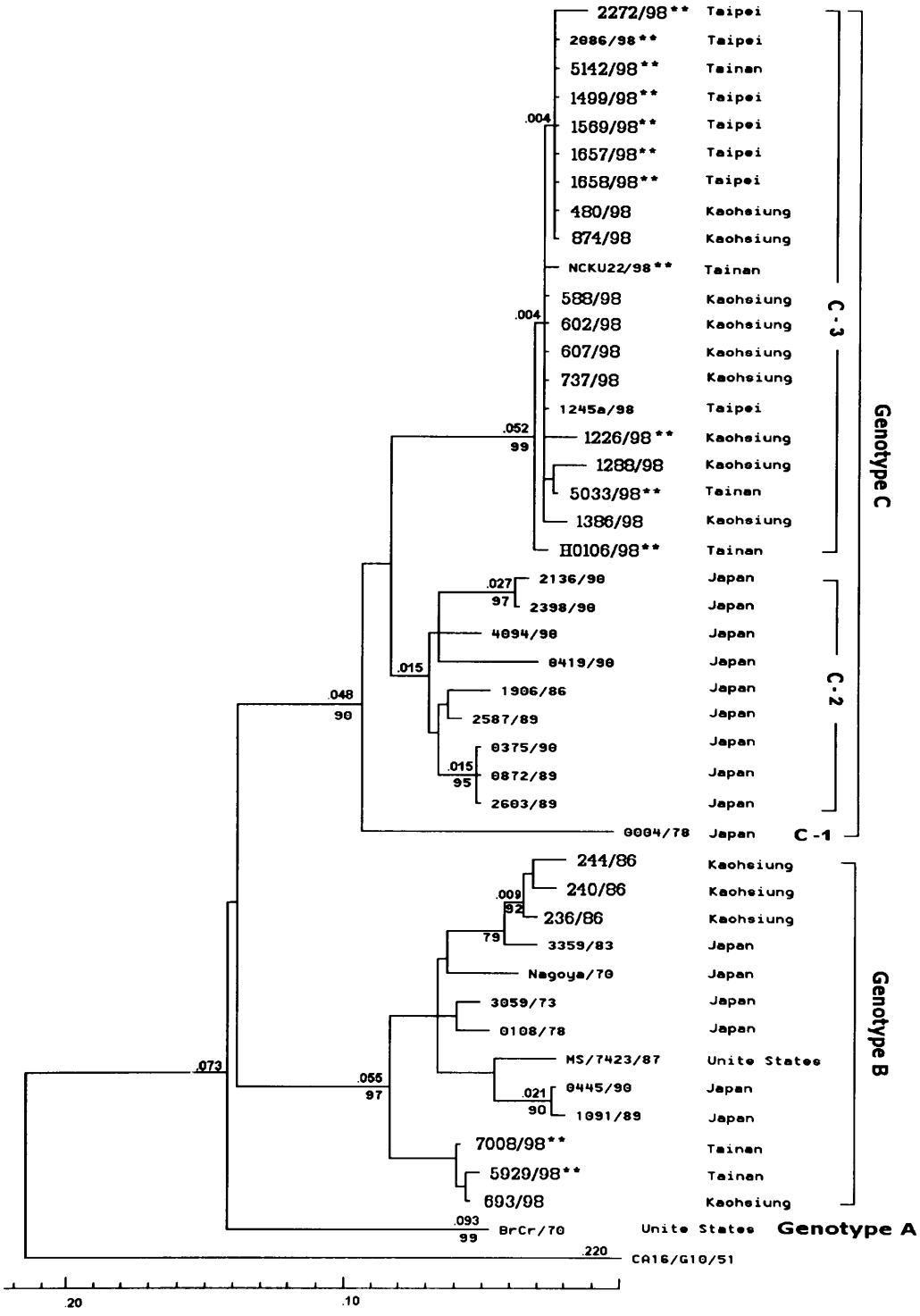

Fig. 2. Phylogenetic analysis of the genetic relationships of 44 EV71 strains worldwide and the strain G-10, CA16 prototype, based on the 207 bp of VP4 region. The upper numbers show the branch length denoted as percentage of difference and the lower numbers indicate the bootstrap value. Strains isolated from this study are highlighted. Strains isolated from fatal cases are marked by double asterisks

in genotype C and 99% in cluster C-3. Prototype BrCr/70 was the only strain in genotype A. Genotype B included strains isolated from Taiwan, Japan and the United States. Most of the strains (10/13) were isolated during the 1970–1990 of period. The exception to this was three strains isolated from the Taiwanese outbreak of 1998: i.e., strains 693/98, 5929/98 and 7008/98. The nucleotide differences among strains in genotype B were 0.5–12.4%. Genotype C consisted of strains isolated from Taiwan and Japan, and was further divided into three clusters. Strain 0004/78 was the only member in cluster C-1 and was the earliest strain reported in genotype C. Cluster C-2 contained isolates from Japan during 1986–1990. All strains of cluster C-3 were composed by strains that were isolated from the Taiwanese outbreak in 1998. Strains isolated from the fatal cases were distributed in both genotypes B and C-3.

All of the isolates from the 1998 outbreak showed identical amino acid sequences with prototype BrCr/70, no matter if they belonged to genotype B or C. Twenty-eight out of thirty strains in genotype C were isolated from Taiwan and Japan during 1989–1998. There was a 0–17.7% nucleotide difference among members within genotype C, while a 19.6–26.1% nucleotide difference was found between genotype C and BrCr/70. The difference between genotypes B and C was 15.9–26.6%. Intriguingly, in genotype B, Taiwanese strains 693/98, 5929/98 and 7008/98 were closely related to strains taken from Japan and the United States from 1987–1990, and strains from the 1986 Taiwanese outbreak were also clustered in this genotype. The nucleotide substitution rates for B and C genotypes were 1.4×10^{-3} and 3.9×10^{-3} per nucleotide substitution per year, respectively.

Discussion

HFMD was the most frequently reported clinical symptom associated with EV71, and these infections usually did not result in life-threatening manifestations [6, 19, 20, 30, 31]. However, in the 1975 Bulgarian and 1978 Hungary outbreaks, the fatal cases showed quick onset of CNS involvement. EV71 directly invaded the spinal cord, causing polio-like paralysis and significant mortality. Minimal cardiopulmonary features were observed in fatal cases. On the other hand, the fatal cases in both 1998 Taiwan and 1997 Malaysia presented with CNS involvement and led to sudden cardiopulmonary collapse [11–14, 32, 33]. It has been reported that EV71 infections have at least two clinical presentations. One of these is a polio-like disease or encephalitis [1–3]. The other presentation is the more prevalent HFMD [6, 30]. It has been postulated that these different clinical patterns could be due to differences in the pathogenicity of viral strains [6, 7, 33]. However, no significant nucleotides difference has been found between the fatal and nonfatal cases, regardless of whether those findings are based on the phenylogenetic analysis of VP4 [34], VP1 [35], or 5′-NCR [10]. Hence, virulence might not be determined by a single viral gene, further analysis of more gene regions or even complete genomes of more viral isolates is necessary. It was found that the outbreak in HFMD epidemic areas, both in Asian and the United States, were always combined with the outbreak of other dermatotropic enteroviruses such as

CA16 [6, 32, 33, 36]. In Taiwan, both EV71 (60.0%) and CA16 (28.9%) were the major causative agents in 1998 [12]. Moreover, the EV71-related cases had a tendency of CNS involvement and accounted for the severe cases requiring hospitalization [12].

Two genotypes of EV71 were found in this outbreak in Taiwan by random sampling, a major genotype (C-3) and a minor genotype (B). Strains from fatal cases were found in both genotypes. Strains 693/98, 5929/98 and 7008/98 were located in genotype B together with isolates taken in 1986 in Taiwan. It has been postulated that some of the EV71 strains in this epidemic might have existed and evolved since 1986. Because three genotypes were found after the analysis of the nucleotide sequences of VP4 region of 44 EV71 strains worldwide. Accordingly, we included BrCr, the prototype in our designation of the genotypes.

An identity of three genotypes was found in the comparison of the phylogenetic analysis based on both VP1 and VP4 nucleotide sequences among 10 strains of EV71 deposited in GenBank (Fig. 3). While compared our results with those reported previously [34, 35], three genotypes were also designated by the phylogenetic analysis. Strain BrCr/70 is the sole member of genotype A. Strains of genotype B were prevalent earlier in 1970s to 1980s and perhaps after a co-circulation of both B and C genotypes, strains of genotype C gained dominance in 1985–1990s [34, 35]. Interestingly, our data showed that genotype C had a higher

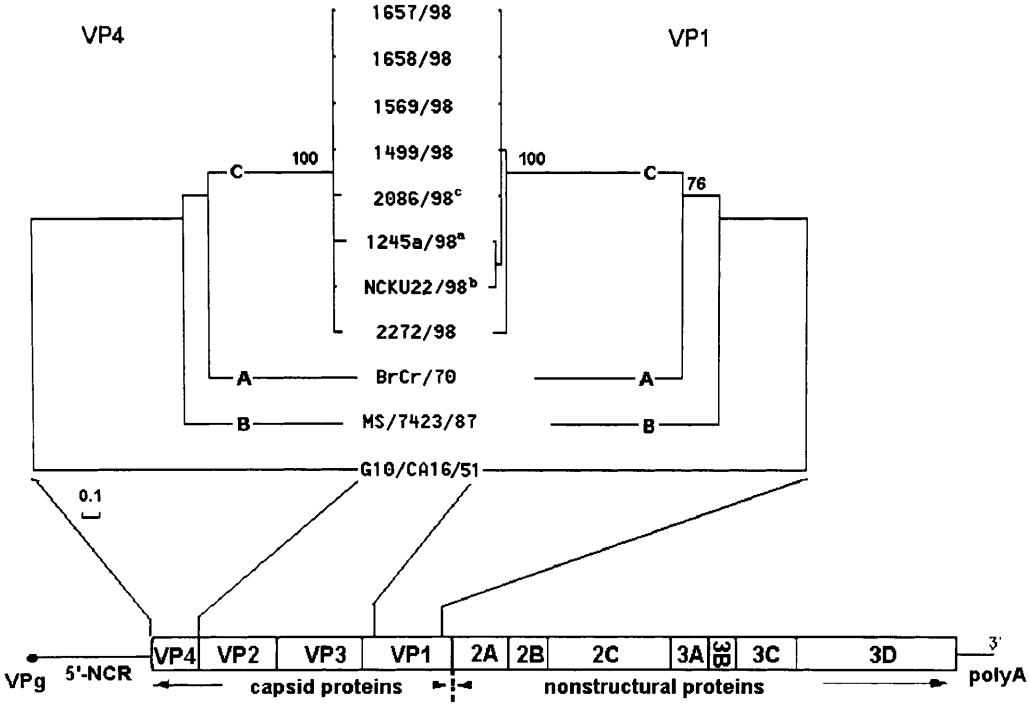

Fig. 3. Comparison of the phylogenetic analysis among isolates in VP1 and VP4 regions. [a.b.c.]: The accession numbers in GenBank are af176044, af136379, and af119796, respectively

evolutionary rate than that in genotype B (3.9×10^{-3} vs. 1.4×10^{-3}), based on analysis of the VP4 region. The bulk of EV71 strains isolated from fatal cases in both Taiwan and Malaysia were genetically and epidemiologically unrelated in the VP4 region. The nucleotide sequences of VP4 in strains in genotype C-3 bore a greater resemblance to strains in Japan in 1997 [34].

In summary, three conclusions can be drawn from our results. First, there are three genotypes of EV71. Though none of the genotypes was related to the pathogenicity and the severity of disease, it seems that genotype B was dominant before 1990 while genotype C became more prevalent after 1990. Second, there were two genotypes participated in the 1998 outbreak in Taiwan. A majority of the isolates were located in cluster C-3 and turned out to be a new variant, while a minor group of viral strains in genotype B might have evolved before 1986. Strains isolated from fatal cases were distributed in both genotypes B and C-3. Third, viruses in genotype C evolved quicker than that in genotype B.

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