Genetic characteristics of human enterovirus 71 isolates from the patients with encephalitis

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Abstract

Introduction: Enterovirus 71 (EV71) was first identifed in 1969 in California and became a common cause of hand, foot, and mouth disease (HFMD) in the world. Some of the children subsquent severe neurologic complications but all seen in partial adult. (Epidemics of enterovirus 71 had caused encephalomyelitis since 1998 that caused large stress on parents and clinical physicans in Taiwan.Clinical presentations in EV 71 CNS infection were HFM dz/herpangina associated myoclonic jerk, ataxia and sympathetic overactivation.Neurological images were characterized of brain stem and cerebellum inflammation.).The central nerve system complications caused by EV71 were also manifestated as brain stem dysfunction and autonomic dysregulation, such as acute flaccid paralysis, aseptic meningitis and rhombencephalitis; rhombencephalitis is one of the most common severe neurologic symptoms in children. The object of this study is trying to clarify the molecular and clinical epidemiology of enterovirus 71 in the middle of Taiwan, and find a disease marker to to predict the disease progression.

Method: Specimens of paients who were diagnosed of herpangina/HFM disease with CNS involvement were collected .Specimens were collected in viral transport medium from sites of throat swabs, stool and CSF. Phylogenetic analysis of the VP4 and VP1 genes of EV71 strains investigated what kind of genogroups of the virus

circulating in Taiwan.

Result: In 27 cases of this study, the results of confirmation PCR were segregated into 5 group from A to E. The patients in specimen group A presented the EV71 PCR positive both in throat tissue and stool; group B only presented PCR negative in CSF; group C presented PCR positive in all specimens, group D only present PCR positive in stool; and group E only presented PCR negative in blood specimen. The further investigate of the genetic relationship among 27 EV 71 isolates in this study by using the 5'-UTR and VP1 regions. Both neighbor-joining trees of 5'-UTR and VP1 regions shown these 27 isolates clustering with isolate SHZH03 in bootstrap number 99% (Fig 2, 3). There were five genogroups, group I to V were deduced in the phylogenetic tree with more than 60% bootstrap number. These 5 genogroup represent the 5 epidemic events among these patients.

Introduction

EV71 history in world and Taiwan

Enterovirus 71 (EV71) was first identifed in 1969 in California and became a common cause of hand- foot- mouth disease (HFMD)/herpangina in the world. Some of the children subsquent neurologic complications but all seen in partial adult (Hamaguchi et al., 2008b). General clinical symptoms of EV71 infections can be diverse, including HFMD, herpangina, pulmonary edema and central nervous system (CNS) complications. EV71 associated HFMD outbreaks with severe CNS complications cases have been increased during recent years (Huang et al., 1999a; Chan et al., 2003; Hamaguchi et al., 2008a). The clinical CNS complications caused by EV71 was also present to many signs, such as acute flaccid paralysis, aseptic meningitis and rhombencephalitis; rhombencephalitis is one of the most common severe neurologic symptoms in children(Huang et al., 1999b). In the most serious EV71-associated HFMD outbreak in Taiwan in 1998, 405 children had severe neurologic complications, pulmonary edema; 78 children died(Ho et al., 1999b).

【I suggest delet this section, because it means nothing.】 CNS was indicated in five types of symptoms. **Those patients with aseptic meningitis had headache and irritability along with cerebrospinal fluid pleocytosis and without an altered**

level of consciousness. The second type involved encephalitis with altered level of consciousness plus CSF pleocytosis. **Cerebellitis was defined as the presence of cerebellar ataxia and dis-coordination.** Poliomyelitislike syndrome was defined as acute limb weakness and decreased reflex and muscle strength**. Finally, patients with encephalomyelitis had the present of both encephalitis and poliomyelitis-like syndrome(Chang et al., 2007)**.

In 1998, the largest outbreak of EV71 infection reported in Taiwan had 34 fatalities (Ho et al., 1999a). Brainstem encephalitis is a major cause of deaths by developing cardiopulmonary (Wang et al., 2000). The previous study demonstrated that the EV71 isolated in Taiwan had both dermatotropic and neurotropic characteristics since 1998 (Liu et al., 2000). Because of the serious morbidity and mortality associated with EV71 infection, EV71 cases identification is an important consideration. Early detecting CNS and starting therapy may reduce the mortality. The previous study suggest that EV71 related encephalitis(EVE) severity varies by serotype, confirm the importance of CSF/brain tissue polymerase chain reaction, and demonstrate that serum IgM findings are of little value in diagnosing EVE. (Fowlkes et al., 2008)

The Enterovirus genus is belong to family Picornaviridae and it consists of five species which isolated from humans: Human enterovirus A (HEV-A), HEV-B, HEV-C, HEV-D and poliovirus. EV71 is one of the HEV-A. The genome of enteroviruses is a single-stranded positive-sense RNA with approximately 7400 nucleotides. The viral genome contains a 5'- and 3'-untranslated regions (UTRs) which are essential for viral RNA replication. The genome structure translated into a polyprotein which including four capsid proteins, VP1 to VP4, and seven nonstructural proteins, 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Palmenberg, 1990). Nucleotide sequencing and phylogenetic analysis have been used extensively as epidemiological and diagnostic tools for poliovirus, the prototype of the Enterovirus genus (Kew et al., 1995). Phylogenetic analysis of the VP4 and VP1 genes of present EV71 strains indicates that different genogroups of the virus have been circulating in the Asia-Pacific region since 1997. The first of these outbreaks, described in **Sarawak (Malaysian Borneo)** in 1997, was caused by genogroup B3. This outbreak was followed by large outbreaks in Taiwan in 1998, caused by genogroup C2, and in **Perth (Western Australia)** in 1999, where viruses belonging to genogroups B3 and C2 cocirculated. Singapore, Taiwan, and Sarawak had HEV71 epidemics in 2000, caused predominantly by viruses belonging to genogroup B4; however, large numbers of fatalities were observed only in Taiwan. HEV71 was identified during an epidemic

of hand, foot and mouth disease in Korea; that epidemic was found to be due to viruses constituting a new genogroup, C3(Cardosa et al., 2003). In pervious phylogenetic study, the viruses have been grouped into different genetic clusters, based upon analysis of sequences at the 5′UTR and VP1 (Huttunen et al., 1996; Huang et al., 2008; Pulli et al., 1995; Poyry et al., 1996; Zell and Stelzner, 1997).

The objectives of this study is trying to understand the molecular and clinical epidemiology of enterovirus 71 in the middle of Taiwan and find a disease marker to to predict the disease progression.

Materials and methods

Specimens collection and processing

We collected 27 specimens from 2005 in China Medical University Hospital .Specimens from throat swabs, stool were collected in viral transport medium. CSF specimen was directly collected in sterile tubes from inpatients or outpatients suspected of having enteroviral infection..

Extraction of EV71 RNA

EV71 was grown in Vero cells and the infected cells were scraped and pelleted by centrifugation when 75% cytopathic effect was seen. The viral RNA was extracted by QlAagen® Viral RNA mini kit (QIAGEN Inc,CA,USA). Purified viral RNA was resuspended in 60μl AVE buffer.The viral RNA was stored at -70℃.

Reverse transcription and PCR

The primer sequences EV-1F (sense): 5’-GGCCCACTGGGCGCTAGCA-3’ (nt 34–53); EV-1R (antisense): 5’-TGTCCCAATGACATACTCT-3’ (nt 1337–1356)) amplified a 1323-bp cDNA which included the 5’ NCR and VP4 of the enterovirus genome. The primer sequences

EV71-1 (sense): 5’-TGGCAGATGTGATTGAGAGTT-3’; EV71-2 (antisense): 5’-TGAACAGCTCCACCTTTC-3’；EV71-3 (sense): 5’-AGTGATGAGAGTATGATTGA-3’; EV71-4 (antisense): 5’-TTATGTCTATGTCCCAGTT-3’amplified VP1 of the enterovirus genome. The cDNA was synthesized and amplified by Omniscript Reverse transcription kit(QIAGEN Inc,CA,USA). Briefly, a 20-ml reaction mixture was which contained 1X buffer RT, 0.5 mM of each deoxynucleoside triphosphates, 20 pmol of EV1337R primer,10units of Rnase inhibitor,4units Omniscript reverse transcriptase, and 5 mg of purified RNA. After incubation at 37°C for 1hr. The PCR mixture contained 50 mM KCl, 10 mM Tris–HCl (pH 8.9), 3.6 mM MgCl2, 0.2 mM (each) deoxynucleoside triphosphates, 100 mg of bovine serum albumin per ml, 20 pmol of primers, and 1 U of Taq DNA polymerase was added after denaturing the cDNA at 94°C for 5 min. DNA amplification was performed with 40 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 56°C, extension for 2 min at 72°C, and then72℃ for 10 min. The PCR products were analyzed by electrophoresis in 2% agarose gels.

Sequencing of PCR products

PCR products were analyzed by a Sanger dideoxy cycle sequencing reaction protocol

using four EV71 specific primers: EV-1F (sense), EV-1R (antisense), EV-2F (sense): 5'-GTCCCAATGACATACTCT-3' (nt 583–602), and EV-2R (antisense): 5'-CTAGCTCAATAGACTCTTCGCA-3' (nt 418–440),EV71-1(sense), EV71-2(antisense), EV71-3(sense), EV71-4(antisense) according to the manufacture's instructions (PE Applied Biosystems). After completion of the cycle-sequencing reactions, products were electrophoresed in an automated DNA sequencer (model 373A; DNA Sequencer, Applied Biosystems).Each PCR product was sequenced in both directions to resolve possible ambiguous nucleotides.

Data analysis

We used ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, V3.1 (Applied Biosystem,CA,USA)for DNA sequence. Sequence data generated by the automated sequencer ABI 3730 XL DNA Analyzer(Applied Biosystem,CA,USA). The nucleotide sequence data were inspected and prepared with the Bioedit (Version 5.0)sequence analysis program. Multiplesequence alignments were performed with the ClustalW program.Phylogenetic analysis was performed using the neighbor-joining method with PHYLIP(version 3.6),and the reliability was evaluated by bootstrap analysis with 1,000 data sets. Cladograms were drawn with the TREEVIEW program.

Phylogenetic analyses of the 5'-UTR and VP4 regions

EV71 genome products were aligned sequences by using BioEdit program. Phylogenetic trees were constructed by the following steps in MEGA program, version 3. The Kimura two-parameter method was used to construct a distance matrix for neighbor-joining analysis. Maximum Composite Likelihood also used to confirm the topology of the phylogenetic trees. The statistical significance of phylogenies was estimated by bootstrap analysis with 1000 replicates.

Result and discussion

Clinical phase

Table 1

Specimen group	PCR positive site
A	T(+),S(+),B(-),C(-)
B	T(+),S(+),B(+),C(-)
C	T(+),S(+),B(+),C(+)
D	T(-),S(+),B(-),C(-)
E	T(+),S(+),B(-),C(+)

T: Throat S: Stool B:Blood C:CSF

In 27 cases of this study, the specimens collection from throat tissue, stool, blood and cerebrospinal fluid (CSF) were collected. The result of confirmation PCR was segregated into 5 group from A to E which shown in Table 1. The patients in specimen group A presented the EV71 PCR positive both in throat tissue and stool; group B only presented PCR negative in CSF; group C presented PCR positive in all specimens, group D only present PCR positive in stool; and group E only presented PCR negative in blood specimen.

Phylogenetic analyses of the 5'-UTR and VP1 regions

The EV71 viral subgenogroup identified by phylogenetic analysis for 40 EV71 strains worldwide was based on BrCr-ts strains sequence (gi60099451 nucleotide

position 34-1352). Bootstrap values(%) in 1,000 replicates were indicated at the branch nodes. This phylogenetic evidence proved the virus isolates in this study belong to subgenogroup C4 which became the predominant virus strain from 2004 (Lin et al., 2006) in Taiwan (Fig.1). The Taiwan C4 subgenogroup was clustered together with 100% bootstrap which different with the virus in China.

The further investigate of the genetic relationship among 27 EV 71 isolates in this study by using the 5'-UTR and VP1 regions. Both neighbor-joining trees of 5'-UTR and VP1 regions shown these 27 isolates clustering with isolate SHZH03 in bootstrap number 99% (Fig 2, 3). There were five genogroups, group I to V were deduced in the phylogenetic tree with more than 60% bootstrap number. In VP1 region, the isolates 0613-15 and 826 were clustered together with bootstrap number 91%. And, the isolates 0607-16 and V973 were clustered together in another genogroup with bootstrap number 87%. Furthermore, genogroup I to V were shown in phylogenetic tree of 5'-UTR region. The sequence variations among group were 0 in group I, IV and V. Group II and III were 0.002 and 0.004 respectively in VP1 region. These 5 genogroup represent the 5 epidemic events among these patients.

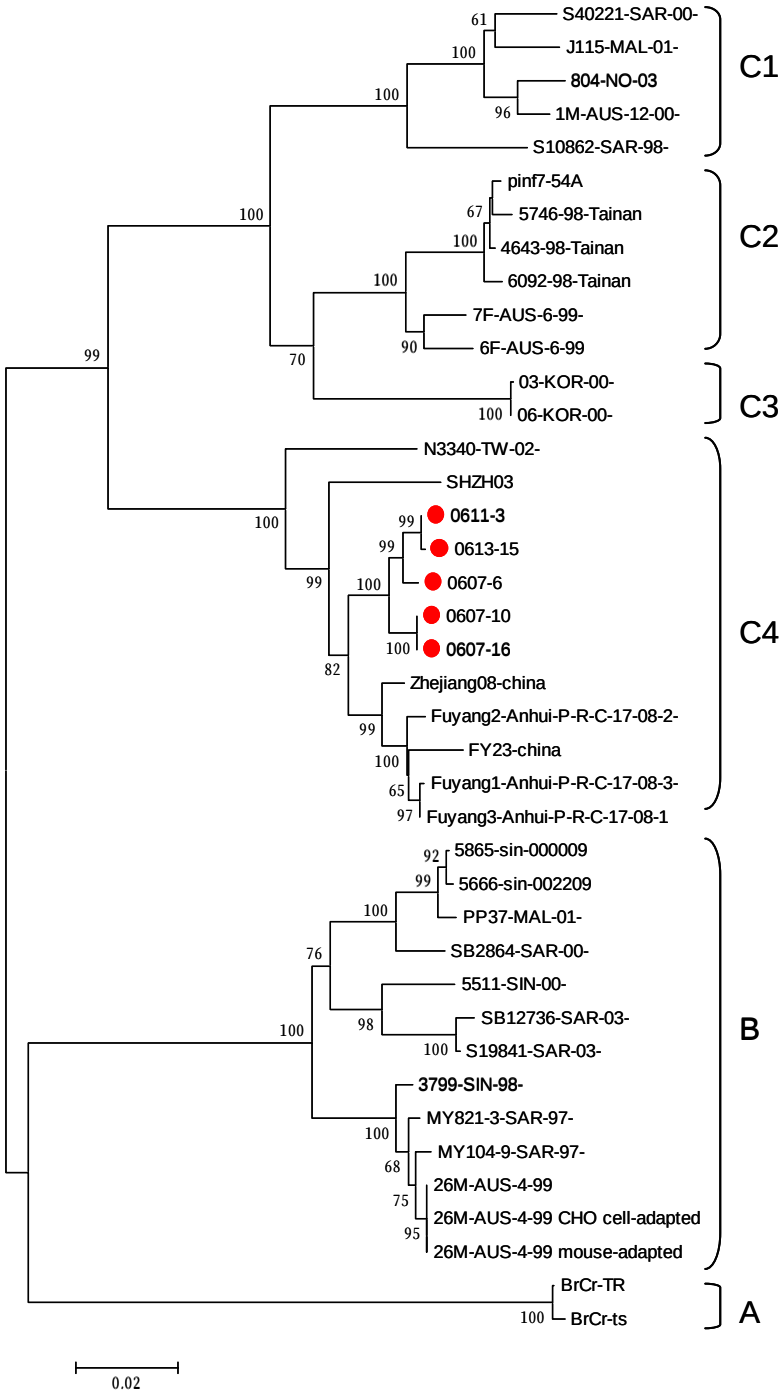


Fig 1 Phylogenetic analysis of 40 EV71 strains worldwide based on BrCr-ts strains sequence (gi60099451 nucleotide position 34-1352). The nucleotide sequence of the prototype BrCr strains were used as an outgroup. Bootstrap values(%) in 1,000 replicates were indicated at the branch nodes.

Fig 2　5’-UTR region

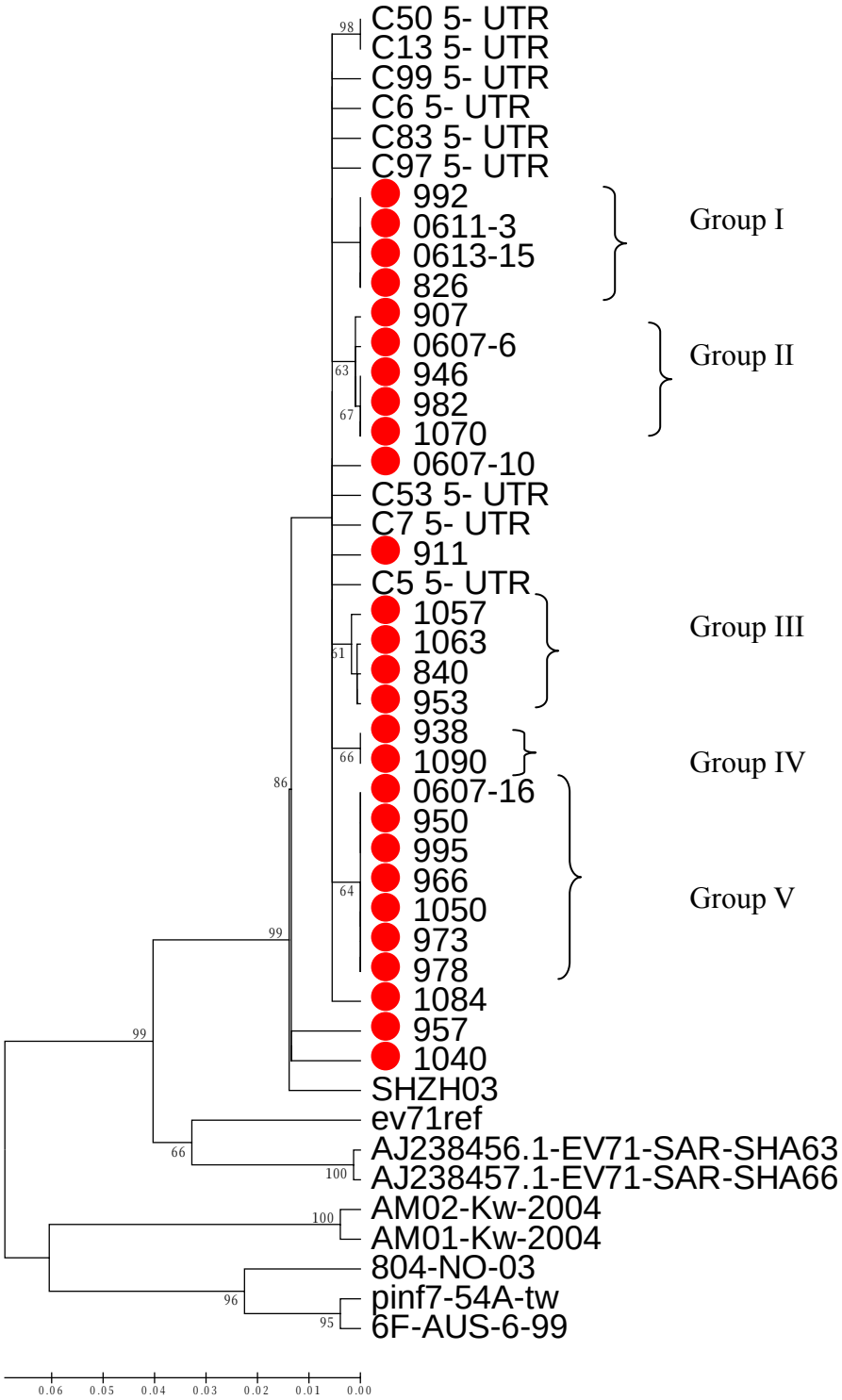
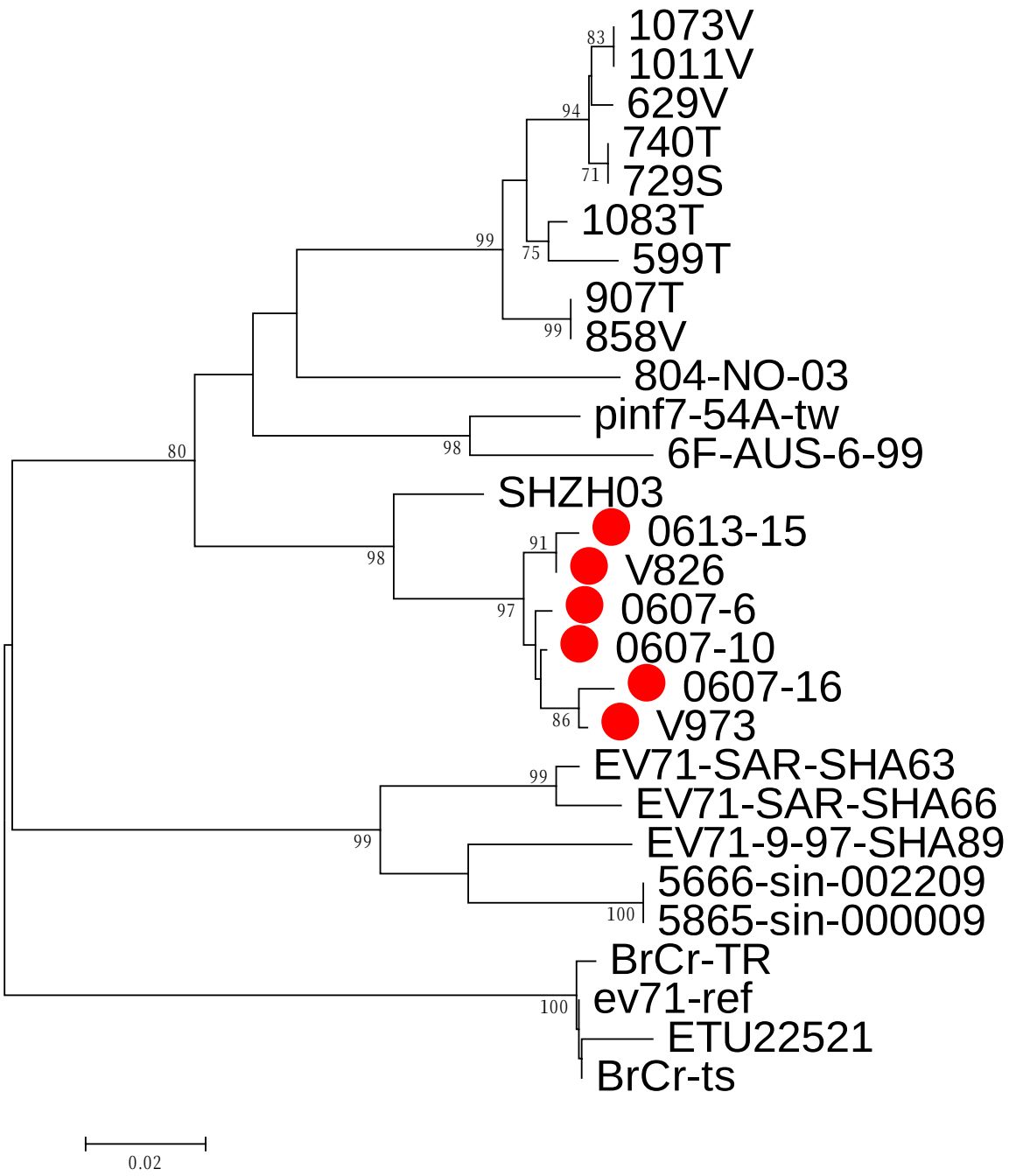


Fig 3　VP1 region



Phylogeny and clinical correlation

Correlations between the phylogenetic cluster (genetic group) of EV 71 virus isolates from patients and the stages of EV71 encephalomyelitis were shown in Table 1.

The clinical severity of the EV 71 neurological illness was classified according to the level of severity: group 1, complicated EV71 ilness with CNS involvement ; group 2, EV 71 encephalomyelitis with autonomic dysfunction; and group 3, EV71 EV 71 encephalomyelitis complicated with cardiopulmonary failure . Patients who were assigned to group 1 were defined as encephalomyelitis with evidence of cerebrospinal fluid (CSF) pleocytosis (>5×10^6 leukocytes per liter) withsubtle neurological signs,includede drowsiness,ataxia and myoclonic jerks. Patients who were assigned to group 2 defined as encephalomyelitis with autonomic dysfunction, included hypertension, cold sweating, hyperglycemia andfrequent myoclonic jerks. Patients who were assigned to group 3 had cardiopulmonary failure with hypotension, complicated pulmonary edema.These children all required the use of inotropic agents,endotracheal intubation, and ventilator support.

EV71 disease progression was split to clinical disease grade from 1-4. Furthermore, we separated the patients' specimens which EV71 viral PCR results positive site into 5 groups from A-E part (Table 2). Genetic group I and II got more serious disease progression in grade 3 and 4. Group III was located in grade 2 and 3.

Group IV and V both had patient in grade 2-4. In PCR specimen positive site classification, Group I and II spread from A to C. Group III and IV had patients located in the A, C and E. Besides A, the patients in genetic group V were including B to E. But, the correlation between the genetic group and two kinds of disease progression markers was no statistical significant.

Furthermore, specimen positive site group A, B and C was belong to Grade 2 and 3. Specimen D with 2 patients both belong to grade 4. And, the relationship between disease progression and PCR specimen was confirmed with the statistically significant (Table 3). This data present EV71 positive specimen group might used as a marker to predict the disease progression grade.

Table2 The correlation of phylogenetic cluster and EV71 illness progression.

		I		II		III		IV		V		P value
Group												0.533
	1	0	(0.0%)	0	(0.0%)	2	(%)	2	(28.6%)	3	(42.9%)	
	2	2	(50.0%)	3	(60.0%)	2	(%)	4	(57.1%)	2	(28.6%)	
	3	2	(50.0%)	2	(40.0%)	0	(%)	1	(14.3%)	2	(28.6%)	
PCR_specimen												0.899
	A	1	(25.0%)	2	(40.0%)	2	(50.0%)	2	(28.6%)	0	(0.0%)	
	B	1	(40.0%)	1	(20.0%)	0	(0.0%)	0	(0.0%)	2	(28.6%)	
	C	2	(50.0%)	2	(40.0%)	1	(25.0%)	4	(57.1%)	3	(42.9%)	
	D	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	1	(14.3%)	
	E	0	(0.0%)	0	(0.0%)	1	(25.0%)	1	(14.3%)	1	(14.3%)	

Table 3 The correlations between EV71 encephalomyelitis stages and PCR specimen.

		Group 1,2		Group3		P value
PCR_specimen						0.039
	A、B、C	10	(100.0%)	1	(33.3%)	
	D、E	0	(0.0%)	2	(66.7%)	

DISCUSSION

The results of EV71 viral subgenogroup was shown the majors strains in Taiwan was C4 which prevalent since 2004 (Lin et al., 2006) in Taiwan (Fig.1). The subgenogroup C4 of EV71 has been identified in Japan and emerged in the surrounding countries, such as China and Taiwan. The genotypic evidence in this study recognized the C4 the spreading and variation among this genotype during 3 years (Fig2). Five existed phylogenetic groups and other sporadic strains presented more than 5 strains circulating in Taiwan now.

In previous reports in clinical study, laboratory confirmation of enterovirus associated neurologic disease is typically perduced by PCR of CSF samples because of the better sensitivity and rapid turnaround time, compared with culture procedure[13, 21]. Several previous studies have demonstrated a rather low virus isolation in CNS cpecimens compared with that in other clinical samples, such as throat swab, and stool samples from EV71-associated cases with HFMD, encephalitis, or both(2,3,11**(www.cdc.gov/eid/content/14/5/8/828.htm))**

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