Minireview

Evolution of re-emergent virus and its impact on enterovirus 71 epidemics

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Abstract

Enterovirus 71 (EV71), a member of the *Enterovirus* genus in the *Picornaviridae* family, has become an emergent infectious disease worldwide, most notably in Asia. As a neurotropic virus, EV71 infection occasionally causes neurological diseases with pulmonary edema, which is fatal for children. In this review, we examine the epidemiology of EV71, with three waves of increased EV71 activity since the 1970s and discuss the genotypic changes in phylogeny between the outbreaks or epidemics. Genetic changes including mutations and recombinations as well as the diversity of antigenic properties among EV71 strains in various outbreaks are described. Furthermore, the impact of genetic changes on viral pathogenesis and vaccine candidate selection are addressed. In conclusion, these genetic and antigenic investigations of EV71 evolution have provided us with new insight into the trend of EV71 epidemiology, which may contribute to a better understanding of the viral pathogenesis and vaccine development.

Keywords: enterovirus 71, epidemiology, phylogenetic analysis, evolution, recombination, antigenicity

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Introduction

Human enterovirus 71 (EV71) belongs to the *Enterovirus* genus in the *Picornaviridae* family, which includes poliovirus, human enterovirus A, B, C and D (HEV-A–D), and more recently, human rhinovirus A, B and C that infect humans. Along with human coxsackievirus (A2-8, A10, A12, A14 and A16) and human enterovirus (76, 89, 90, 91 and 92), EV71 is classified as a human enterovirus A species based on its genome sequence.[1,2] EV71 and coxsackievirus A16 (CAV16) are major causative agents of hand, foot and mouth disease (HFMD); however, EV71 is occasionally associated with severe neurological sequelae, such as aseptic meningitis, brainstem encephalitis, poliovirus-like paralysis, shock and cardiac dysfunction, although the neurovirulent determinants of EV71 are not well clarified.[3] In 2009, two investigations reported two different cellular receptors for EV71. Yamayoshi *et al.*[4] identified scavenger receptor class B member 2 as a functional receptor of EV71, which supports EV71 propagation and infection. In another study, Nishimura *et al.*[5] have reported that EV71 binds to human P-selectin glycoprotein ligand-1 (PSGL-1) and infects PSGL-1 expressed cells. The results suggest that other molecules are not excluded from playing important roles in EV71 binding to cells and other cellular and viral factors may contribute to enterovirus infectivity and might cooperatively contribute to the neurotropism of EV71.[6,7]

EV71, like other members of the genus *Enterovirus*, is a non-enveloped, positive-sense, single-stranded RNA virus which consists of 60 copies each of four capsid proteins (VP1, VP2, VP3 and VP4) that form a symmetrical icosahedral structure. The capsid proteins VP1, VP2 and VP3 are exposed on the virus surface and the smallest VP4 is arranged inside the icosahedral lattice. Consequently, the external capsid proteins play the roles of not only receptor binding on the surface of susceptible cells but also contain the antigenic determinants. The viral genome is approximately 7500 nucleotides in length and is packaged within the viral capsid. Instead of a cap structure, a small protein VPg (3B protein) is covalently conjugated to the 5′ terminus of RNA. As soon as the RNA is released from the protection of the viral capsid into the cytoplasm of the host cells, the translation of virus proteins commence after binding of the translation initiation factors and

ribosomes to the internal ribosomal entry site of the 5′-untranslated region (5′-UTR). In addition to the 5′-UTR, the RNA genome contains a single open reading frame (ORF) and a 3′-untranslated region (3′-UTR) at the 3′ terminus. The translated product of the ORF is a large polyprotein that is co- or post-translationally cleaved by translated viral proteases into P1, P2 and P3 regions. The P1 region is the part of the polyprotein which generates the four structural proteins (VP1, VP2, VP3 and VP4). P2 and P3 regions contain the non-structural proteins, 2A, 2B, 2C, and 3A, 3B, 3C, 3D, respectively. In a poliovirus study, Nicklin *et al.*[8] found that the 2A protease can cleave the P1 capsid precursor from the nascent polyprotein, and 3CD protease can cleave the P1, P2 and P3 precursors into mature structural proteins for virus assembly and non-structural proteins for virus replication, apoptosis induction, innate immunity repression and shutting down host cell translation.

Two viral proteases, 2A protease ($2A^{pro}$) and 3C protease ($3C^{pro}$), are encoded by the non-structural protein coding region. In addition to participating in the dissociation of the P1 capsid polyprotein from the P2 and P3 non-structural polyproteins, $2A^{pro}$ is also involved in the cleavage of the eukaryotic initiation factor 4G during an EV71 infection,[9] which is important for host protein synthesis. $3C^{pro}$ is also involved in the proteolytic processing of the viral polyprotein and assists in the interaction of the 5′-UTR with the RNA-dependent RNA polymerase ($3D^{pol}$) for viral RNA replication. In addition, $3C^{pro}$ can inhibit retinoic acid inducible gene I (RIG-I)-mediated type I interferon response, thereby halting the innate immune response in infected cells and facilitating virus replication.[10] Moreover, transient expression of the two proteases can induce cell apoptosis.[9,11] Consequently, $2A^{pro}$ and $3C^{pro}$ are suggested to have multiple roles in virus replication. In addition to viral proteins, the binding of two cellular proteins, heterogeneous nuclear ribonucleoprotein A1 and heterogeneous nuclear ribonuclear protein K, to the 5′-UTR is required for viral replication.[12,13] In contrast, another cellular factor, far upstream element binding protein 2, binds to the 5′-UTR and negatively regulates viral protein translation by EV71.[14] Aside from binding to 5′-UTR, reticulon 3 is a cellular protein identified to be associated with the replication complex by binding to 2C protein and increases protein and RNA synthesis by EV71.[15] Therefore, any substitution acquired in EV71 evolution may change the complex interactions of viral and cellular proteins and influence virus replication and pathogenesis.

Recently, a report by WHO Western Pacific region emphasized EV71 emerging as a serious concern to public health and anticipated more cases of HFMD being reported in the future.[16] In this paper, we review the epidemiology, as well as the genetic and antigenic evolution of EV71 from past outbreaks. The effects of these genetic and antigenic changes on EV71 pathogenesis and vaccine development are emphasized.

Epidemiology and clinical manifestations of enterovirus 71

Enterovirus 71 (BrCr strain) was first identified and isolated from a patient with a central nervous system disease in 1969 in the United States.[17] In 1974, a total of 20 isolates from cases with encephalitis or meningitis since 1969 were reported. In the 1970s, several outbreaks with HFMD in young children were described in the USA, Australia, Japan, Hungary, Sweden, France and Bulgaria.[17–26] The clinical manifestations associated with EV71 infections in these outbreaks included aseptic meningitis, meningoencephalitis, respiratory disease, gastroenteritis and HFMD.[27] Lesions in the grey matter of the medulla, spinal cord or brain stem were observed in histopathological examinations.[23] In addition, when the virus was inoculated into cynomolgus monkeys, these animals showed either paralysis or weakness in the hind limbs and lesions in the central nervous system.[28] After the 1970s, another wave of EV71 activity occurred in the 1980s in Asia, Brazil, Netherlands and the United States, with cases with neurological involvement identified until 1990.[29–35]

A third wave of increased activity of EV71 was identified after 1997. A HFMD and herpangina outbreak was initially reported in Malaysia, with 31 fatal cases of encephalomyelitis[36] (Table 1). In 2000 and 2003, there were two additional outbreaks of HFMD, with EV71 being the predominant enterovirus serotype detected.[37] In 1998, a large EV71 outbreak occurred in Taiwan, which caused 78 child or infant deaths.[3] Following this outbreak, one, 25 and 26 fatal cases were reported in 1999, 2000 and 2001, respectively.[38] Lin *et al.*[39] reported an increase of EV71 cases with five and 16 deaths in 2004 and 2005, respectively, in Taiwan. Three years later, another large outbreak of EV71 activities caused 14 fatal cases in Taiwan.[40] Hosoya *et al.*[41] reported an EV71 surveillance result from 1983 to 2003 in Japan, another neighboring country to Malaysia and Taiwan, indicating the occurrence of HFMD outbreaks in 1984, 1987, 1990, 1997 and 2000, with a large outbreak observed in 2003. In addition, two large outbreaks of HFMD associated with EV71 were identified in Singapore in 2000 and 2006.[42,43] Moreover, EV71 was the predominant strain in 2001, 2003 and 2005/2006 in Singapore.[44] Contributing to the waves of increased EV71 activity, the EV71 pandemic in mainland China was first reported in 2004.[45] The affected region extended from southern China before 2004 to the middle and northern regions, such as Anhui, Shandong, Shanghai and Beijing provinces in 2008. Zhang *et al.*[46] reported a HFMD outbreak with 99 severe cases and 13 fatalities in Fuyang City, Anhui province in 2008. In the same time period, EV71 caused large HFMD outbreaks with occurrences of severe neurological disease in other countries in the Asian-Pacific region and other parts of the world such as in Perth, Australia in 1999,[47] southern Vietnam in 2005,[48] Brunei in 2006,[49] India in 2007[50] and the Netherlands in 2007.[35] A longer period of surveillance will be needed to determine whether EV71 in these countries will re-emerge again every two to three years. Taken together, these facts suggest that EV71 has circulated continually since 1997 and has become a predominant strain with two- to three-year cycles resulting in enterovirus outbreaks and cause peaks of EV71 activity, which threaten children's lives. The data suggest that EV71 has established a continuous circulation in the Asian-Pacific countries, and may also expand to other regions of the world in the future.

Table 1 Genotype changes of EV71 throughout the world from 1997 to 2010

	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Malaysia	C1,C2,B3*, B4	C1	C1	B4, C1		C1	B5, C1		B5, C1	B5				
Singapore	B3, B4	B3, C1	B3	B4	B4	B4,C1				B5				
Taiwan		B4, C2, C4	B4	B4	B4	B4,C4	B4, B5	C4	C4	C5	B5, C5	B5	B5	C4
Japan	C2,B3, B4	C2	C2	C2, B4	C2	B4, C2, C4	C4, B5	C4		C4	C4			
China		C4		C4	C4	C4	C4	C4			C4	A, C4	C4	
Vietnam									C1, C4, C5					
Australia		C2	B3, C2	B4, C1	B4, C1	C1	C1	C4						
Korea				C3										
Netherlands	C1, C2		C2	C2	C1	C1, C2		C1, C2	C1, C2		C1, C2	C2		
UK		C1	C1, C2	C1	C1	C1		C1		C1,C2				
Norway						C1	C1							
Austria					C1	C1	C1	C4						

*The underlined genotypes were the predominant genotypes occurring in outbreaks

Changes of genotypes within or between epidemics

According to the comparison of VP1 sequences, EV71 can be phylogenetically divided into three distinct genotypes: A, B and C.[51] Genotype A is composed of the EV71 strain (BrCr-CA-70) identified in 1970 in the USA but was not detected afterwards until 2008.[51] Yu *et al.*[52] reported the emergence of five isolates that are closely related to genotype A in central China. In contrast, genotypes B and C continued to circulate around the world after the 1970s and the 1980s, respectively. Genotypes B and C can be further divided into five subgenotypes, respectively – genotypes B1–B5 and C1–C5. Among these subgenotypes, genotype C3 was only seen in Korea in 2000[53] and others were found to spread either globally or regionally (Table 1). In the 1970s, only genotypes B1 and B2 were reported to play roles in HFMD epidemics in America and Europe, including the United States,[51] Netherlands,[35] Bulgaria[21] and Hungry[54] (reviewed in van der Sanden *et al.*[35]). In the 1980s, genotype B2 became the predominant genotype and this EV71 lineage continued circulating in Japan, Taiwan, the United States, Netherlands and Australia.[35,39,41,55] However, a new genotype C1 appeared and replaced the predominant genotype B2 and became the major genotype in the late 1980s and the early 1990s.

Since 1997, the largest wave of EV71 activity in the history has caused hundreds of child deaths around the Asian-Pacific region. According to the phylogenetic results, multiple genotypes including genotypes C2, B3 and B4 were introduced into populations in 1997 and co-circulated in this region. In contrast to previous genotypes B2 and C1 in the early 1990s, the genetically diverse genotypes B3 and C2 became the dominant strains in these HFMD outbreaks in the last three years of the 1990s (Table 1).

In the first HFMD outbreaks in the Asian-Pacific region, various genotypes including genotypes C1, C2, B3 and B4 were observed in Malaysia in 1997, and genotype B3 was the predominant strain in the outbreak resulting in several fatalities.[36] In 2000 and 2003, genotypes B4 and B5 replaced genotype B3 and became the predominant genotypes in the later outbreaks, respectively, according to the result of sentinel surveillance from 1998 to 2005 in Sarawak, Malaysia.[37] Genotype C1 appeared sporadically between the three outbreaks in 1997, 2000 and 2003. In short, intragenotype B shifts were observed from the late 1990s to early 2000s in Malaysia, including genotypes B3 and B4 (during 1997–2000) and B4 to B5 (during 2000–2003), and genotype C1 is the only genotype that had continually circulated in Sarawak.

In the same period, the two outbreaks in 2000 and 2006 were detected in Singapore, which is a neighbor of Malaysia. After the Malaysia outbreak in 1997, only a limited number of genotypes (B3, B4 and C1) viruses were isolated from 1997 to 1999 in Singapore. Afterwards, the first large outbreak caused by genotype B4 in 2000 was reported in Singapore.[56–59] Genotypes B4 and C1 co-circulated after the outbreak in 2000 and an intragenotype B shift (genotypes B4 to B5) appeared; this genotype B5 replacement resulted in an outbreak in 2006.[44]

Instead of an intragenotype B shift, an intergenotype shift between genotypes B and C have been shown between HFMD outbreaks in Taiwan. In 1998, Taiwan observed the largest HFMD outbreak in the Asian-Pacific region, in which 129,106 severe cases were reported and 78 child cases

were fatal.[3] A genetic analysis showed that genotype C2 was the predominant strain, but approximately 10% of EV71 isolates in the same period belonged to genotype B4.[60] Although the number of cases of enterovirus infections dramatically decreased in Taiwan in 1999, another large HFMD outbreak occurred in 2000 and 2001. A genotype shift was observed in EV71 in Taiwan in which the predominant strain changed from genotype C2 to B4.[61] Afterwards, genotype B4 circulated in Taiwan until 2004 and genotype C4 replaced B4 as the predominant strain between 2004 and 2005.[39] In contrast to the low EV71 activity between 2006 and 2007, genotype B5 activity significantly increased in 2008 and dozens of fatal cases with neurological diseases were reported.[40] After the 2008 outbreak, EV71 returned to low activity in Taiwan. Interestingly, genotype changes exhibited a pattern from genotype B to C or C to B in Taiwan, genotype C2 to B4 between 1998 and 2000, B4 to C4 between 2001 and 2004, and C4 to B5 between 2005 and 2008. Note that the predominant genotypes of the outbreaks, including genotype B4 in 2000 and 2001, genotype C4 in 2004, and genotype B5 in 2008, were detected in the sporadic cases from roughly two to five years before a large outbreak occurred in Taiwan. Recently, similar results were also shown by Tee *et al.*,[62] who estimated the dates of origin of each subgenotype of EV71 by Bayesian relaxed molecular clock method. They found that diverse genotypes of B may appear to circulate for approximately two to five years before causing large HFMD outbreaks. Similar time intervals were shown in the estimated dates of origin of subgenotype C. The subgenotype C viruses can be detected about one to five years before they cause the occurrence of outbreaks. Before an outbreak occurs, a virus may exhibit at low prevalence so that only a few or no severe case was reported.[63] In summary, in contrast to the intragenotype B shift in Malaysia and Singapore, the intergenotype shifts between genotypes B and C were observed in Taiwan, and the predominant genotype in the outbreaks could be detected two to five years prior to large HFMD outbreaks.

Shimizu *et al.* reported a HFMD outbreak with severe cases in Japan in 1997. Sequence comparison showed that genotype C2 was more prevalent than genotype B4 in Japan, contrasting to the occurrence of predominant genotype B4 in Malaysia in the late 1990s.[64] Fujimoto *et al.*[65] and Mizuta *et al.*[66] reported increased prevalence of EV71 in 2000 in Hyogo Prefecture and Yamagata, respectively, and a genotype shift change in Japan occurred from genotype C2 to B4. A similar genotype shift reappeared in the Yamagata outbreak in early 2003, but this time the genotype changed from B4 to C4. Interestingly, in the October of 2003, the predominant genotype shifted again from C4 to a new genotype B5.[66] Comparing the trend of intergenotypes B and C shifts in Taiwan and Japan after 1997 shows that the pattern of intergenotype changes that occurred in these outbreaks were identical: genotypes C2 to B4 (1997–2000 in Japan and 1998–2000 in Taiwan), B4 to C4 (2000–early 2003 in Japan and 2001–2004 in Taiwan) and C4 to B5 (early 2003–late 2003 in Japan and 2005–2008 in Taiwan).

In the other countries of the Asian-Pacific region, the EV71 outbreak did not show a regular pattern similar to the countries mentioned above. In China, only a single genotype C4 (which contains two minor genetically diverse lineages C4a and C4b) continually circulated and resulted in the HFMD outbreak in 2008.[45,46,67] In Vietnam, genotypes C1, C4 and C5 co-circulated and C5 was the predominant one in the HFMD outbreak in 2005.[48] In Australia, genotype B3 showed high viral activity and resulted in an EV71 outbreak in 1999 accompanied with sporadic cases of infection by genotypes B4, C1, C2 and C4.[47,55,56] Outside of the Asian-Pacific region, only one EV71 outbreak with 58 severe hospitalized cases was reported in Netherlands in Europe in 2007 to our knowledge and genotype C2 showed a high prevailing strain in this outbreak.[35] Whether the other genotypes of EV71 have been introduced into these countries or the occurrence of intra- and intergenotype shift between outbreaks should be further investigated.

Occurrence of inter- and intragenic recombination among EV71

A range of various enteroviruses circulating in populations at any point of time is shown in Table 1. In the course of an enterovirus circulation, recombination between parts of genome may occur when different viruses infect and replicate in the same cell. This process allows enteroviruses to create and maintain their genetic diversity.[68] To analyze the occurrence of recombinant events, the complete EV71 genome has been sequenced and phylogenetically analyzed via swapping through the whole genome.[69] Until now, several reports have shown their evidences for EV71 recombination. It is determined that four genotypes had undergone recombination after analyzing all circulating EV71 isolates in public domain (Genbank): two intra- and two intertypic recombinations. To our surprise, most of the predominant strains in the HFMD outbreaks around the Asian-Pacific region after 1997 were found to be an intertypic or an intratypic recombinant virus, involving genotypes B3, C2, B4 or C4. Genotype B3 caused the first HFMD outbreak in Malaysia in 1997 and in Perth, Australia in 1999. Phylogenetic evidence shows that the intertypic recombination occured in genotype B3 (Figure 1a), in which the 3D polymerase and 3′-UTR are closely related to CAV16 instead of the prototype of EV71.[70] Another intertypic recombination appeared in genotype C2, which was the major genotype in EV71 outbreaks in Japan and Taiwan in 1998.[40,64,71] Bootscan analysis of the sequences of EV71 genotype C2 in Asia and other HEV-A shows the presence of EV71 genotype C and coxsackievirus A8 (CAV8) genome sequences within the genome of genotype C2 isolates (Figure 1b). According to the similarity analysis, the 5′-UTR, non-structural protein coding region and 3′-UTR of genotype C2 reveal higher similarity to CAV8 than other HEV-A viruses including EV71 prototype strain BrCr-CA-70, indicating the recombination event between sequences of CAV8 non-structural genes and EV71 structural genes.

The appearance of genotype B4 was initially reported among some sporadic cases in Malaysia and Singapore in 1997 (Table 1). In the following year, genotype B4 was found in the HFMD outbreak in Taiwan and Japan, although it only showed low prevalence until 2000. In 2000, genotype B4 widely spread in the Asian-Pacific region and became a

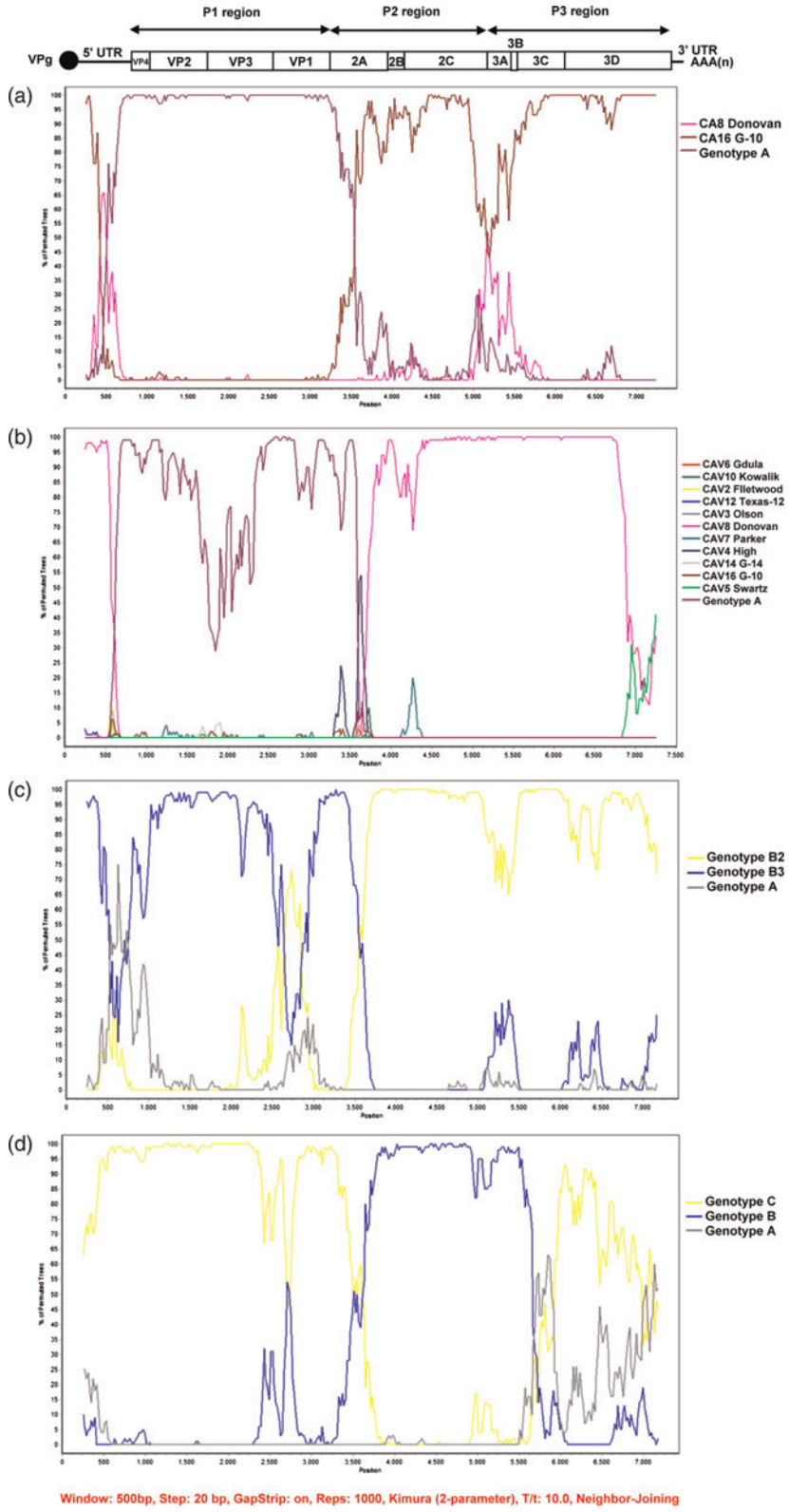

Figure 1 EV71 genetic recombination. Complete genome sequences of EV71 of various genotypes were analyzed by BootScan analysis, including (a) genotype B3 (EV71/SAR/SHA63, accession number AM396588), (b) genotype C2 (4643-TW98, accession number AF304458), (c) genotype B4 (N7008-TW99, accession number FJ357375) and (d) genotype C4 (S0584-TW04, accession number FJ357373). The enterovirus 71 genetic map is shown on the top panel. UTR, untranslated region; CAV, coxsackievirus. (Reprinted from Huang *et al.*[40] with kind permission from American Society for Microbiology).

predominant genotype in the large outbreak in Japan, Taiwan, Singapore and Malaysia; however, only sporadic isolates were identified in Australia. In Taiwan, this genotype caused the second peak of HFMD activities with hundreds of severe cases in 2001 and then has continued to circulate until 2003. In contrast to the intertypic

recombination that occurred in genotypes B3 and C2 EV71, both phylogenetic and BootScan examinations indicate that the genotype B4 of EV71 viruses in Taiwan and Malaysia are similar to genotype B3 in the 5′-UTR and the capsid protein coding region and genotype B2 in the P3 region (Figure 1c), suggesting the possible intratypic recombination between genotypes B3 and B2 in the genotype B4 evolution.

Genotype C4 represented another intratypic recombination event in the EV71 evolution. Similar to the other EV71, genotype C4 has been circulating for several years in the Asian-Pacific region and initially became the predominant genotype in the outbreaks in Japan in 2003. Afterwards, genotype C4 re-emerged in Taiwan and exhibited two peaks of EV71 activities in 2004 and 2005. Genotype C4a, a linage of genotype C4, shows the genetic diversity in contrast to previous strains belonging to genotype C4b in China and became predominant in 2008 in China. Bootscan analysis reveals that genotype C4 virus in China and those in Taiwan in 2004 and 2005 outbreaks have a similar P1 region to EV71 genotype C but the 2B to the 3B protein coding regions are similar to genotype B (Figure 1d).[40,71–73] In addition, Chan and co-workers[71,73] reported that the 3C to 3D protein coding region of genotype C4 shows high similarity to CAV16. In summary, these recombination reports show the evidence of genome recombination of genotype C4 sequences between EV71 genotype C structural genes and non-structural genes derived from genotype B and CAV16.

According to the EV71 full genome sequences available in Genbank, Chen *et al.*[74] mapped the EV71 recombination break points and verified the frequencies of recombination events. They reported that the recombination break points do not randomly occur throughout the genome. Although the location of break points in the genome are diverse among the strains from different countries, the frequency and location of recombination results show that the 3D polymerase coding region represents the highest frequency which the recombination region occurs as a recombinant unit. Accompanying the 3D polymerase coding region, the P1 region is suggested as another recombinant unit by measuring the topological differences between phylogenetic trees constructed from various parts of the EV71 genome.[74] Therefore, these investigations provide a new insight to the dynamics of recombination and the evolution in the EV71 emergence.

Genetic changes resulted in antigenic changes in epidemics or outbreaks

Phylogenetic results show that EV71 are clustered into genotypes A, B and C, which can then be classified into 10 subgenotypes including B1 to B5 and C1 to C5. To analyze the antigenicity of EV71, Mizuta *et al.*[75] examined the cross-reactivity among the various genotypes by guinea pigs antiserum against genotypes B2 and C1. Neutralization results displayed that antiserum against genotypes either B2 or C1 with the higher neutralization antibody titers against genotypes B2, B4 and B5 than those against genotypes A and C1, C2 and C4, suggesting some differences of antigenic properties among the various genotypes. In addition to the cross-reactivity in guinea pig antiserum, van der Sanden *et al.*[76] verified cross-neutralization activity of rabbit antisera against genotypes B2 and C1. The results of rabbit antisera show that the B2 antiserum can neutralize genotypes B0, B1 and B2 with high average titers but reacted with genotype C1, C2 and A with lower ones. In contrast, rabbit antisera against genotype C1 exhibited higher cross-neutralization activity with genotype A, B and C of EV71. Although slightly different results are shown in rabbit antisera in contrast to those found in guinea pig antisera, both of the two reports indicate the antigenic diversity between different genotypes of EV71. To investigate the cross-reactivity in human serum sample against this human pathogenic EV71, we previously titrated human antisera from EV71-infected patients during the period of 1998–2008 against the EV71 clustered in various genotypes for understanding the antigenic property of viruses in human.[40] An antigenic map was constructed from the seromicroneutralization data to quantitatively analyze the antigenic evolution of the EV71 isolates (Figure 2); the genotype B1 and B4 viruses (blue circles) were clustered together while genotype C2 from 1998 (green circle) were found in another antigenic cluster distinct from the genotype B viruses. In comparison to genotype B, genotype C4 viruses (red circles) are more closely related antigenically to genotype C2. The re-emergent genotype B5 viruses in 2008 in Taiwan mapped in a separate cluster neither closer to genotype B1/B4 nor to genotype C2/C4 in the antigenic map, indicating that the re-emergent genotype B5 viruses are antigenically different from the other genotype B and C viruses in the study. This antigenic cartography shows the antigenic diversity among the genotype B and C viruses, which may explain the intergenotypic shift between B and C that occurred in Taiwan and Japan. These genotypic shifts, accompanied with antigenic changes, may be beneficial for EV71 to escape herd immunity and result in another outbreak as the seroprevalence is reduced.

Impact of genetic changes on viral pathogenesis and vaccine candidate selection

The evolution of EV71 is engaged in a never-ending struggle; EV71 evolves (i) naturally as part of the replication cycle (ii) as a result of immune response. In an evolution, recombination is the fastest way to obtain new genomic regions with high diversity in contrast to the original genome, which may mediate the change of the virus characteristics including the viral infection property and pathogenesis. For instance, to investigate the possible genetic diversity between the EV71 strains before and after 1998, we previously compared the sequences of 3D polymerase in a non-structural region of EV71.[77] We have identified a T251I substitution in this region of the 1998 strains that is different from the 1986 strains, and this mutation can change the temperature susceptibility of genotype C2 from susceptible to resistance at 39.5°C. In addition, the T251I substitution increases the viral virulence and presents more severe clinical symptoms in a

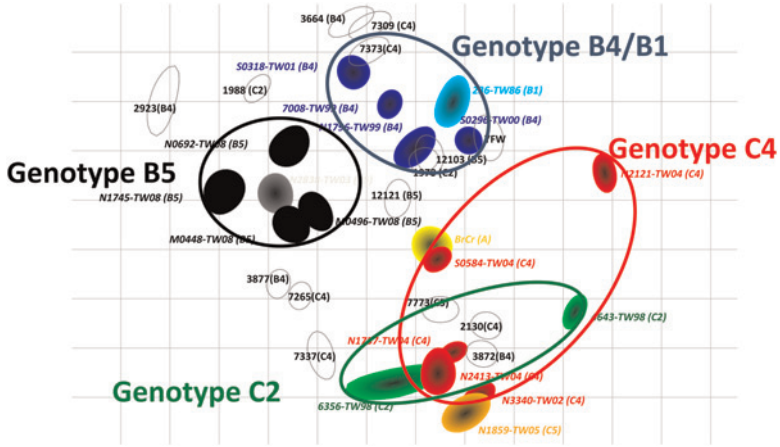

Figure 2 Antigenic map of EV71. The relative positions of strains (colored shapes) and antisera (uncolored shapes) were adjusted such that the distances between strains and antisera in the map represent the corresponding neutralization assay measurements with the least error. The vertical and horizontal lines represent antigenic distance, and, because only the relative positions of antigens and antisera can be determined, the orientation of the map within these axes is free. The spacing between grid lines is one unit of antigenic distance corresponding to a two-fold dilution of antiserum in the neutralization assay. Isolate number and genotype of viruses or serum numbers and genotype of infected viruses were indicated, respectively. (Reprinted from Huang *et al.*[40] with kind permission from American Society for Microbiology)

mouse infection model *in vivo*. Interestingly, our result of EV71 recombination shows that the non-structural protein coding region of genotype C2 strain, the predominant strain in the large outbreak in Taiwan in 1998, is closely related to the CAV8 prototype strain.[40] Analysis of the sequence of CAV8 indicates that the amino acid located in the corresponding position of position 251 in EV71 3D polymerase is Thr, which is identical to the 1998 genotype C2 EV71. Accordingly, we believe that the T251I substitution may be introduced into genotype C2 by recombination with CAV8 in the non-structural protein coding region, which changed the temperature susceptibility of EV71 to be resistant at high temperature *in vitro* and increases the virulence of EV71 *in vivo*. The genetic change thereby may change the virus infection ability and even viral pathogenesis.

A consequence of the low fidelity of 3D RNA-dependent RNA polymerase results in a high divergence of EV71 circulating in human population. Specifically, the immunodominant protein of enteroviruses, the VP1 coding region shows a high diversity in contrast to other regions of genome and becomes the target of selection by herd immune response.[78] Several investigations have examined the selective pressure on the VP1 protein coding region of EV71 by estimating the ratio of non-synonymous to synonymous substitution. Shi *et al.*[79] initially found four sites of VP1 protein that are positively selected: position 58 of the Australia strains, position 98 of the Malaysia strains, position 145 of the Malaysia, the Singapore, the Taiwan and the US strains, and position 241 of the US strains. Moreover, the position 145 of VP1 protein was reported as positive selection site by Chen *et al.*[74] as well, and our previous finding also suggests that the positions 98 and 145 of VP1 protein in those genotype B are under positive selection.[40] Tee *et al.*[62] recently analyzed the selective pressure in VP1 protein among different subgenotypes, and position 98 in genotypes B4 and C1, position 145 in genotypes B2, B3, B4 and C1, position 237 in genotype B3, and position 241 in genotype B4, and C2 are detected in the evolution pressure of positive selection. In contrast to the capsid VP1 protein facing the positively selective pressure, no positive selection site was detected in the non-structural protein $3D^{Pol}$ since it is not exposed on the virus surface, which an antibody may recognize (data not shown). Tee *et al.*[62] also characterized the phylogeny of EV71 subgenotypes in a ladder-like structure similar to influenza virus evolution. A maximum likelihood phylogenetic tree shows in a temporal structure that the recent strain continually replace the previous strains through time. One of the explanations of the observed shape of EV71 phylogenetic tree is that EV71 may be in the process of antigenic evolution like the influenza virus and immune escape mutants are continually generated under positive selection. Therefore, the identified positive selection sites may determine antigenic property of EV71 as target sites that neutralization antibody may recognize.

In addition, antigenic diversity in various genotypes shown by antigenic cartography is suggested to be correlated with the phylogenetic evolution of EV71. The phylogenetically classified genotype B1/B4, genotype B5 and genotype C based on the VP1 protein coding region are segregated into three different antigenic clusters in the antigenic map. The VP1 coding sequence comparisons displayed that Glu43, Thr58, Thr184 and Ser240 are specific signature amino acids for genotype B including B1, B4 and B5, while Arg22, Asp31 and Ile249 are presented only in genotype C2. Genotype B5 has aspartic acid at position 164 similar to genotypes C2 and C4 but not in B1 or B4. In simple terms, these genetic changes may contribute to the distinct antigenic properties of genotypes, although the other capsid protein-coding regions still require further examinations.

Throughout the Asian-Pacific region, EV71 shows an increased activity and continues to cause epidemics; however, the EV71 vaccine has not been available yet. As to developing an EV71 vaccine, there are challenges selecting a proper immunogen to elicit neutralization antibody reactivity against EV71 epidemics. First, each subgenotype virus is continually positively selected by herd immune

pressure so that it is hard to choose a vaccine strain as the strain replacement continues to occur. Second, the continuous inter- and intragenotypic shifts among EV71 outbreaks and the circulating subgenotypes seemed to exhibit at least three different antigenic properties to our knowledge. Choosing only one strain as a vaccine immunogen may not provide enough protection for various genotypes of EV71. Therefore, searching a common epitope between antigenically different strains for a suitable vaccine candidate to elicit neutralization antibody against diverse antigenic properties may be a possible solution to generate a vaccine with broadly neutralization antibody protection. The positive selection sites and antigenic determinants should be brought into consideration for vaccine development.

Throughout these investigations, we have gained better understanding of EV71 epidemiology and viral evolution. However, the continuous surveillance by the whole genome sequence is still necessary to provide more insights on the dynamic evolution of EV71 in the future. Furthermore, the natural selection and antigenic changes among EV71 viruses should be considered for the EV71 vaccine development.

Author contributions: As principal investigator, J-RW had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. J-RW and S-WH were involved in study concept and design. DJS and DK were involved in acquisition and analysis of data.

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