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Introduction of a strong temperature-sensitive phenotype into enterovirus 71 by altering an amino acid of virus 3D polymerase

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ABSTRACT

In 1998, an enterovirus 71 (EV71) epidemic in Taiwan resulted in 78 deaths; however, the molecular basis of EV71 pathogenicity remains poorly understood. Comparison of the deduced amino acid sequences in 3D polymerases of EV71clinical isolates showed the T251V or T251I substitution from 1986 and 1998 outbreaks. An EV71 replicon system showed that introducing an I251T mutation did not affect luciferase activities at 35 °C when compared with wild type; however, lower luciferase activities were observed when they were incubated at 39.5 °C. In addition, the I251T mutation in the EV71 infectious clone not only reduced viral replication at 39.5 °C *in vitro* but also decreased the virulence of the mouse adaptive strain MP4 in neonatal mice in an *i.p.* infection model. Therefore, these results suggested that the threonine at position 251 results in a temperature sensitivity phenotype of EV71 which may contribute to the attenuation of circulating strains.

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Introduction

Enterovirus 71 (EV71) belongs to the Picornaviridae family and is the causative agent of hand, foot and mouth disease (HFMD) and herpangina. In addition, EV71 can cause severe neurological diseases including encephalitis, meningitis, acute flaccid paralysis and death in children. The pathogenicity of EV71 is not fully understood. Currently, vaccine therapy and antiviral agents are not available for EV71 infection.

EV71 was first isolated and characterized from patients with neurological disease in California in 1969, and subsequent outbreaks occurred in numerous countries including Bulgaria, Hungary and Malaysia (Ho, 2000). The most severe outbreak occurred in 1998 resulting in 78 deaths in Taiwan and subsequently an average of 40 fatalities occurred each year between 2001 and 2005. Prior to the outbreak in 1998, sporadic cases of EV71 infection were reported in 1980 and 1986 (Lin et al., 2006). In 1980, EV71 infected children had poliomyelitis-like flaccid paralysis, HFMD or herpangina; however, these cases were not associated with death (Ho et al., 1999). EV71 were isolated from patients with HFMD or herpangina in 1986. It was suggested that aside from host factors, the increased virulence of EV71, which resulted in severe clinical manifestations in 1998, was due in part to the evolution of the viral genome (Lin et al., 2003). Unfortunately, the molecular determinants of EV71 virulence have not been defined.

Previous studies have shown the importance of the 3D region, which encodes the RNA dependent RNA polymerase, in virus replication. Besides incorporation of nucleotides during RNA elongation (Baltimore, 1964), the 3D polymerase is responsible for uridylylation of the protein primer, Vpg, which is essential for the initiation of replication (Flanegan and Baltimore, 1977; Paul et al., 1998). Furthermore, the 3CD proteinase, which is the precursor of 3D polymerase, processes the viral polyprotein (Jore et al., 1988; Ypma-Wong et al., 1988), stabilizes RNP complex with the 5′ cloverleaf and 3AB (Bedard and Semler, 2004) and promotes viral protein maturation and RNA synthesis, respectively. The nuclear localization signal in the 3D region directs 3CD to the host cell nucleus and 3C shut off the host cell transcription machinery while it is excised from 3CD (Weidman et al., 2003). Recent studies also suggested that the low

Abbreviations: EV71, Enterovirus 71; HFMD, hand foot and mouth disease; UTR, untranslated region; TR, temperature resistant; TS, temperature sensitive.

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fidelity of 3D polymerase is essential for the high rate of mutation which aids in the survival of the virus population in the presence of selective pressure (Pfeiffer and Kirkegaard, 2005). Mutations in 3D region of poliovirus resulted in a changing growth rate, viral RNA accumulation, temperature susceptibility and viral attenuation in mice (Agut et al., 1989; Burns et al., 1989; Diamond and Kirkegaard, 1994; Toyoda et al., 1987). Therefore, we reasoned that the 3D region of EV71 may be important in altering the virulence of the virus.

The replicon system, in which the coding sequence of the structural genes were replaced with a reporter gene, has been used to study the replication and translation of various plus-stranded RNA viruses, such as hepatitis C virus, West Nile virus, yellow fever virus and human enterovirus (Kaplan and Racaniello, 1988; Molenkamp et al., 2003; Moradpour et al., 2004; Yamshchikov et al., 2001; Yi et al., 2002). The replicon system has been used to analyze poliovirus virus replication, tissue tropism and construct foreign gene delivery vectors (Ansardi et al., 1994a, 1994b; Arita et al., 2006; Bledsoe et al., 2000; Johansen and Morrow, 2000; Porter et al., 1998; Porter et al., 1997). These replicon systems have been a useful tool to test vaccine candidates or to analyze biological properties of viruses.

In this study, we constructed an EV71 replicon with the capsid region replaced by the firefly luciferase reporter gene. In addition, recombinant viruses produced from the EV71 infectious clone were used to study the viral phenotype without interference of other mutations generated during viral passage. Our results showed that 3D-I251T mutation resulted in a strong temperature-sensitive phenotype. This phenomenon suggested the presence of the I251T mutation within the 3D region might contribute to attenuation in virulence of clinical virus isolates.

Results

Analysis of the 3D regions of EV71 clinical isolates

The 3D regions of EV71 clinical isolates from 1986, 1998 and after 1998 outbreaks were sequenced and compared. The result of 3D amino acid sequence alignment indicated a T to I or T to V substitution at position 251 among isolates from and after 1998 outbreaks (Fig. 1). All of the clinical isolates from 1986 had threonine at position 251 in 3D, and their biological properties are distinct from those isolates containing a valine or isoleucine at position 251. We next generated 3D-I251T mutation into the EV71 replicon and infectious clone systems to examine the effect of this substitution on viral biological characteristics.

Replication of 3Dpol mutant replicon

To examine the effect of the 3D mutation on RNA replication in cultured SK-N-SH cells in the absence of virus production, EV71 wild-type replicons were constructed (Fig. 2A). An infectious cDNA clone containing EV71 genome from 1998 isolate was obtained, and the P1 structure region was replaced with firefly luciferase gene (Figs. 2B and C). This pT7-driven replicon RNA could be transcribed *in vitro* and displayed a time-dependent increase of luciferase activity when transfected into SK-N-SH cells with a peak at 8 h posttransfection (data not shown). The results revealed the EV71 replicon RNA could replicate and translate well in SK-N-SH cells, simulating the life cycle of enterovirus after uncoating. The I251T mutant 3Dpol coding sequences were subcloned into the EV71-luciferase replicon (Fig. 2C) to determine the effect on replication. In addition to the 3D-I251T mutant replicon, a 3Dpol gene-deleted construct was also generated which could be translated but could not replicate while transfected into cells. Thus, a basal level of luciferase activity was still expected and would reflect an input RNA translation level (data not shown). In addition, another mutation D328H which results in a distinct replication defect in poliovirus was also introduced to the EV71 replicon. RNA of this construct was transcribed and translated *in vitro* and displayed high luciferase activities with no difference from wild type (Fig. 3A). The results suggested that the *in vitro* transcribed RNA could be translated into protein successfully and there was no discrepancy in the translation of RNA derived from those construct. Transfection of 3D-deleted construct and D328H replicon RNA into SK-N-SH cells resulted in low luciferase activities (Fig. 3B). Moreover, there was no significant difference of luciferase activities between wild-type and 3D-I251T mutant replicon at 35 °C (Fig. 3B). However, the 3D-I251T mutant

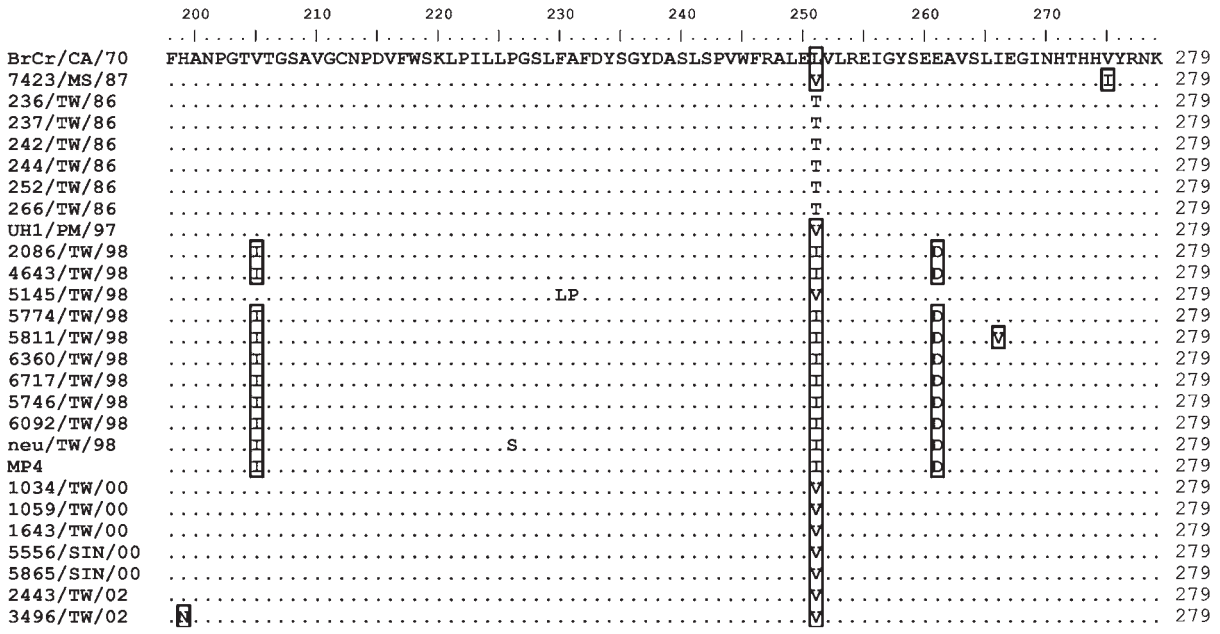

Fig. 1. Alignment of partial 3D amino acid sequences of EV71 clinical isolates from 1986, 1998 and post-1998 outbreaks. The amino acid sequences were deduced from nucleotide sequences and aligned using Vector NTI, and the figure was generated with BioEdit. The I251T mutation is highlighted by the black frame. Viruses were designated as isolate number/location/year.

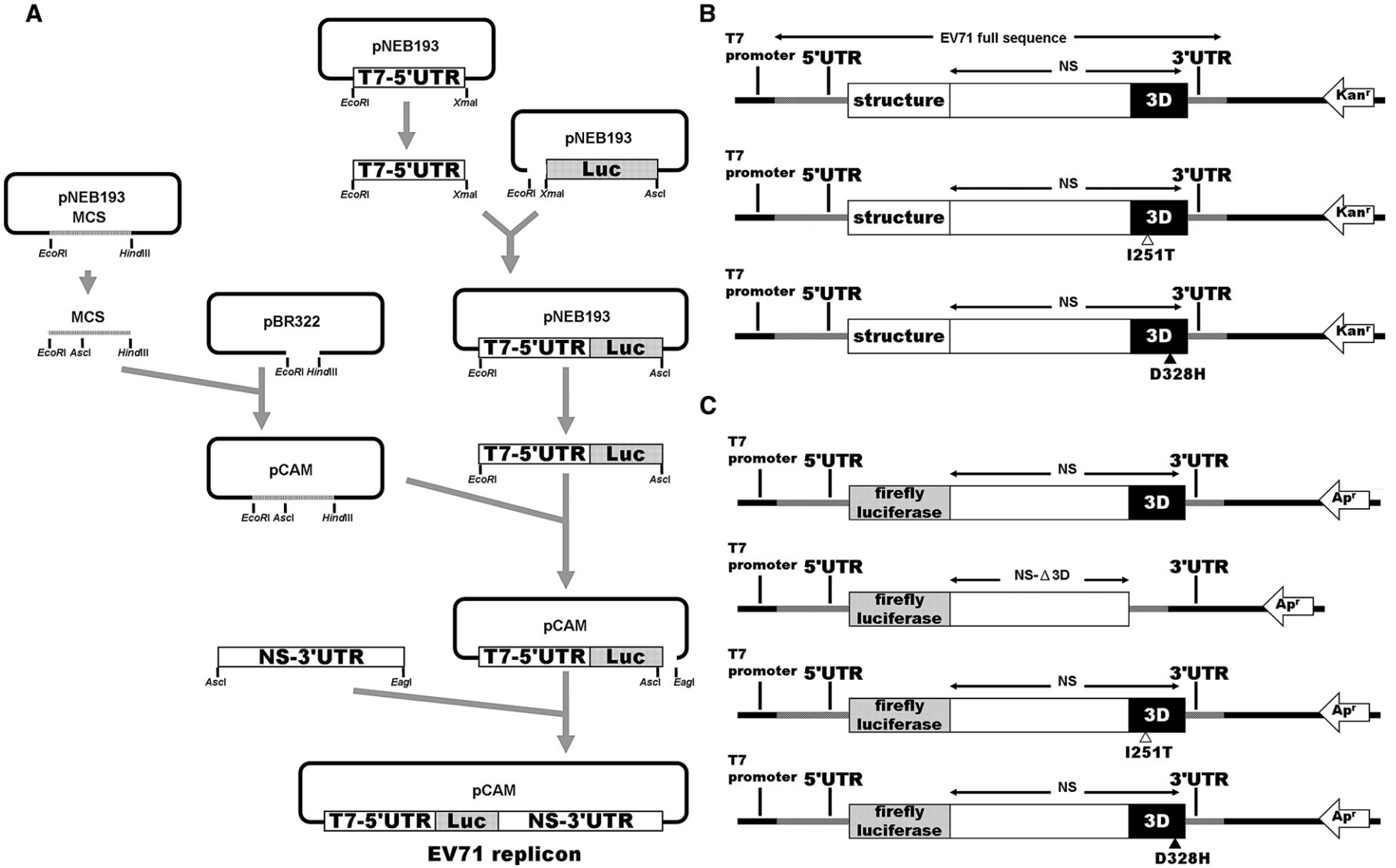

Fig. 2. Schematic diagram of EV71 infectious clones and replicons. (A) Construction scheme for wild-type EV71 replicon. Luc, firefly luciferase; NS, nonstructural region; Kanr, kanamycin resistant; Apr, ampicillin resistant; Δ3D, 3D region deleted. Schematic diagram of EV71 replicon (B) and infectious clone (C) were shown. Black and white triangles represent the position of mutations in 3D region.

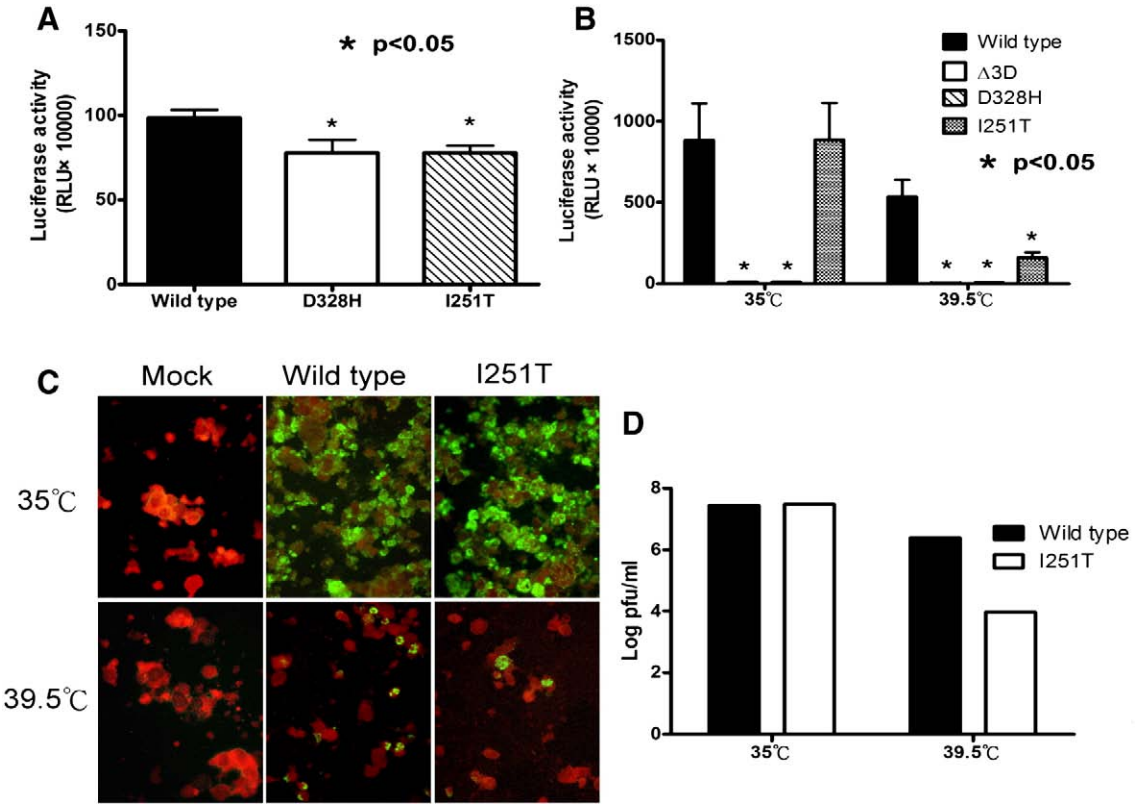

Fig. 3. Translation and replication of EV71 replicon RNA as measured by luciferase activities. (A) Luciferase activities of EV71 replicons following *in vitro* translation using the rabbit reticulocyte lysate system. (B) Luciferase activities of EV71 replicons following RNA transfection into SK-N-SH cells for 8 h at varying temperatures. Transfection of EV71 infectious clones in SK-N-SH cells at (C) 35 °C and 39.5 °C. Virus titer of the supernatant determined by plaque assay was presented in (D). The data shown were from triplicate experiments, expressed as the mean ± SD. *$p<0.05$ in a *t* test.

replicon showed an approximately 4-fold decrease in luciferase activities when compared with wild type at 39.5 °C (Fig. 3B), suggesting that 3D-I251T mutant might influence the 3D polymerase activities at the higher temperature.

Effects of I251T polymerase mutations on EV71 infectious clone

To test the ability of the mutant polymerases to support replication *in vivo*, the mutant 3Dpol genes were subcloned into an infectious clone of EV71 and the RNA was transfected into SK-N-SH cells. After transfection, the RNA from wild-type EV71 infectious clone RNA at 35 °C displayed a typical cytopathic effect of enterovirus in SK-N-SH cells at day 3 posttransfection. Production of recombinant EV71 was confirmed by immunofluorescence staining using EV71 monoclonal antibody (Fig. 3C). The cell culture supernatant was also collected and inoculated into SK-N-SH cell monolayer to insure the generation of infectious virus particles. The virus production of 3D-I251T mutant infectious clone was not different from wild type when cultured at 35 °C. However, the 3D-I251T mutant infectious clone presented a 100-fold decrease in virus progeny at 39.5 °C (Fig. 3D). These results indicated that 3D-I251T mutant polymerase might not effectively support replication at a higher temperature.

Phenotypes of EV71 carrying I251T polymerase mutation

To further investigate the effect of 3D mutation on EV71 biological characteristics, viruses were harvested from cell culture transfected with RNA from the infectious clone. Growth kinetics and plaque phenotype showed no difference between wild-type and the 3D-I251T mutant viruses at 35 °C (Fig. 4A). Interestingly, the growth kinetics of 3D-I251T mutant viruses displayed lagging growth rates and smaller plaques than the wild type when grown in SK-N-SH cells at 39.5 °C (Figs. 4B and C). Temperature-sensitivity tests were also carried out by measuring the virus titer in SK-N-SH cells at 35 or 39.5 °C. The wild-type neu strain EV71 displayed a temperature-sensitive phenotype, while the 3D-I251T mutation conferred a strong temperature-sensitive phenotype (Table 1). Notably, EV71 isolates from 1986 (237-TW-86 and 242-TW-86) also displayed a strong temperature-sensitive phenotype. These results indicated that 3D-I251T might serve as the temperature sensitivity determinant in the genetic context of EV71.

Next, we investigated whether the I251T correlated with EV71 virulence in addition to its role as a determinant of temperature sensitivity. We generated the infectious clone MP4/y-5 using mouse-adapted EV71 strain MP4 as backbone and introduced the I251T mutation into MP4/y-5 to examine the attenuated effect of the I251T mutation to mice. The recombinant MP4 viruses were inoculated into 1-day-old ICR mice intraperitoneally and the virulence of the virus in neonatal mice was analyzed. The results revealed that 86% of the mice were dead on day 4 postinfection and none survived on day 5 postinfection (Fig. 4D). In contrast, mice infected with MP4 carrying I251T mutation survived up to 5 days postinfection, an indication of the attenuation of the virus in neonatal mice. Moreover, the results of clinical score indicated that the I251T mutation significantly delayed the paralysis progression whereas the average clinical scores decreased from 5 to 4 on day 4 postinfection (Fig. 4E). According to these results, we suggested that 3D-I251T mutant in the genetic context of EV71 would increase the viral temperature susceptibility in neuron cells and attenuate the virulence of EV71 in the neonatal mice infection model.

Discussions

EV71 has circulated in Taiwan for more than one decade. Two mild EV71 epidemics that occurred in 1980 and 1986 resulted in HFMD, herpangina and a few cases of poliomyelitis-like flaccid

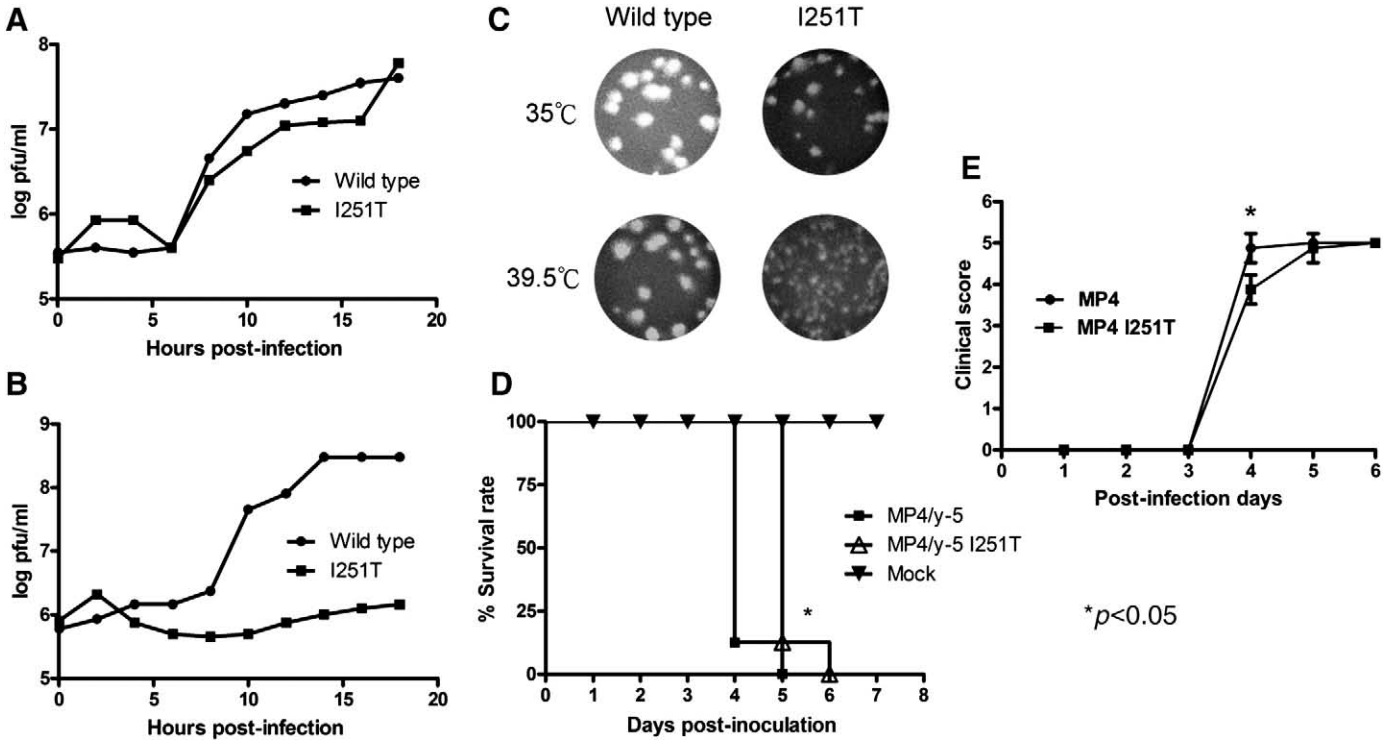

Fig. 4. Biological characteristics of the EV71 3D mutant. (A) Growth kinetics of EV71 wild-type and I251T mutant at 35 °C, MOI = 10. (B) Growth kinetics of EV71 wild-type and I251T mutant at 39.5 °C, MOI = 20. (C) Plaque phenotype of EV71 wild-type and I251T mutant at 35 and 39.5 °C. L, Large-plaque phenotype; M, medium-plaque phenotype; S, small-plaque phenotype. (D) Inoculation of EV71 wild-type and I251T mutant into one-day-old ICR mice intraperitoneally. The survival rates are indicated.

paralysis with no case fatalities (Ho et al., 1999). In contrast, the outbreak in 1998 resulted in 78 deaths and 405 severe cases with neurological symptoms, pulmonary edema and acute flaccid paralysis (Ho et al., 1999). Serological evidence showed that age-specific pre-epidemic EV71 seroprevalence rates were inversely correlated with age-specific enterovirus-related mortality rates (Chang et al., 2002). Also, clinical observations during the outbreaks revealed that small children were infected more frequently and had more severe clinical manifestations than older children and adults, suggesting that pre-existing neutralizing antibodies might offer protective immunity against EV71 infection (Lu et al., 2002), and that numerous host factors were associated with clinical presentations (Lin et al., 2003). On the other hand, increasing viral virulence due to evolution of the virus might be one of the primary contributors to serious epidemics. Currently, the genetic determinants of EV71 virulence are poorly understood. The goal of this study was to determine the molecular determinant(s) of EV71 virulence which may have contributed to the 1998 outbreak. The 5′-UTR of poliovirus, necessary for RNA replication and translation in many organs, was reported to determine virulence phenotype (Kauder and Racaniello, 2004; Kawamura et al., 1989). Determinants of coxsackievirus B3 cardiovirulence phenotype have been localized to the 5′-UTR (Lee et al., 1997; Tu et al., 1995) and capsid region (Knowlton et al., 1996). A mutation in the 2A protease of swine vesicular disease virus affecting eIF4GI cleavage resulted in a change in virulence of the virus (Inoue et al., 2005; Kanno et al., 1999). Protease 3C was responsible for triggering apoptosis in poliovirus-infected cells by a mechanism that involved caspase activation (Barco et al., 2000). The Y73H mutation in 3D polymerase of poliovirus type 1 contributed to virus attenuation in mice (Tardy-Panit et al., 1993).

Table 1
Temperature sensitivity of mutant EV71 strains.

Viruses	Titer		ΔLog	Phenotype
	35 °C	39.5 °C		
237-TW-86	7.33[a]	1[a]	6.33	Strong TS
242-TW-86	7.33[a]	1.33[a]	6	Strong TS
neu-TW-98	6.24[b]	3.73[b]	2.51	TS
neu-TW-98-I251T	6.68[b]	2.05[b]	4.63	Strong TS

[a] Virus titer represents $\log_{10}(TCID_{50})$ in 10-μl virus suspension.
[b] Virus titer represents $\log_{10}$(PFU) in 1-ml virus suspension. Δlog ≤2, temperature resistant, TR; Δlog >2, temperature sensitive, TS; Δlog >2.75, strong temperature sensitive, strong TS.

Our study focused on variations of the 3D region of EV71 strains isolated between 1986 and 1998. Sequence alignment of the 3D regions revealed numerous amino changes between EV71 isolates from 1986 and those from or after 1998. The majority of amino acid changes were inter-genotypic variations except position 251. EV71 isolated from 1986 had a hydrophilic amino acid, threonine, in position 251 in contrast to those isolated from 1998 with a hydrophobic amino acid, either isoleucine or valine. Therefore, our primary objective was to identify the effect of introducing a 3D-I251T mutation to the EV71 replicon and infectious clone systems with the 1998 strain as the backbone. Moreover, a well-defined substitution at 3D position 328 was used as a control. The D328H mutation seemed to have no effects on replicon RNA translation *in vitro* suggesting that D328H might not act at the level of viral RNA translation. Transfection of the 3D-D328H mutant replicon or infectious clone RNA into SK-N-SH cells caused low-level luciferase activities and no infectious viral progeny, respectively. The luciferase activities brought out by D328H and Δ3D replicon RNA transfection was similar to basal translation level, indicating RNA replication was abolished. Previous studies showed D328 was the first aspartic acid located in the GDD motif of the palm subdomain of RNA-dependent RNA polymerase, which was highly conserved within plus-stranded RNA viruses (Jablonski and Morrow, 1995). The D residue was suggested to be exposed on the loop region of β-turn-β conformation by structural prediction (Argos, 1988). The two aspartic acids in the GDD motif have been postulated to be involved in metal ion coordination at or near the catalytic active site of the enzyme (Argos, 1988; Delarue et al., 1990). The substitution

of the first D residue resulted in nonfunctional polymerase *in vitro* or *in vivo* (Jablonski and Morrow, 1995; Ribas and Wickner, 1992; Sankar and Porter, 1992). Taken together, our results were agreed with these previous findings, indicating that D328 was required for polymerase activity of EV71 and most plus-stranded RNA viruses.

In this study, we identified a novel genetic determinant at position 251 which was never studied in other enteroviruses. A single point mutation of this position reduced EV71 replication rated up to 100-fold at high temperature. In addition, EV71 1998 strain became strong temperature sensitive when the 3D 251 position had an I to T substitution and this substitution was also identified in the 1986 strains. This result revealed the substitution of position 251 in 3D was the region responsible for partial change of temperature susceptibility from 1986 to 1998. Position 251 is located in the α-helix between the fingers subdomain and the A motif of the palm subdomain (O'Reilly and Kao, 1998). Structure prediction displayed no obvious alteration in 3D polymerase structure when I change to T at position 251 (by PS^2 software) (data not shown). Studies of poliovirus showed that mutations in the highly conserved regions, 5′-UTR, 3D and 3′-UTR (Kawamura et al., 1989; Omata et al., 1986), acting together or independently could affect temperature susceptibility (Bouchard et al., 1995). Introducing those temperature-sensitive determinants of Sabin 1 into EV71 BrCr-TR strain also caused defective viral replication at nonpermissive temperatures. EV71 demonstrated a strong temperature-sensitive phenotype as a result of three mutations, Y73H and C363I in 3D and A7049G in the 3′-UTR (Arita et al., 2005). According to those findings, PV1 and EV71 shared a common genetic determinant of temperature sensitivity.

Although none of the *in vitro* phenotypic markers alone can serve as a good indicator of neurovirulence or attenuation (Omata et al., 1986), temperature sensitivity of PV vaccine strains serves as an *in vitro* attenuation marker in general. A total of 53 strains isolated from 1998 were analyzed for the phenotype of temperature susceptibility. The results showed that 22.6% of the viruses were temperature resistant, while 77.4% of the viruses isolated were temperature sensitive (data not shown). According to our data, 36.7% (11/30) of EV71 clinical strains isolated from patient with CNS involvement or death were temperature resistant; in contrast, there was only about 4.3% (1/23) which were temperature-resistant among those isolates from HFMD. These results suggested that EV71 with temperature-resistant phenotype could replicate more efficiently in neuron cells in the patients with fever; thus, they were more virulent and caused more severe neurological diseases. In the another study of EV71 infection in mice, a G145E mutation in VP1 capsid protein was found to be essential for mouse-adapted EV71 virus to infect NOD/SCID mouse and to cause paralysis of the infected mice and suggested that the capsid proteins played an important role for EV71 to cross the species barrier between human and mouse (Arita et al., 2008). The same mouse virulence determinant was also found in newborn BALB/c mice infection model (Chua et al., 2008). Therefore, to examine the attenuation effect of position 251 mutation of 3D polymerase in mouse infection model, we further used mouse-adapted strain MP4 as template and constructed the MP4/y-5 infectious clone which also has glutamate at position 145 in VP1 and the generated virus displayed virulence to neonatal mice. The MP4/y-5 virus with I251T substitution resulted in attenuation and prolonged the survival time of infected mice. Although this mutation only delayed the death of infected mouse for one day, we suggested the I251T mutation affected the virulence of EV71 which correlated with lower viral replication at high temperature *in vivo* in neurons. Therefore, with the cooperation of the capsid region for cell entry, we speculated that I251T mutation might be one of the potential determinants for the viral virulence. This finding might explain the increase in viral virulence in the 1998 outbreak in Taiwan as a result of the change from threonine to isoleucine at position 251. In addition, these results might be useful for EV71 vaccine development.

In conclusion, we set up an EV71 replicon system with the firefly luciferase reporter gene which could rapidly estimate the level of the viral replication. After comparing the 3D regions of EV71 strains from 1986 and 1998, we identified a novel determinant at position 251, which was probably responsible for alternation of temperature susceptibility and viral virulence. I251T substitution might explain the increase in viral virulence of the virus in the 1998 outbreak.

Materials and methods

Cells and viruses

SK-N-SH cells (ATCC no. HTB-11) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL, Grand Island, NY) (supplemented with 10% FBS, 100 u/ml penicillin, 100 μg/ml streptomycin) and incubated at 35 °C with 5% CO2. EV71 clinical isolates and recombinant viruses were propagated in SK-N-SH cells. Virus titration, growth kinetics and temperature sensitivity tests were also performed using SK-N-SH cells.

RNA extraction, RT-PCR and sequencing

EV71-infected SK-N-SH cells with 75% cytopathic effect were scraped and pelleted by centrifugation. Viral genomic RNA was extracted from the pellet of infected cells by using a Viral Nucleic Acid Extraction Kit (Geneaid, Taipei County, Taiwan). RT-PCR was performed by using MMLV reverse transcriptase (Promega, Madison, WI) and the 3D-7360R primer for reverse transcription. Primers including 3D-5809F and 3D-7360R were used to amplify the 3D region by using Taq DNA polymerase (New England Biolabs, Beverly, MA). PCR products were purified by using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced. The amino acid sequences were deduced from the nucleotide sequences and aligned using Vector NTI. The 3D region of the following EV71 strains were deduced from sequences retrieved from GenBank: BrCr-CA-70 (Q66478), 7423/MS/87 (Q66479), UH1/PM/97 (CAL36656), 2086/TW/98 (AAD22046), 4643/TW/98 (AAG24906), 5746/TW/98 (AAG24905), 6092/TW/98 (AAG24907), Neu/TW/98 (AAY59418), 5556/SIN/00 (AAK30618) and 5865/SIN/00 (AAK13008). Sequencing was performed for the following strains: 236/TW/86 (FJ357379), 237/TW/86 (FJ357380), 242/TW/86 (FJ415467), 244/TW/86 (FJ357381), 252/TW/86 (FJ357383), 266/TW/86 (FJ357384), 5145/TW/98 (FJ415462), 5774/TW/98 (FJ415463), 5811/TW/98 (FJ415464), 6360/TW/98 (FJ415466), 6717/TW/98 (FJ415465), MP4 (mouse-adapted enterovirus 71 4643/TW/98 strain) (Wang et al., 2004), 1034/TW/00 (FJ415457), 1059/TW/00 (FJ415458), 1643/TW/00 (FJ415459), 2443/TW/02 (FJ415460) and 3496/TW/02 (FJ415461).

Construction of EV71 replicon and infectious clone

The complete 5′-UTR region of the EV71 4643 strain and firefly luciferase gene were amplified using 4643-F (containing T7 promoter upstream of 5′-UTR), 5′UTR-*Xma*I and Luc-*Xma*I, Luc-*Acs*I primers, respectively (Table 2). The PCR-amplified T7-5′UTR and firefly luciferase (Luc) fragments were cloned into pNEB193 vector individually. The T7-5′UTR fragment of pNEB193-T7-5′UTR plasmid was subcloned into pNEB193-Luc plasmid following *Eco*RI and *Xma*I digestion. In order to change the plasmid backbone to pBR322 vector, a novel vector with *Acs*I restriction enzyme site was generated. The multiple cloning sites (MCS) of pNEB193 vector was digested with *Eco*RI and *Hin*dIII and then cloned into pBR322 vector. The new vector containing pBR322 vector backbone and pNEB193 vector MCS was named pCAM. T7-5′UTR-Luc fragment was digested with *Eco*RI and *Acs*I and then ligated into pCAM. Nonstructural region (NS) and the 3′UTR from the EV71 infectious clone neu strain was subcloned

Table 2
EV71 primers used in this study.

Primer name	Sequence (5'-3')	Purpose
3D-5809F	ccchacnaarscdrgdcagt	3D PCR
3D-6237R	cttcttccatgctcatctt	3D sequencing
3D-6655R	aaccacactgggctgagac	3D sequencing
3D-6793R	garttgaaratggadgtdcc	3D sequencing
3D-7360R	cagtcattaacacgaccaga	3D PCR
4643-F	ctagaattcagctaatacgactcactatagttaaaacagcctgtgggttg	5'UTR amplification
4643-R-T50	cctacggccgttttttttttttttttttttttttttttttttttttttttttttttttttttttttgctatt ctggttataacaaatttac	RT and 3'UTR amplify
5'UTR-XmaI	tttccccccgggtgtggacacttgtgagcccat	5'UTR amplification
Luc-XmaI	tttccccccgggatggaagacgccaaaaacat	Luc gene amplification
Luc-AscI	tttggcgcgccacacggcgatctttccgc	Luc gene amplification
neu-I251T-F	ggttcagggcgctggagaCagtcctgcgggaaattgg	3D mutation
neu-I251T-R	ccaatttcccgcaggactGtctccagcgccctgaacc	3D mutation
neu-D328H-F	ctgaacatggtggcctacgggCatgatgtgttggctagttacc	3D mutation
neu-D328H-R	ggtaactagccaacacatcatGcccgtaggccaccatgttcag	3D mutation

Sequences underlined represent restriction enzyme cutting site; hollow square shows T7 promoter and block letters indicate the positions of nucleotide substitutions.

into the pCAM-T7-5′UTR-Luc plasmid using *Acs*I and *Eag*I restriction sites (Shih et al., 2004). The pCAM-T7-5′UTR-Luc-NS-3′UTR plasmid was sequenced and confirmed to contain the EV71 full-length genome with the structural region replaced by firefly luciferase gene. A mouse-adapted infectious clone, MP4/y-5, was constructed using the strain MP4. Viral genomic RNA was extracted and RT-PCR was performed by using primer 4643-R-T50 and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) for reverse transcription, and viral cDNA was next amplified using primer 4643-F and 4643-R-T50 by using the Advantage cDNA polymerase mix (BD Biosciences, Palo Alto, CA) for PCR. The products were purified by phenol–chloroform extraction and then directly cloned using the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA).

Construction of 3D mutant replicons and infectious clones

Mutations, 3D-I251T and 3D-D328H, were introduced into the replicon or infectious clone using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutant oligonucleotide I251T-F and I251T-R changed nucleotide 6690 (T to C) of wild-type EV71, resulting in alternation of isoleucine acid to threonine (I251T); and D328H-F and D328H-R change nucleotide 6923 (G to C), resulting in alternation of aspartic acid to histidine (D328H). The obtained mutant 3D fragment was digested with restriction enzymes (*Dra*III and *Pml*I, *Mlu*I and *Nru*I for replicon and infectious clone, respectively) and subcloned into the wild-type replicon and infectious clone. Primers mentioned above are listed in Table 1.

In vitro transcription

EV71 replicon and infectious clone plasmids were linearized with *Eag*I and *Sma*I, respectively. Transcription was performed with T7 RNA polymerase by using a RiboMAX™ Large Scale RNA Production Systems (Promega, Madison, WI). For translation experiments, transcription reactions were terminated by addition of RQ1 RNase-Free DNase. RNA was extracted with phenol–chloroform and analyzed by electrophoresis under denaturing condition in 2.2 M formaldehyde according to Maniatis et al. (1982) using the MOPS buffer system and stained with ethidium bromide.

Transfections and luciferase assays

Approximately 90% confluent monolayers of SK-N-SH cells grown in 6-well plates were rinsed twice with phosphate-buffered saline (PBS). One-microgram RNA was added to TransMessenger Transfection Reagent (Qiagen Valencia, CA) to a final volume of 100 μl. After incubation for 10 min at room temperature, 0.9 ml of Dulbecco's modified Eagle's medium (DMEM) was added, and cultures were incubated at 35 °C for 4 h. Medium was then replaced with DMEM supplemented with 10% fetal bovine serum (FBS), and plates were allowed to incubate at 35 °C for 8 h. At the indicated times, cells were washed twice with PBS. Cell culture lysis buffer (0.5 ml) (Promega, Madison, WI) was added to each well, and cells were collected in a 1.5-ml Eppendorf tube. Aliquots (20 μl) of the supernatants were used in the Dual-Glo™ Luciferase Assay System (Promega, Madison, WI), and light emission was measured with a MiniLumat LB 9506 luminometer (Berthold, Bad Wildbad, Deutschland). Their relative light units (RLU) were calculated accordingly.

Immunofluorescent staining

At the indicated times, cells were scraped and fixed on glass slides with acetone for 10 min. The slides were stained with mouse anti-EV71 monoclonal antibody (mab979, Chemicon, Billerica, MA), FITC-conjugated goat anti-mouse IgG antibody and then examined under a fluorescent microscope.

Plaque assay

The plaque assay was performed in 6-well plates (TPP, Trasadingen, Switzerland) containing SK-N-SH cell monolayer (Dolan et al., 1968). Ten-fold dilutions of virus suspension were inoculated at 400 μl/well and incubated for 1 h at 35 °C. Virus suspension was discarded and 2 ml DMEM containing 2% FBS and 1% methylcellulose (Sigma-Aldrich, St. Louis, MO) was added per well and incubated at 35 °C. After incubation for 4 days, overlay medium was discarded, and cells were fixed in 10% formaldehyde and then stained with 1% crystal violet.

Replication kinetics

SK-N-SH cell monolayers in culture tube (2×10^5 cells) were incubated with virus for 1 h at 35 °C. The cells were washed and then cultivated in Dulbecco's modified Eagle's medium containing 2% fetal bovine serum at 35 °C and 39.5 °C, respectively. Virus titers were determined at different intervals by a plaque assay using SK-N-SH cell monolayers in 6-well plates.

Temperature sensitivity

SK-N-SH cell monolayers in culture tube (1×10^5 cells) were infected with virus at a multiplicity of infection (MOI) of 10^{-4} at 35 °C and 39.5 °C. Virus titers were determined by $TCID_{50}$ using SK-N-SH cells after 96 h incubation. Temperature sensitivity was expressed as

difference of $TCID_{50}$ values at 35 and 39.5 °C ($\Delta TCID_{50}$). We defined a less than 2.0 logarithmic difference as a temperature resistant (tr) phenotype, those from 2.0 to 2.75 logarithmic difference as a temperature-sensitive (ts) phenotype and those with more than 2.75 logarithmic difference as a strong ts phenotype (Arita et al., 2005).

In vivo mouse model

Specific pathogen-free, ICR mice (newborn, the Laboratory Animal Center, National Cheng Kung University) were inoculated with 100 μl (i.p.) of different EV71 viruses as indicated. Control animals were given medium instead of virus. Animals were observed daily for weight gain or lost, clinical signs and mortality until postinfection 7 days. According to the clinical signs, disease progressions were scored as follows: 0 = health; 1 = ruffled fur and hunchbacked appearance; 2 = wasting; 3 = limb weakness; 4 = limb paralysis; and 5 = moribund and death (Chen et al., 2007). The institutional animal care and use committee approved all animal protocols.

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