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# Complete genome sequencing and analysis of six enterovirus 71 strains with different clinical phenotypes

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## Abstract

**Background:** Hand, foot and mouth diseases (HFMD) caused by enterovirus 71 (EV71) presents a broad spectrum of clinical manifestations ranging from mild febrile disease to fatal neurological disease. However, the mechanism of virulence is unknown.

**Methods:** We isolated 6 strains of EV71 from HFMD patients with or without neurological symptoms, and sequenced the whole genomes of the viruses to reveal the virulence factors of EV71.

**Results:** Phylogenetic tree based on VP1 region showed that all six strains clustered into C4a of C4 sub-genotype. In the complete polypeptide, 298 positions were found to be variable in all strains, and three of these positions (Val<sup>P814</sup>/Ile<sup>P814</sup> in VP1, Val<sup>P1148</sup>/Ile<sup>P1148</sup> in 3A and Ala<sup>P1728</sup>/Cys<sup>P1728</sup>/Val<sup>P1728</sup> in 3C) were conserved among the strains with neurovirulence, but variable in strains without neurovirulence. In the 5'-UTR region, it showed that the first 10 nucleotides were mostly conserved, however from the 11th nucleotide, nucleotide insertions and deletions were quite common. The secondary structure prediction of 5'-UTR sequences showed that two of three strains without neurovirulence (SDLY11 and SDLY48) were almost the same, and all strains with neurovirulence (SDLY96, SDLY107 and SDLY153) were different from each other. SDLY107 (a fatal strain) was found different from other strains on four positions (C<sup>P241</sup>/T<sup>P241</sup>, A<sup>P571</sup>/T<sup>P571</sup>, C<sup>P579</sup>/T<sup>P579</sup> in 5'-UTR and T<sup>P7335</sup>/C<sup>P7335</sup> in 3'-UTR).

**Conclusions:** The three positions (Val<sup>P814</sup>/Ile<sup>P814</sup> in VP1, Val<sup>P1148</sup>/Ile<sup>P1148</sup> in 3A and Ala<sup>P1728</sup>/Cys<sup>P1728</sup>/Val<sup>P1728</sup> in 3C), were different between two phenotypes. These suggested that the three positions might be potential virulent positions. And the three varied positions were also found to be conserved in strains with neurovirulence, and variable in strains without neurovirulence. These might reveal that the conservation of two of the three positions or the three together were specific for the strains with neurovirulence. Variation of secondary structure of 5'-UTR, might be correlated to the changes of viral virulence. SDLY107 (a fatal strain) was found different from other strains on four positions, these positions might be related with death.

**Keywords:** Enterovirus 71, Virulent determinant, Hand, foot and mouth disease

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## Background

Enterovirus 71 (EV71) belongs to the Enterovirus genus of the family Picornaviridae. It is one of the pathogens that are associated with hand, foot and mouth disease (HFMD). In most cases, EV71 infections are generally mild. However, this virus has also been implicated to cause severe neurological manifestations including aseptic meningitis, polio-like paresis and possibly fatal encephalitis [1].

Since 1969, when EV71 was first isolated in California, USA [2], EV71 associated outbreaks have been reported worldwide [3-10]. In recent years, it has gained more attention as there is an upward trend in the prevalence of EV71 in Asia [11]. EV71 infection is a serious threat to the health of infants and young children; therefore, it is necessary to understand the mechanism of central nervous system involvement. Zheng et al. reported nucleotide differences in 5'-UTR between strains isolated from patients with and without neurological symptom, and proposed that such variation may be correlated with different clinical presentations [12]. Shih-Cheng Chang reported that a significant amino acid change was observed in more than one of high virulent strains [13]. Melchers et al. suggested that point mutations in 3'-UTR can result in a lethal phenotype [14]. All these points were located in different regions of the genome, therefore, it is necessary to search for potential points associated with neurovirulence in complete genome.

## Results

### Virus identification and segmented amplification

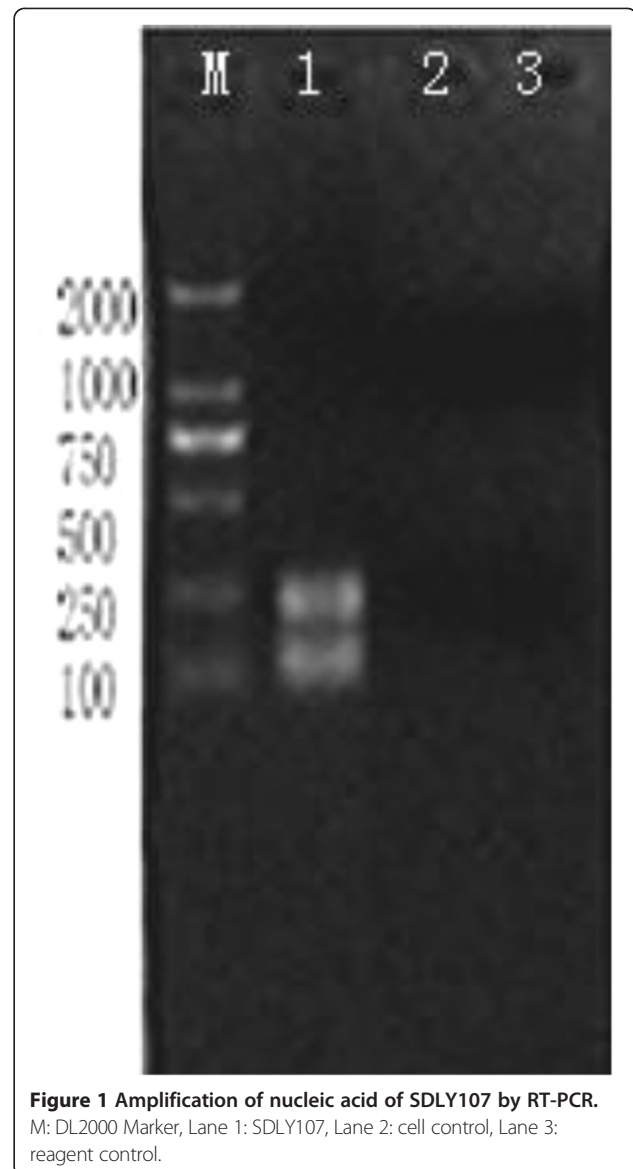
All the six strains were proved to be EV71 by RT-PCR (Figure 1), and were amplified with the nine pairs of overlapping primes (Figure 2).

### Sequence analysis of the genomes

The sequences of the six strains were deposited in GenBank (GenBank accession number JX244182, JX244183, JX244184, JX244185, JX244186, JX244187). The genomes of strains SDLY11, SDLY48, SDLY96 and SDLY107 were all 7405 bp in length, whereas strains SDLY1 and SDLY153 were 7408 bp in length. All six strains had one ORF which encoded a polypeptide of 2193 amino acids.

Pair-wise nucleotide and amino acid sequence comparisons showed that the genetic variation among the six strains was limited. The nucleotide homology of the genomes was 95.5% ~ 99.7%. The amino acid homology of the polyproteins was 98.5% ~ 99.5%. The nucleotide homology of 5'-UTR and 3'-UTR were 97.2% ~ 99.6% and 95.3 ~ 100.0%, respectively. They shared 77.5%-99.0% nucleotide homology of the genomes with reference strains, and 98.6% to 89.6% at the amino acid level (Table 1).

Phylogenetic analysis of the six strains and reference strains based on the nucleotide sequences of the complete

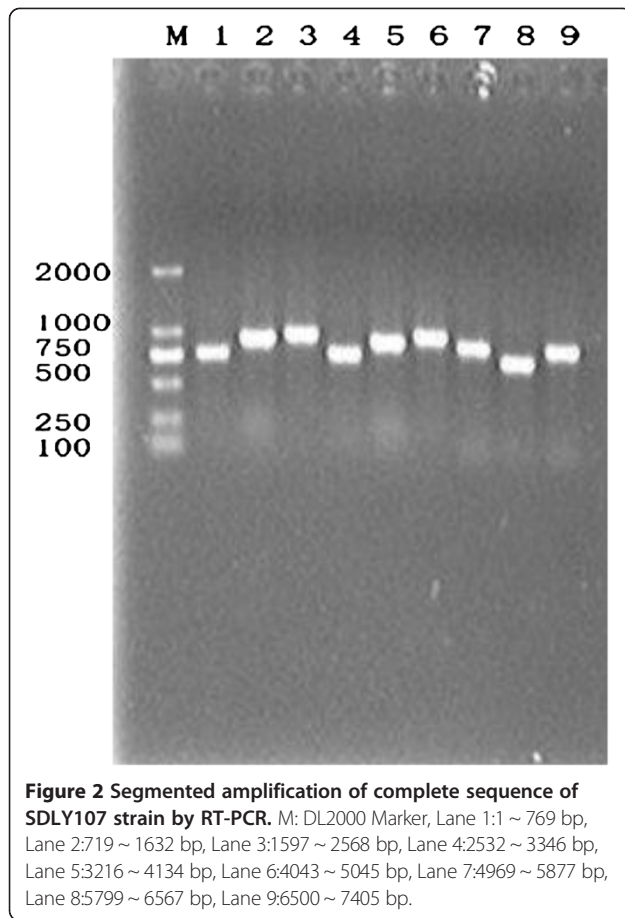


VP1 region showed that all the six strains clustered in the C4a of C4 sub-genotype (Figure 3).

### Analysis of polyprotein

The polyprotein consists in three regions [15]: P1 containing capsid proteins (VP ~ VP4), P2 and P3 containing non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) which are crucial for virus replication.

The complete genome sequence of 58 strains of EV71 were available in GenBank, but only 25 strains had information of clinical symptoms of the patients. 13 of the 25 strains isolated from patients with neurological symptom, and 12 of the strains were isolated from patients without neurological symptom. As we aimed to correlated sequences of defined clinical symptoms, we only



analyzed the 25 genomes of EV71 strains from GenBank that had description of clinical symptoms and the 6 strains sequenced in this study (Table 2). In the complete polyprotein, 298 positions were found to be variable and three of these positions were statistical significant (Table 3). The three points were Val<sup>P814</sup>/Ile<sup>P814</sup> (Fisher's Exact Test,  $P = 0.018$ ,  $\alpha = 0.05$ ), Val<sup>P1148</sup>/Ile<sup>P1148</sup> (Fisher's Exact Test,  $P = 0.043$ ,  $\alpha = 0.05$ ) and Ala<sup>P1728</sup>/Cys<sup>P1728</sup>/Val<sup>P1728</sup> (Fisher's Exact Test,  $P = 0.018$ ,  $\alpha = 0.05$ ).

#### Analysis of 5'-UTR

5'-UTR sequences of 31 strains were aligned. It showed that the first 10 nucleotides were mostly conserved, however from the 11th nucleotide, nucleotide insertions and deletions were quite common. No position was found statistical significantly different between strains with and without neurological symptom. Whereas SDLY107 (a fatal strain) was found different from other strains on three positions (C<sup>P241</sup>/T<sup>P241</sup>, A<sup>P571</sup>/T<sup>P571</sup>, C<sup>P579</sup>/T<sup>P579</sup>), suggesting that these positions might be related to death.

5'-UTR sequences of 6 strains isolated in our study were aligned by BioEdit 7.09 software (Figure 4), and no significantly difference was found.

Phylogenetic analysis of 31 strains based on the nucleotide sequences of 5'-UTR showed no definite regularity of these strains, revealing that there was no distinction on evolution between strains with different symptoms (Figure 5).

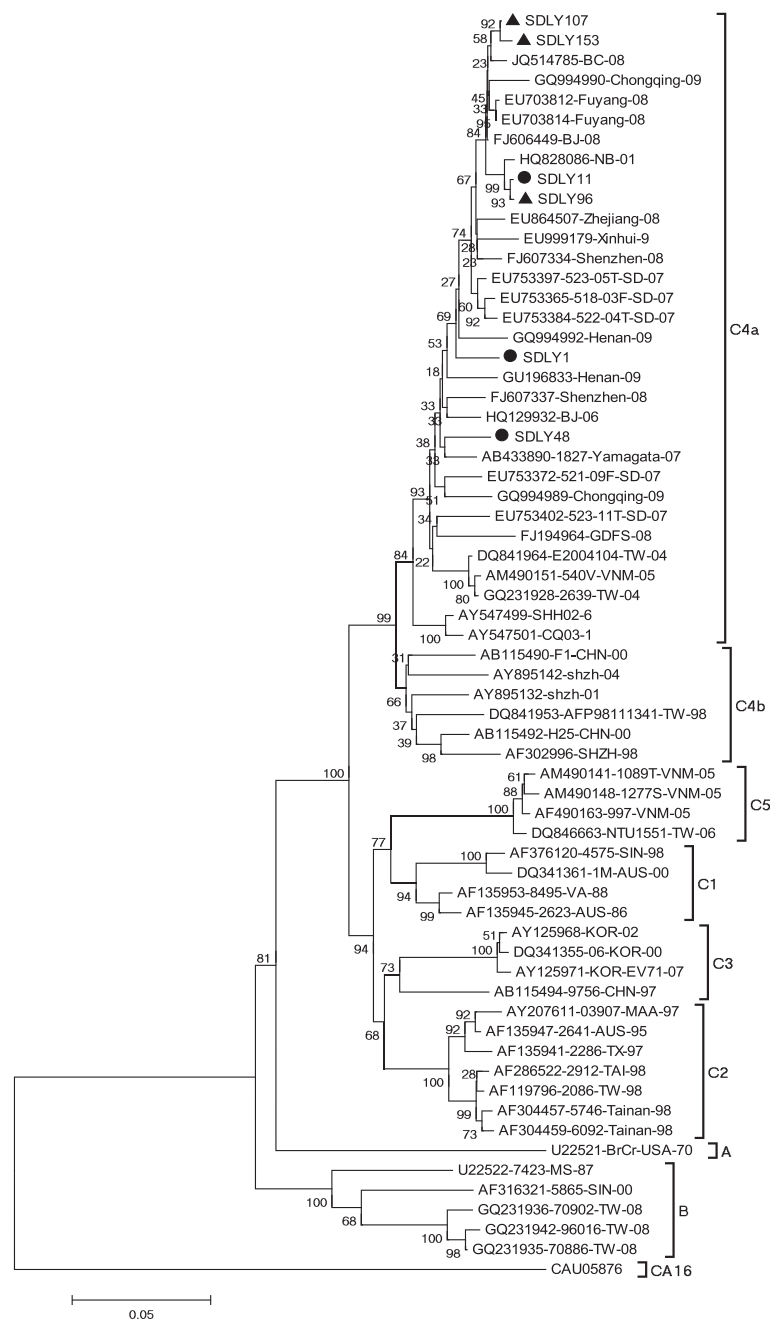
The 5'-UTR of EV71 could be divided into two regions: the 5' terminal cloverleaf and the IRES element [16]. IRES initiated genome translation by a cap-independent mechanism mediated [17]. IRES includes five stem loop (domain I ~ V), all of these five domains are essential to viral RNA replication and translation. The secondary structure prediction of the complete 5'-UTR sequences showed that two of three strains from patents without neurological symptom (SDLY11 and SDLY48) were almost the same, and all strains with neurovirulence (SDLY96, SDLY107 and SDLY153) were different from each other (Figure 6). In IRES element, domain III and II were relatively conservative regions, however, domain I, IV and V were marked variation. This information suggested that variety of secondary structure of 5'-UTR, especially domain I, IV and V might influence virulence.

#### Analysis of 3'-UTR

The 3'-UTR of EV71 was a highly conserved region and point mutations in the 3'-UTR could result in a lethal phenotype [14]. Alignment of 3'-UTR sequences of 31

**Table 1 The nucleotide and amino acid homology of the six strains with reference strains(nucleotide/amino acid)**

|               | SDLY1     | SDLY11    | SDLY48    | SDLY96    | SDLY107   | SDLY153   |
|---------------|-----------|-----------|-----------|-----------|-----------|-----------|
| U22521(A)     | 80.1/95.1 | 79.9/95.1 | 79.9/95.2 | 79.9/95.0 | 79.9/95.0 | 79.9/94.5 |
| ETU22522(B)   | 81.9/95.9 | 81.9/95.8 | 81.9/95.9 | 81.9/95.8 | 82.0/95.7 | 81.7/95.2 |
| DQ341361(C1)  | 82.3/96.3 | 82.2/96.2 | 82.5/96.4 | 82.3/96.2 | 82.3/96.2 | 82.1/95.6 |
| AF119795(C2)  | 81.8/93.0 | 81.4/93.0 | 81.9/93.0 | 81.6/93.0 | 81.3/93.0 | 81.3/92.6 |
| DQ341356(C3)  | 81.8/96.2 | 81.4/96.2 | 81.9/96.2 | 81.6/96.2 | 81.3/96.2 | 81.3/95.6 |
| EU703813(C4a) | 97.2/99.2 | 98.9/99.6 | 96.4/99.1 | 99.0/99.6 | 98.6/99.7 | 98.4/99.1 |
| AF302996(C4b) | 91.3/95.9 | 91.0/95.9 | 91.4/95.8 | 91.0/95.9 | 90.8/95.8 | 90.8/95.2 |
| EU527983(C5)  | 82.1/96.4 | 82.0/96.4 | 82.3/96.3 | 82.1/96.4 | 82.1/96.5 | 82.1/95.9 |
| U05876(CA16)  | 77.9/90.3 | 77.6/90.2 | 77.7/90.2 | 77.7/90.2 | 77.9/90.2 | 77.5/89.6 |



**Figure 3 Phylogenetic tree hylogenetic analysis based on EV71 VP1 nucleotide sequences (891 bp).** ● strains isolated from patients without neurovirulence in this study, ▲ strains isolated from patients with neurological symptom in this study. The phylogenetic tree was drawn using the neighbor joining method. Bootstrap values are shown as percentages derived from 1000 samplings and the scale reflects the number of nucleotide substitution per site along the branches.

strains by BioEdit 7.09 software did not reveal significant position associated with virulence. However, SDLY107 (a fatal strain) was found different from other strains on position  $T^{P7335}/C^{P7335}$ , suggesting that this position might be correlated to death.

Phylogenetic analysis of 31 strains based on the nucleotide sequences of 3'-UTR (Figure 7) showed strains

with different symptoms were mixed up, suggesting that there was no distinction on evolution between strains with or without neurological symptoms.

The secondary structure prediction of the complete 3'-UTR sequences showed that except strain SDLY48, the other five strains were almost the same (Figure 8).

**Table 2 Complete genome sequences of 31 strain used in this study**

| Strains with neurovirulence |                        | Strains without neurovirulence |                        |
|-----------------------------|------------------------|--------------------------------|------------------------|
| GenBank No.                 | Source                 | GenBank No.                    | Source                 |
| U22522                      | GenBank                | EU753384                       | GenBank                |
| AF316321                    | GenBank                | HQ129932                       | GenBank                |
| JQ514785                    | GenBank                | AF304459                       | GenBank                |
| EU753365                    | GenBank                | GQ994989                       | GenBank                |
| GQ231942                    | GenBank                | FJ607334                       | GenBank                |
| HQ828086                    | GenBank                | GQ231936                       | GenBank                |
| GU196833                    | GenBank                | AF119796                       | GenBank                |
| EU753397                    | GenBank                | AF304457                       | GenBank                |
| GQ994992                    | GenBank                | GQ231935                       | GenBank                |
| GQ231928                    | GenBank                | GQ994990                       | GenBank                |
| EU703814                    | GenBank                | FJ606449                       | GenBank                |
| FJ607337                    | GenBank                | DQ341361                       | GenBank                |
| EU703812                    | GenBank                | SDLY1                          | Isolated in this study |
| SDLY96                      | Isolated in this study | SDLY11                         | Isolated in this study |
| SDLY107                     | Isolated in this study | SDLY48                         | Isolated in this study |
| SDLY153                     | Isolated in this study |                                |                        |

## Discussion

EV71 is one of the most virulent enteroviruses and can cause mortality in children [1]. Defining virulent positions on molecular level is considered as one of the most important aspects of disease prevention. In our study, complete genomes of six EV71 strains with different clinical phenotypes were sequenced and analyzed. Together with other strains isolated in Shandong in recent years, the six strains clustered into C4a of C4 sub-genotype [18].

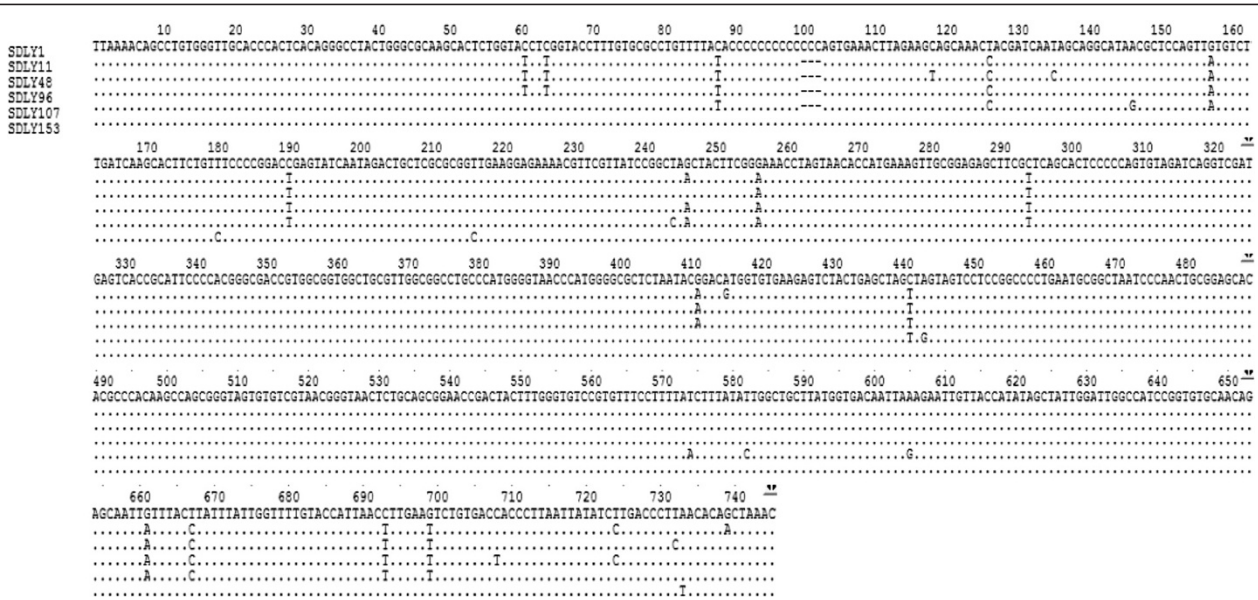
At present, molecular neurovirulence determinant of EV71 remains unclear, though virulence factors of other enteroviruses have been reported. Nucleotide 480, 481 and 472 on 5'-UTR of poliovirus were identified as neurovirulence determinants of poliovirus [19-21]. Minetaro et al. reported that mutation of the EV71 standard strain BrCr in 5'-UTR showed attenuated neurovirulence in the cynomolgus monkey model [22]. In this study, insertions and deletions were frequently found in 5'-UTR region. Two of three EV71 strains

(SDLY11 and SDLY48) from patients without neurovirulence had almost the same secondary structure of 5'-UTR, and all strains with neurovirulence (SDLY96, SDLY107 and SDLY153) were different from each another. In IRES element, domain III and II were relatively conserved regions, however, domain I, IV and V are very variable. These suggest that variation of the secondary structure of the 5'-UTR, especially domain I, IV and V might be correlated to the virulence. When aligned the strain isolated from a fatal patient (SDLY107) with other five strains, three position of 5'-UTR ( $C^{P241}/T^{P241}$ ,  $A^{P571}/T^{P571}$ ,  $C^{P579}/T^{P579}$ ) might be related to the virulence. Li et al. reported that four amino acids ( $Gly^{P710}/Gln^{P710}/Arg^{P710}$  and  $Glu^{P729}$ ) in the DE and EF loop of VP1, one ( $Lys^{P930}$ ) in the surface of protease 2A were potentially associated with EV71 virulence [23]. In our study, three positions,  $Val^{P814}/Ile^{P814}$  in VP1,  $Val^{P1148}/Ile^{P1148}$  in 3A and  $Ala^{P1728}/Cys^{P1728}/Val^{P1728}$  in 3C, were different between two phenotypes. These results suggest that three positions are potential virulent positions.

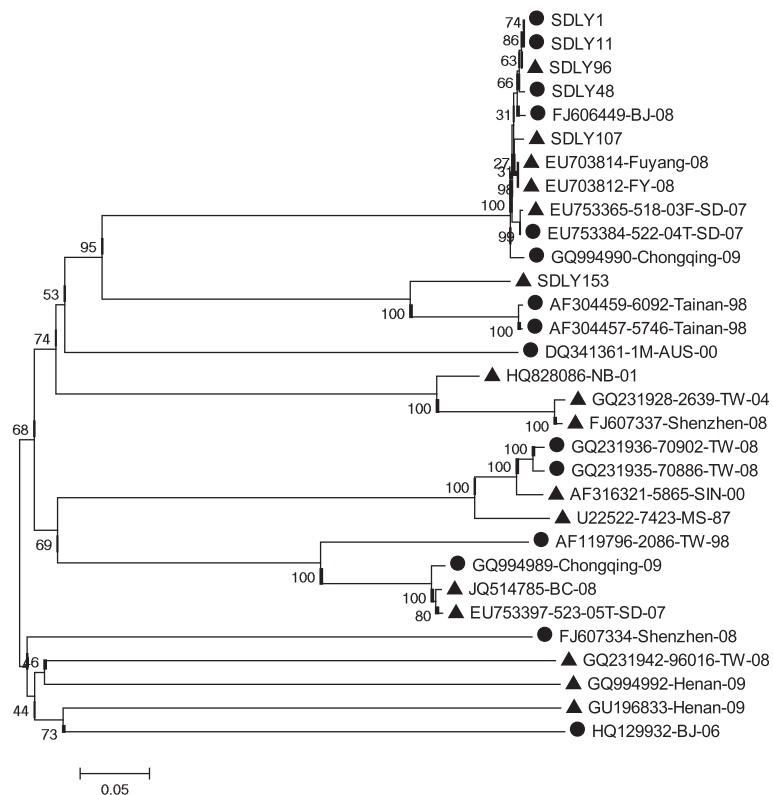
**Table 3 Significant positions of polyprotein of 31 strains**

| Region | Position | Amino acid                  |                                |
|--------|----------|-----------------------------|--------------------------------|
|        |          | Strains with neurovirulence | Strains without neurovirulence |
| VP1    | 814      | Val/Ile(16/0)*              | Val/Ile(10/5)                  |
| 3A     | 1148     | Val/Ile(16/0)               | Val/Ile(11/4)                  |
| 3C     | 1728     | Ala/Cys/Val (16/0/0)        | Ala/Cys/Val (10/1/4)           |

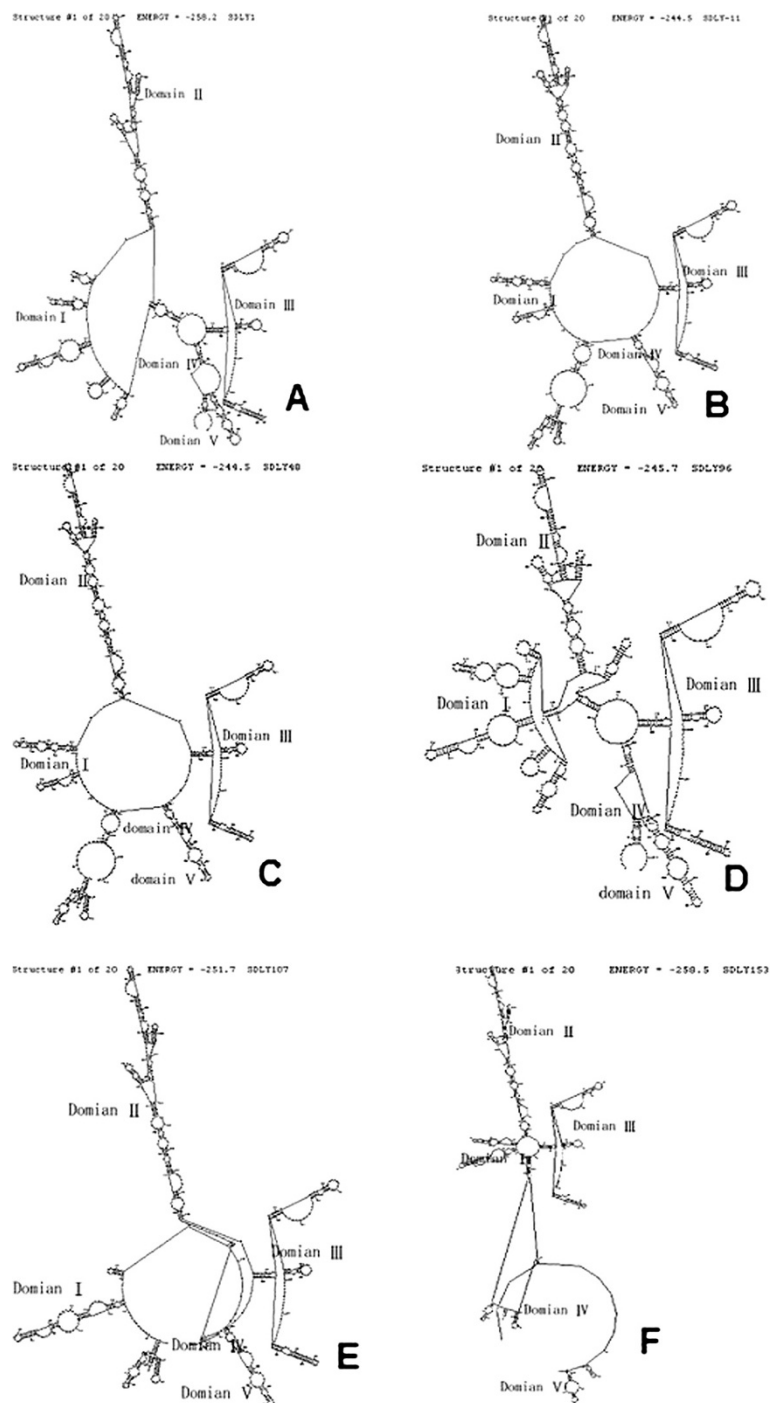
\*Val/Ile(16/0): in all 16 strains with neurovirulence, 16 of them were Val<sup>P814</sup> and none of them was Ile<sup>P814</sup>



**Figure 4** Sequence alignment of 5'-UTR sequences of 6 strains isolated in our study.



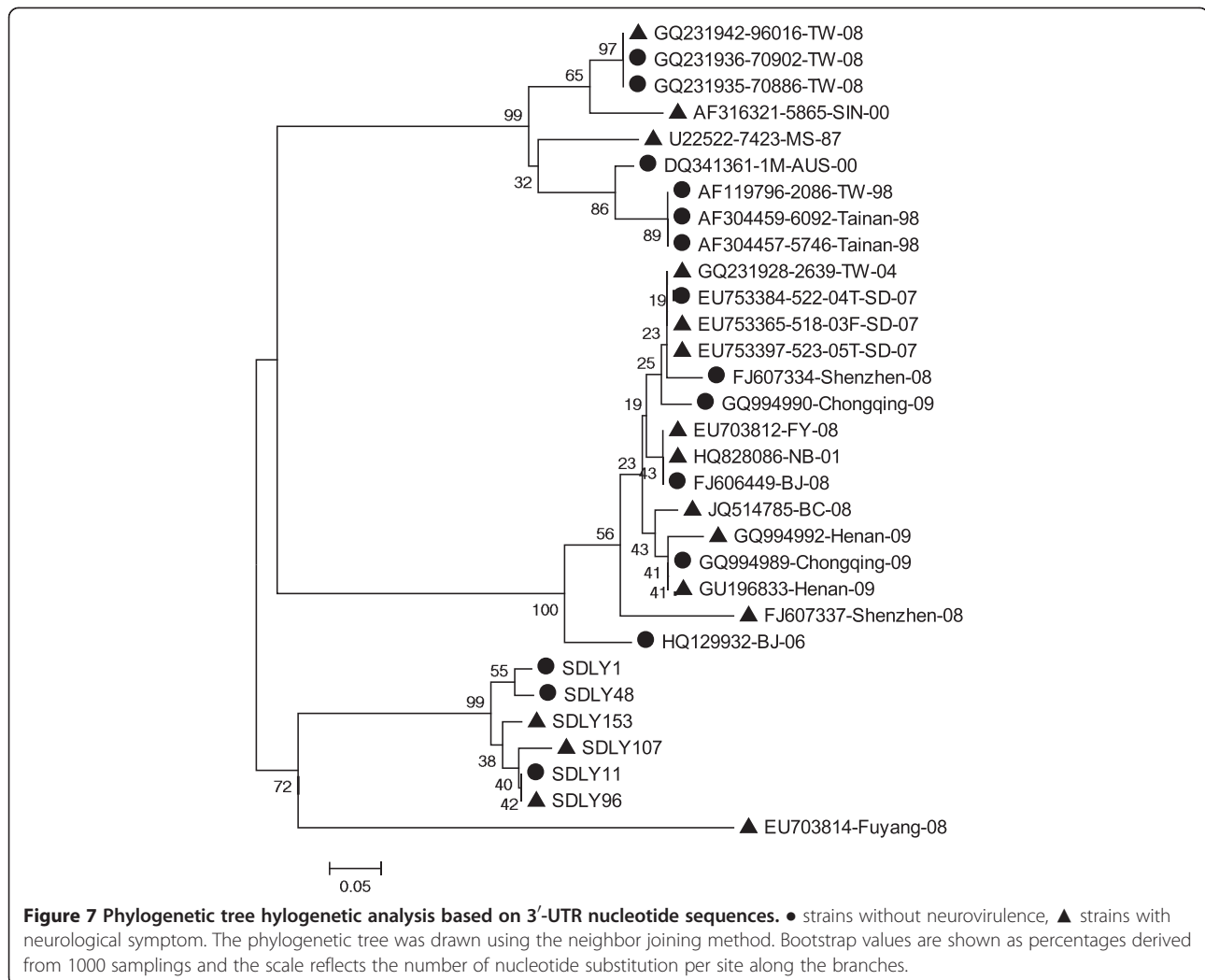
**Figure 5** Phylogenetic tree hylogenetic analysis based on 5'-UTR nucleotide sequences. ● strains without neurovirulence, ▲ strains with neurological symptom. The phylogenetic tree was drawn using the neighbor joining method. Bootstrap values are shown as percentages derived from 1000 samplings and the scale reflects the number of nucleotide substitution per site along the branches.



**Figure 6** The secondary structures of 5'-UTR. **A:** SDLY1, **B:** SDLY11, **C:** SDLY48, **D:** SDLY96, **E:** SDLY107, **F:** SDLY153.

The position 814 locates in C-terminal part of the VP1 protein which locates on the surface of the virus, mediates the initiation of infection by binding to receptors on the host membrane [24]. C-terminal part of the VP1 protein were supposed to be capable of eliciting neutralizing antibodies against EV71 [25].

Variations in VP1 region may influence the ability of the virus binding to host cell and eliciting neutralizing antibodies. Protein 3A plays a role in inhibiting cellular protein secretion and mediating presentation of membrane proteins during viral infection. Variations in 3A region may affect the process of viral infection. Protein



3C can cleave numerous factors and regulators that are associated with cellular DNA-dependant RNA polymerase I, II and III, and may be involved in the virus-induced blockage of host transcription. Variations in 3C region may affect activity of RNA polymerase and host cellular transcription. The three positions were conserved in strains with neurovirulence, and variable in strains without neurovirulence. These also reveals that the conservation of two of the three positions or the three together maybe specific for the strains with neurovirulence.

The 3'-UTR is a highly conserved domain and mutations in the 3'-UTR may cause change of phenotype. However, in our study, analysis of nucleotides of 3'-UTR showed no virulence associated nucleotides.

To test our aforementioned findings, site-directed mutagenesis need to be performed on these positions in the future study, and infectious cDNA clones with different potential virulent positions need to be constructed and evaluated at *ex vivo* and *in vitro*.

## Methods

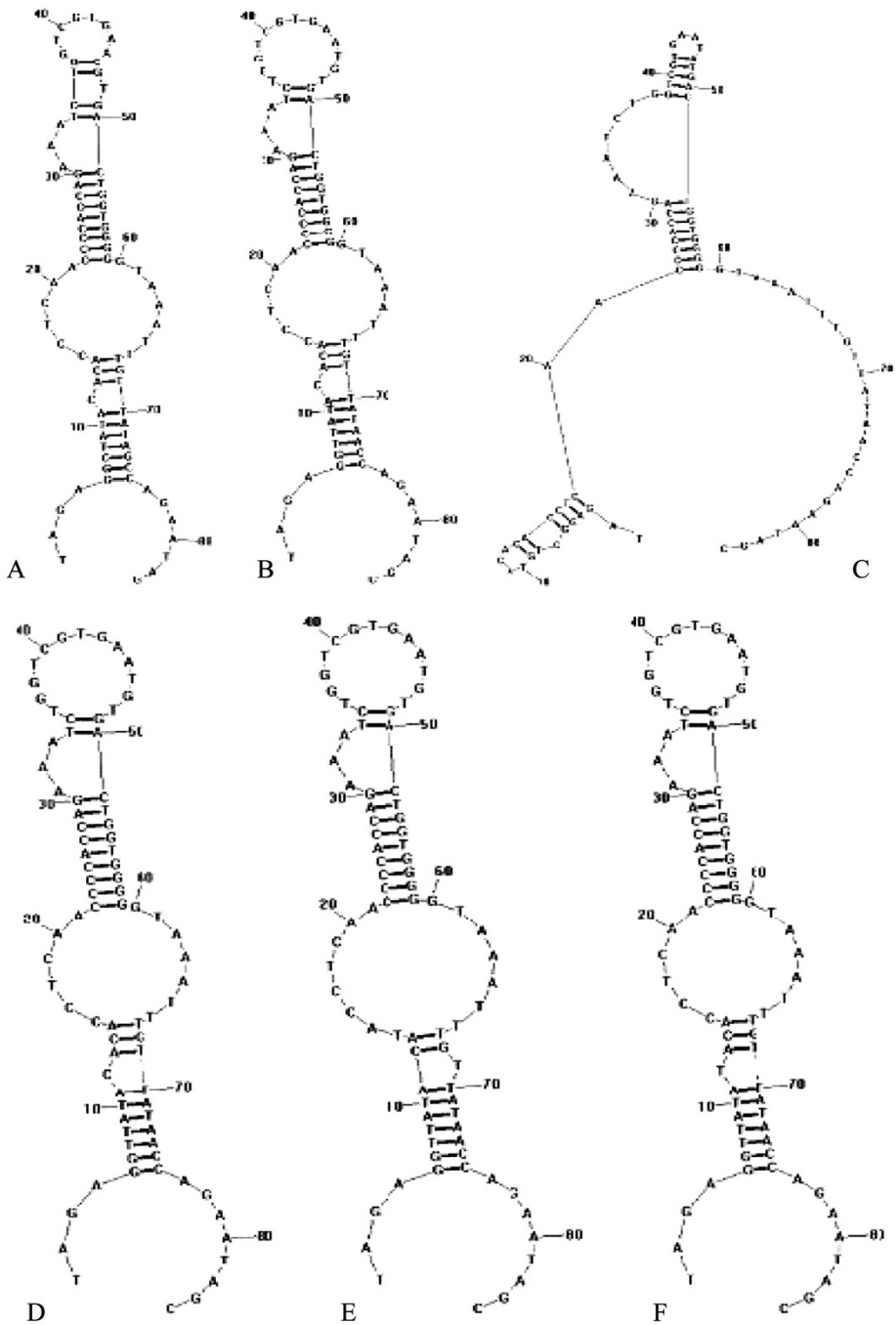
### Cells and viruses

EV71 strains SDLY1, SDLY11, SDLY48, and SDLY96 were isolated from stool samples of four patients without neurovirulence. SDLY107, SDLY153 were isolated from anal swabs samples of two patients. Among these strains, SDLY1, SDLY11 and SDLY48 were isolated from patients with mild symptoms. SDLY96 and SDLY153 were isolated from patients with neurological symptom, and SDLY107 was isolated from a fatal patient. All six patients were from Linyi City, Shandong Province, China. Human rhabdomyosarcoma (RD) cells were maintained in DMEM supplemented with 10% FBS. Viruses were propagated on RD cells to increase the titer for use in subsequent assays.

### RNA extraction and virus identification

Total virus RNAs were extracted from EV71-infected cell culture supernatants using a RNA extraction kit (OMEGA) following the manufacture's instructions. Virus





**Figure 8** The secondary structures of 3'-UTR. A: SDLY1, B: SDLY11, C: SDLY48, D: SDLY96, E: SDLY107, F: SDLY153.

**Table 4 Primers used for amplifying the genome**

| NO. | Name of primer | Sequence                   | Amplification(bp) |
|-----|----------------|----------------------------|-------------------|
| 1   | P1+            | TTAAACAGCCTGTGGTTGCACC     | 769 bp            |
|     | P769-          | GTGTAGACACTTGC GAACC       |                   |
| 2   | P719+          | C TTGACCC TTAACACAGCTA     | 914 bp            |
|     | P1632-         | GCTCCTTGGTCGTAGTCTAG       |                   |
| 3   | P1597+         | GTGCCTATTAGCCCACTAGAC      | 973 bp            |
|     | P2568-         | ACCTTGCCTGTATCCAGTCGA      |                   |
| 4   | P2532+         | ACAGGTGAGCAGTCATCGACT      | 815 bp            |
|     | P3346-         | CTGTTGTCCAAATTTCCCAAGA     |                   |
| 5   | P3216+         | GTGGATACCTCGCCCGATGC       | 919 bp            |
|     | P4134-         | CACTCTAACCCCTTAGCGGCGT     |                   |
| 6   | P4043+         | CCATCTTAGGTATCCCTATCGC     | 1003 bp           |
|     | P5045-         | TTGTGTTGCCAATGGCGGACC      |                   |
| 7   | P4969+         | GTCAGATACAGTGTGGATACG      | 909 bp            |
|     | P5877-         | CCACCAATGTGAATACCGACAA     |                   |
| 8   | P5799+         | CAACTTCTACTAAAGCAGGAC      | 769 bp            |
|     | P6567-         | CCCACGGCTGATCCAGTTATCG     |                   |
| 9   | P6500+         | TGGCTTTCGGACATTTGTATGA     | 906 bp            |
|     | P7405-         | GCTATTCTGGTTATAACAAATTTACC |                   |

types were identified by One-Step RT-PCR described previously [26].

#### Segmented amplification of the complete genomes

Nine overlapping clones covering the whole viral genome were obtained by RT-PCR (QIAGEN, OneStep RT-PCR Kit). RT-PCR amplifications were carried out with the primers in Table 4. RT-PCR products were purified using Gel Extraction Mini Kit (OMEGA) and were cloned to the pMD19-T plasmid (TaKaRa). The recombinant vectors were transformed into competent *E. coli* DH5 $\alpha$ . Positive clones were sequenced by Biosune Biotechnology Co. Ltd.

#### Sequences analysis

The nucleotide sequences of six complete genomes and the derived amino acid sequences were analyzed by BioEdit 7.09 software. The genotype and subgenotype were determined by comparing sequences with reference strains from GenBank. The secondary structures of 5'-UTR and 3'-UTR were predicted by RNA structure 4.0 software. The phylogenetic tree was constructed using MEGA 4 software based on the nucleotide sequences of the complete VP1 region.

#### Ethics statement

This study was approved by the ethical committees of School of Public Health, Shandong University, Jinan, Shandong 250012, China (permit number 20080301).

Written consents were obtained from all children's parents involved in the study.

#### Abbreviations

EV71: Enterovirus 71; HFMD: Hand, foot and mouth diseases; RT-PCR: Reverse transcription-polymerase chain reaction; ORF: Open reading frame; IRES: Internal ribosome entry site; DMEM: Dulbecco's modified Eagle's medium; FBS: Supplemented with 10% fetal bovine serum.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

HLW, ZYW and SBH conceived the study and designed the experiments. LYS, XJY, FLC, CXS performed the experiments. HLW and LYS analyzed the data and wrote the manuscript. FG contributed in sample collection. All authors read and approved the final manuscript.

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