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

Yen-Hua Kung [a], Sheng-Wen Huang [b], Pin-Hwa Kuo [e], David Kiang [h], Mei-Shang Ho [f], Ching-Chung Liu [c], Chun-Keung Yu [d], Ih-Jen Su [g], Jen-Ren Wang [a,b,g,*]

[a] Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, Tainan, Taiwan
[b] The Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan
[c] Department of Pediatrics, National Cheng Kung University, Tainan, Taiwan
[d] Department of Microbiology and Immunology, National Cheng Kung University, Tainan, Taiwan
[e] Department of Pathology, National Cheng Kung University Medical Center, Tainan, Taiwan
[f] Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan
[g] Division of Infectious Diseases, National Health Research Institutes, Tainan, Taiwan
[h] Viral and Rickettsial Disease Laboratories, California Department of Public Health, CA, USA

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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.

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.

* Corresponding author. Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, One University Road, Tainan, 701, Taiwan. Fax: +886 6 2760695.
E-mail address: jrwang@mail.ncku.edu.tw (J.-R. Wang).

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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

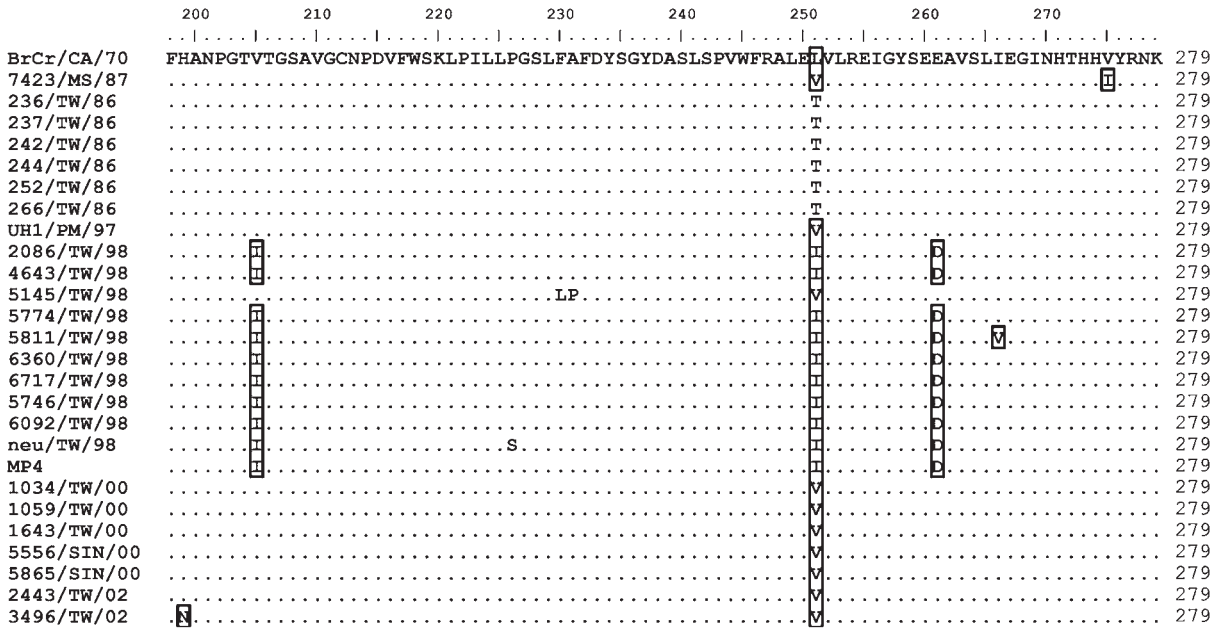

Fig. 1. Alignment of partial 3D amino acid sequences of EV71 clinical isolates from 1986, 1998 and post-1998 outbreaks. The amino acid sequences were deduced from nucleotide sequences and aligned using Vector NTI, and the figure was generated with BioEdit. The I251T mutation is highlighted by the black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