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Original Article

Association of EV71 3C polymorphisms with clinical severity

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Abstract *Objectives:* Enterovirus 71 (EV71) may cause neurological and fatal cases. EV71 3C plays an important role on viral replication and possess proteolysis activity. To delineate pathogenesis of EV71 virulence, we studied EV71 3C genetics, protease activity and correlated the results with clinical severity.
Methods: EV71 cases were collected; 3C of EV71 was sequenced and linked with clinical severity. 3C protease activity, viral replication rates of EV71 infectious clones with different 3C and 3C interaction with host proteins were analyzed.
Results: The polymorphisms of EV71 3C at the 79th amino acid were associated with clinical severity. About 26% (62/234) patients infected by EV71 with wild-type 3C (T79) had neurological involvement but 78% (25/32) patients infected by EV71 with mutant 3C (T79V) did ($p < 0.001$). There was no significant difference of protease activity among the different 3C variants. EV71 with mutant 3C (T79V) had the highest viral replication rate and the mutant 3C (T79V) had weaker interaction with TRIM21, a component of antibody-dependent intracellular neutralization, than the other mutants (T79I and T79A).
Conclusion: We found that 3C polymorphisms were associated with clinical severity and viral replication, which might be related to 3C interaction with important host proteins such as TRIM21.

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Introduction

Enterovirus 71 (EV71) is associated with a spectrum of diseases including hand-foot-mouth disease (HFMD), herpangina, aseptic meningitis, encephalitis and poliomyelitis-like syndrome.[1] An epidemic caused by EV71 occurred in Taiwan from April to July 1998, when 78 children died from cardiopulmonary failure among the 405 severe cases with central nervous system involvement.[2] Since 2008, the virus has continued to circulate in China as well as the other Asian countries and caused severe cases and deaths among children.[3] The neurovirulence associated with significant mortality is specific for EV71,[4] which might result from the specific pathogenesis of the virus.

EV71 is a positive-stranded RNA virus encoding a polyprotein containing 2200 amino acids, which are processed into structural proteins and nonstructural proteins. Several viral proteins were hypothesized to be related to clinical severity, such as viral protein 1 (VP1), 5′-non-coding region (5′-NCR),[4,5] 3A and 3C protein etc. 3C protein is a viral protease responsible for viral protein processing. In addition, 3C protease was also reported to cleave some host cellular proteins,[6] such as cleavage stimulation factors subunit 64KD (CstF-64) or Poly(A)-binding protein (PABP), to further affect host machinerises.[7,8] Previous studies found that the substitution of 147th amino acid or 40th amino acid might disrupt 3C protease activity and affected host protein expression.[7,9] It remains unknown whether the polymorphisms of 3C protease affect host cellular protein processing, viral replication rates, or host protein interaction, which further leads to different clinical severity and outcomes. Our study aimed to find out the association of the EV71 3C polymorphisms with clinical severity and to clarify possible interactions of EV71 3C protease with host proteins responsible for viral virulence.

Methods

Identification of EV71 cases and definition of clinical severity

This study was approved by the institutional review board with the approved number of 9361701136 at National Taiwan University Hospital. We collected EV71 cases and their clinical isolates from National Taiwan University Hospital and Chang Gung Children's Hospital between 1998 and 2003.

All the EV71 cases had viral culture confirmation of EV71 by the standard laboratory procedure in both hospitals and had clinical manifestations of either hand-foot-and-mouth disease (HFMD), herpangina or febrile illness. We divided clinical severity into 3 categories: (1) 244 uncomplicated cases, (2) 148 cases with central nervous system (CNS) involvement but without cardiopulmonary failure/pulmonary edema, (3) 27 cases with both CNS involvement and cardiopulmonary failure/pulmonary edema. Severe disease was defined as having CNS involvement with or without cardiopulmonary failure/pulmonary edema. CNS involvement included aseptic meningitis, encephalitis, poliomyelitis-like syndrome or encephalomyelitis. The case definition is as our previous report.[1]

Sequencing of EV71 3C

Viral RNA from clinical EV71 isolates was extracted using a viral RNA extraction kit (QIAamp Viral RNA MiniKit, Qiagen). RNA samples underwent amplification by one-step RT-PCR kit (Titan One Tube RT-PCR System, Roche Diagnostics, Indianapolis, IN, USA) using the standard primer (forward: 5′TTYCARGGWGCDTAYTCY, reverse: 5′ TGATGTTCAACCTGCCAGTTTCTTT or 5′ AYCCAYTGGATCTCWCCTTG) for EV71 3C and the products were purified by High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN, USA). Later, cycle sequencing was performed by using the purified PCR products, the ABI Prism BigDye® Terminator cycle sequencing kit and ABI Prism 3730 DNA sequencer (Model 3730 version 3.1, Applied Biosystems, Foster City, CA, USA). According to the frequency of different polymorphisms of 3C at the 79th amino acid, we defined the most frequent type (the 79th amino acid to be tryptophan, T79) to be the wild-type 3C and others to be mutant 3C as the 79th amino acid to be A, I or V (alanine T79A, isoleucine T79I, or valine T79V).

We compared clinical severity among the EV71 cases with different 3C variants according to the polymorphisms of the 79th amino acid. Later, we examined differences in protease activity, viral replication rates and host protein interactions among different 3C variants.

Protease activity of wild-type or mutant 3C

Modification and expression of the plasmids

pET28a-3C was constructed with wild 3C and *E. coli* plasmid pET28a. We digested the coding sequence of 3C protease from previously produced pET28a-3C and inserted to pcDNA3.0-HA with BamHI and XhoI restriction sites to create different EV71 3C mutants. These plasmids of pcDNA3-HA-3C variants were transfected into SF268 cells, which were glioblastoma cells, using *Trans*IT®-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA). After 30 and 48 h post-transfection, the total cell lysates were harvested and analyzed by western blot with mouse anti-HA antibody (Merck Millipore, Massachusetts, USA) to check expression of HA-3C variants.

Analysis of 3C protease activity

For analysis of protease activity among different 3C variants, we did western blotting of the cell lysates with the antibodies of 3C substrates to check whether these substrates were cleaved. The 3C substrates included IκBα (inhibitor of κBα, Epitomics, Abcam®, Cambridge, MA, USA), CstF64 (cleavage stimulation factor, 64 kDa, Abcam, Cambridge, MA, USA), IRF7 (interferon regulatory factor 7, Epitomics, Abcam®, Cambridge, MA, USA), PARP [poly(ADP-ribose) polymerase, Cell Signaling Technology, Danvers, MA, USA], eIF5B (eukaryotic initiation factor 5B, Abnova, Walnut, CA, USA), PABP [poly(A)-binding protein, Merck

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