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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

Millipore, Massachusetts, USA] and COX-2 (cyclooxygenase-2, Epitomics, Abcam®, Cambridge, MA, USA).

Growth curve of EV71 containing 3C variants

The EV71 infectious clone pCR®2.1-TOPO-EV71 was modified by site-directed mutagenesis to encode either alanine, isoleucine, or valine at the 79th codon using commercial kits with standard primers (NEB Q5 Site-Directed Mutagenesis Kit, T79A forward: 5′ AACGCTTGATgCTAACGAAAAATTTAG, reverse: 5′ ACCAGTGTGAGCTCTAAG; T79I forward: 5′ ACGCTTGATAtTAACGAAAAATTTAG, reverse: 5′ TACCAGTGTGAGCTCTAAG; T79V forward: 5′ AACGCTTGATgtTAACGAAAAATTTAGAGAC, reverse: 5′ ACCAGTGTGAGCTCTAAG) in an Applied Biosystems 2720 thermal cycler. The plasmids were purified using Viogene DNA/RNA extraction kit (Viogene Bio Tek Corp. New Taipei City, Taiwan). RD cells were transfected with 0.01 m.o.i plasmids DNA complex (2 μg pCR®2.1-TOPO-EV71 wild 3C or mutant 3C, 2 μg pAR3132 for T7 polymerase) using transfection reagent (TransIT-LT1, Mirus Bio LLC, Madison, WI, USA). After one to five days post-transfection, the supernatants were harvested and analyzed by plaque assay to characterize the growth curve.

Interaction of 3C variants with host proteins

After 24 h post-transfection, the SF268 cells transfected with HA tagged wild-type 3C and mutant 3C were lysed. We identified host proteins which interacted with 3C by anti-HA antibody with Pierce crosslink immunoprecipitation kit (Thermo Fisher Scientific Inc, Grand Island, NY, USA). These immunoprecipitation fractions were analyzed by SDS-PAGE gel and visualized by silver staining. We further analyzed the significantly different immune-precipitated bands between the mutant 3C fractions and the wild-type 3C by ESI LC-MS/MS (LTQ Orbitrap Velos, Thermo Fisher Scientific Inc, Grand Island, NY, USA). We confirmed the host protein interacting with EV71 3C protein by western blotting of immunoprecipitation gel with antibody.

Statistical analysis

We examined the association of clinical severity among the EV71 cases with different 3C polymorphisms by chi-square test and compared growth curves of EV71 infectious clones containing either wild-type 3C or mutant 3C by repeated measures ANOVA test.

Results

Association of EV71 3C polymorphisms with EV71 clinical severity

The polymorphisms of 3C at the 79th amino acid are associated with clinical severity as demonstrated in the Table 1. We found that about 26% (62/234) patients infected by EV71 with wild-type 3C (T79) had severe diseases. However, 78% (25/32) patients infected by EV71 with mutant 3C (T79V) had severe diseases: 72% patients with CNS involvement and 6% with CNS involvement plus cardiopulmonary failure/pulmonary edema. Another EV71 3C mutant T79I was also associated with a higher proportion of severe disease and 59% (88/150) of the infected patients had severe disease. Due to different clinical severity caused by EV71 with different 3C polymorphisms, we next tried to compare difference of some viral factors including protease activity, viral replication rates and host protein interactions to find out the possible pathogenesis among different 3C variants. Since many previous studies reported 3C protease cleavage activity of various host proteins, we first examined whether there was difference of the 3C protease activity among different 3C variants.

Protease activity among 3C variants

We confirmed the expression of reconstructed plasmids with 3C variants in the SF268 cells with western blotting. After 30 and 48 h post-transfection, we examined the levels of different 3C protease substrates by western-blotting to assess the protease activity. Fig. 1 shows that there was no difference between the levels of 3C protease substrates in both the wild-type 3C and mutant 3C transfected cell, such as IκBα, CstF64 (R7), IRF7, PARP, eIF5B, and PABP. Therefore, 3C protease activities for currently known substrates were the same in the wild type 3C as in the mutant types. However, the expression of cyclooxygenase-2 (COX-2) was only up-regulated in cells expressing wild-type 3C but not mutant 3C which had the same expression level as the cell transfected with the vector, as shown in Fig. 2. Since there was no significant difference in the protease activity of different 3C proteins, we further investigated that whether

Table 1 Number and percentage of EV71-infected patients with different severity correlating with the polymorphisms of 3C protease at the 79th amino acid of EV71 isolates.

Clinical severity	3C protease polymorphisms[c]				p value
	A	I	T	V	
Uncomplicated (N = 244)	3 (100%)	62 (41%)	172 (74%)	7 (22%)	
CNS[a] (N = 148)	0 (0%)	72 (48%)	53 (23%)	23 (72%)	<0.001
CNS + PE[b] (N = 27)	0 (0%)	16 (11%)	9 (4%)	2 (6%)	

[a] CNS: EV71 infection with central nervous system involvement.
[b] CNS + PE: EV71 infection with central nervous system involvement with cardiopulmonary failure/pulmonary edema.
[c] Level of significance for the association between clinical severity and the 3C protease polymorphisms of EV71 isolates, $p < 0.001$.
A denotes alanine, I isoleucine, T tryptophan and V valine. T is wild type and the others are mutant.

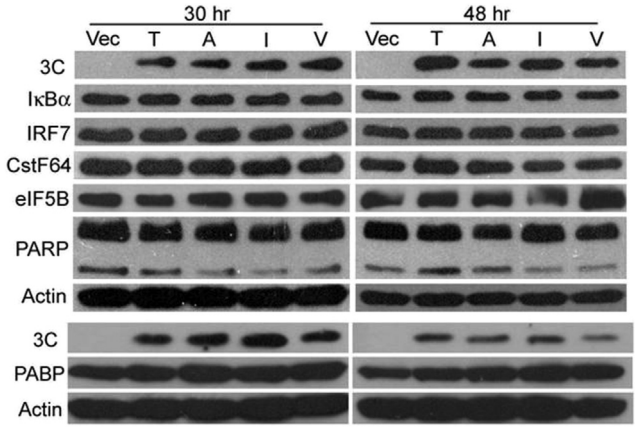

Figure 1. The protease activity of 3C. After 30 and 48 h post-transfection, there were no differences in the levels of different 3C protease substrates previously known, i.e., IκBα (inhibitor of κBα), CstF64 (cleavage stimulation factor, 64 kDa), IRF7 (interferon regulatory factor 7), PARP [poly(ADP-ribose) polymerase], eIF5B (eukaryotic initiation factor 5B) and PABP [poly(A)-binding protein] in the presence between wild-type 3C and mutant 3C protease.

the viral replication rates were different among EV71 with different 3C variants.

Significantly higher replication rate of EV71 with mutant 3C (T79V)

We confirmed that the infectious clones, pCR®2.1-TOPO-EV71 with different 3C variants, were successfully transfected into RD cells by western blotting with anti-EV71 antibody. We measured the virus growth curves of EV71 infectious clones by plaque assay. Fig. 3 showed the plaque numbers at 1-day through 5-day of EV71 with either wild-type 3C or mutant 3C and significant difference was found ($p < 0.05$). At 5-day post-infection, the plaque number of the infectious clone containing T79V was about 2 folds more than those containing other 3C mutants (T79A, T79I). Because most cases (78%, 25/32) infected by EV71 containing mutant 3C (T79V) had severe diseases with CNS involvement or cardiopulmonary failure/pulmonary edema, we hypothesized that the higher viral replication rates of EV71 with the mutant 3C (T79V) might be resulted from the different interaction with host proteins. Therefore, we

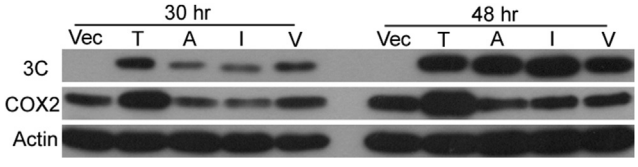

Figure 2. Cyclooxygenase-2 level in SF268 cells transfected with 3C variants. After SF268 cells were transfected with wild-type 3C protease and mutant 3C protease, significantly higher level of COX-2 (cyclooxygenase-2) was found only in the cells transfected with wild-type 3C protease.

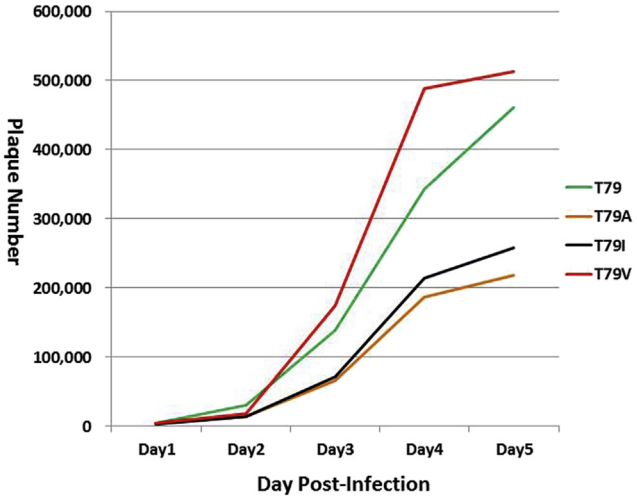

Figure 3. The growth curves of EV71 containing 3C variants. The growth curves of EV71 with different 3C variants assessed by plaque assay on Day 1 through Day 5 post-infection ($p < 0.05$ with repeated measures ANOVA test).

further investigated the virus-host protein interactions by immunoprecipitation.

Different interaction of EV71 3C with TRIM21

We confirmed the successful production of HA-tagged 3C proteins. The Supplementary Figure shows the immunoprecipitation result analyzed by SDS-PAGE with silver stain. There were 7 bands (A through G) showing significant difference between host cells infected by wild-type or mutant EV71. The Supplementary Table lists the 18 possible host proteins identified by ESI LC-MS/MS. We further confirmed the interaction by western blotting with antibody to verify these 18 host proteins. At last, within the 18 possible proteins, Fig. 4 shows that the only host protein confirmed to interact with EV71 3C protein is E3 ubiquitin-protein ligase TRIM21 (tripartite motif-containing protein 21), which targeted virions for proteasome and valosin-containing protein (VCP)-dependent degradation in a process known as antibody-dependent intracellular neutralization. The mutant type T79V had weaker interaction with TRIM21 than the other 3C mutants (T79A, T79I).

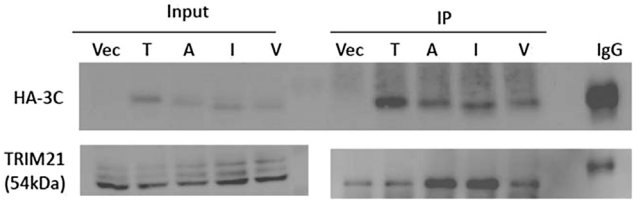

Figure 4. Interaction of EV71 3C protein with TRIM21. Western blotting with anti-TRIM21 (tripartite motif-containing protein 21) antibody revealed that interactions between TRIM21 and different EV71 3C variants are different. EV71 with 3C protease with T79A and T79I variants had stronger interaction then those with T79 and T79V.

Discussion

Our study shows that different EV71 3C polymorphisms at 79th amino acid were associated with clinical severity. EV71 with the mutant 3C (T79V) was linked with the worst clinical severity and the higher viral replication rate of EV71 with mutant 3C (T79V) might explain this phenomenon. The high viral replication rate of EV71 with mutant 3C (T79V) did not seem to result from the increased 3C protease activity, since there was no difference of the 3C protease activity among EV71 3C variants with polymorphisms at 79th amino acid in our study. EV71 3C protein may not only have protease activity but also other functions to affect the clinical severity. From our results, we hypothesize that the higher viral replication rate in EV71 with mutant 3C (T79V) may be due to different interaction of 3C with host proteins to help the virus evade host intracellular immune system.

Previous studies mainly focused on the cleavage of host proteins, such as CstF-64, PABP, by EV71 3C protein. However, we did not find any difference of 3C protease activity among different variants.[7,8] Here we demonstrated the interaction of EV71 3C with a new target host protein, TRIM21, which is not through the protease cleavage pathway. The different interaction of 3C with TRIM21, a component of antibody-dependent intracellular neutralization, may lead to different host immune defense,[10] and thus result in different replication rate and different clinical severity. TRIM21 was an intracellular cytosolic protein with a high affinity for IgG.[10] TRIM21 rapidly recruited incoming antibody-bound virus and targeted it to the proteasome via its E3 ubiquitin ligase activity.[11] TRIM21 was also proven to restrict adenovirus, herpes simplex virus infection in previous studies.[10,12] Our study demonstrated the interaction of EV71 3C protein with TRIM21. The interactions were strong in EV71 with 3C variants T79I and T79A but the interactions were weaker in EV71 with T79V and wild-type (T79). Since TRIM21 was involved in the intracellular viral neutralization,[10,12] the weaker interaction between TRIM21 and EV71 3C variants (T79, T79V) might contribute to lesser extent of EV71 intracellular neutralization and made EV71 replicate better as the results of plaque assay showed that EV71 3C variants (T79V, T79) had the higher plaque numbers. The decreased intracellular neutralization effect of EV71 3C variant (T79V) might cause much more severe clinical presentations in our EV71 cases. T79 (wild type) grew better but had less complications, which might be related to the other factors such as the other host immune response. The evidence is that we observed up-regulation of COX-2 by EV71 with wild-type 3C, which indicated better immune response.

Our study found some different results compared with previous studies. First, in contrast to previous studies, our study failed to demonstrate different 3C protease activities between different EV71 3C variants. Previous studies revealed the direct 3C protease cleavage of CstF-64, PABP, eIF5B, IRF7 in EV71 with wild-type 3C but not mutant 3C.[7,8,13,14] Also, some studies demonstrated induced cleavage of host protein by 3C protease, such as TRIF.[15] These might be due to the different mutation site of EV71 3C protease in our study. Previous studies revealed that the mutations of 3C protein at 40th amino acid, 189th amino acid or 190th amino acid were associated with different protease cleavage activities.[14] Our mutation site of 3C protease was at the 79th amino acid, which might not be the active site for protease cleavage. Therefore, the different clinical severity of EV71 with 3C variants at 79th amino acid might be associated with different virus-host protein interaction rather than protease activity.

Second, we observed up-regulation of COX-2 by EV71 with wild-type 3C protease, but not the other variants. COX-2 was an enzyme catalyzing the biosynthesis of prostaglandins and thromboxanes, which are related to inflammation, angiogenesis or carcinogenesis.[16,17] The expression of COX-2 was rapidly induced by proinflammatory or mitogenic activators, which resulted in production of hyperalgesic and pro-inflammatory prostaglandins. Viral infections were shown to stimulate COX-2 mRNA accumulation and PGE2 expression,[18] which was true in coxsackievirus B3.[19] Up-regulation of COX-2 by wild 3C but not mutant 3C in this study might be a hint of a better host immune response and thus less complications in cases with wide 3C. However, further studies are needed to prove this hypothesis. This also help explain the reason why T79 (wild type) grew better but caused less complications, which might be related to the other factors such as host immune response.

Conclusions

Our study found that polymorphisms of 3C protease at the 79th amino acid were related to clinical severity. EV71 with 3C mutant (T79V) had the most severe clinical presentations, which might be related to a high viral replication rate possible through the weaker interaction with host protein TRIM21 and thus poorer immune response, while EV71 with wide type 3C (T79) induced up-regulation of COX-2, which was a hint of stronger host immune response and thus less complications in cases with wide 3C.

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Conflict of interest statement

All the authors declared that they did not have conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2016.12.006.