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BRIEF REPORT

Association of Toll-like receptor 3 gene polymorphism with the severity of enterovirus 71 infection in Chinese children

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Abstract Enterovirus 71 (EV71) infection has become one of the major threats to children globally in recent years. Toll-like receptor 3 (TLR3) plays an essential role in host defense against EV71 infection. This study was designed to assess the possible association between the TLR3c.1377C/T polymorphism and disease severity in Chinese children with EV71 infection. The TLR3c.1377C/T gene polymorphism was identified in EV71-infected patients (n = 177), including mild cases (n = 99) and severe cases (n = 78) as well as healthy controls (n = 225), using improved multiplex ligation detection reaction (iMLDR) technology. Serum levels of IFN-γ and IL-4 were measured using enzyme-linked immunosorbent assays. The presence of the TT genotype ($p = 0.030$) and the T allele (OR, 1.8; 95% CI, 1.2-2.8; $p = 0.010$) was significantly more frequent in severe cases. The plasma levels of IFN-γ and the IFN-γ/IL-4 ratio were significantly lower with the TT (102.0 ± 24.2 pg/mL, $p < 0.01$ and 14.2 ± 2.8, $p < 0.001$) and CT genotypes (114.1 ± 26.2 pg/mL, $p < 0.05$ and 18.0 ± 3.1, $p < 0.001$) than with the CC genotype (135.5 ± 36.8 pg/mL and 24.9 ± 4.7), but the plasma levels of IL-4 with the TT (7.3 ± 1.7 pg/mL, $p < 0.01$) and CT genotypes (6.4 ± 1.3 pg/mL, $p < 0.05$) were significantly higher than with the CC genotype (5.5 ±1.3 pg/mL). These findings suggest that the TLR3c.1377T allele is associated with susceptibility to severe EV71 infection in Chinese children.

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Introduction

Enterovirus 71 (EV71) is a positive-sense single-stranded RNA virus belonging to the species *Enterovirus A* of the genus *Enterovirus* within the family *Picornaviridae* [1]. EV71 was first isolated in California in 1969 from an infant with severe encephalitis [2]. Since then, many EV71 outbreaks have been reported among children throughout the world [3]. Although the virus is present globally, the largest EV71 outbreaks have been predominantly found in the Asia-Pacific region [4, 5]. The clinical spectrum of EV71 infection is wide, ranging from a mild and self-limiting hand, foot, and mouth disease (HFMD) to aseptic meningitis, poliomyelitis-like acute flaccid paralysis, brainstem encephalitis, and other rare fatal manifestations with high fatality rates and severe sequelae [5, 6]. Currently, the pathogenesis of EV71 infection and its varied clinical outcomes remain elusive, but in a recent study, Zhu et al. found that TLR3-mediated responses are involved in protective immunity against EV71 infection in a mouse model [7].

Toll-like receptor 3 (TLR3) is one of the pattern-recognition receptors (PRRs) that play a fundamental role in recognition of invading pathogens and subsequently activate host innate immunity. TLR3 specifically

recognizes double-strand RNA (dsRNA) [8], a replicative intermediate generated during most viral infections, including EV71 infection [9]. A deficiency of TLR3 was found to increase viral replication and alter the type of immune response, resulting in progressively impaired cardiac function in mice following coxsackievirus B3 infection [10]. Genetic polymorphism of the TLR3 gene has been reported to be associated with a number of diseases, including those caused by hepatitis B virus [11], coxsackievirus B3 virus [12], and Crimean-Congo hemorrhagic fever (CCHF) virus [13]. A common single-nucleotide polymorphism of TLR3c.1377C/T (rs3775290) is located in exon 4 of TLR3 on chromosome 4, and this C-to-T change results in a silent mutation at phenylalanine residue 459, which might affect the receptor-ligand interaction by altering the TLR3 ectodomain and functionally impairing the receptor [14]. Based on this background information, we examined whether the TLR3c.1377C/T gene polymorphism is associated with the severity of EV71 infection.

Materials and methods

Study population

All participants were enrolled from the Pediatric Department of the Affiliated Hospital of Qingdao University Medical School and Pediatric Department of the Qingdao Women & Children Hospital, China. A total of 177 patients (aged from 1.0 to 6.7 years) with EV71 infection were included in this study. EV71 infection was identified by reverse transcription polymerase chain reaction (PT-PCR). RNA was extracted from throat swabs, rectal swabs, vesicular fluid, cerebrospinal fluid (CSF) and vesicular fluid, which were collected from each patient on the day of admission. The control group consisted of 225 unrelated healthy subjects (aged from 1.0 to 6.0 years) who presented for check-up in the same period, without any clinical evidence of EV71 infection or having had contact with EV71-infected patients, which was confirmed by testing RNA extracted from throat swabs.

Hand, foot, and mouth disease (HFMD) was diagnosed based on clinical manifestations of typical oral ulcers and skin eruptions on the hands and feet, using the hand, foot and mouth disease diagnosis and treatment guidelines (2010) published by the Chinese Ministry of Health [15]. The HFMD cases were divided into a mild group and a severe group according to the guidelines. Mild cases were characterized by typical lesions on the hands, feet, mouth and buttocks, with or without fever. EV71-infected patients with the following characteristics were regarded as severe cases: (1) persistent high fever; (2) muscle weakness, limb jitter, spasm, disorder of consciousness, weak or absent tendon reflexes, positive meningeal stimulation; (3) pallor, tachycardia, impaired peripheral circulation, abnormal blood pressure; (4) dyspnea or irregular breathing rhythm, cyanosis, increased lung wet rale, signs of lung consolidation; (5) high ($>15 \times 10^9$/L) or low ($<2 \times 10^9$/L) peripheral blood leukocyte count; (6) significantly elevated blood glucose (>9 mmol/L); (7) abnormal chest X-ray [15, 16]. All patients received a general physical examination, as well as laboratory tests within 3 days after admission. Clinical baseline data and laboratory parameters were recorded.

This study was approved by the Clinical Research Ethics Committee of the Affiliated Hospital of Qingdao University. All participants' guardians provided written informed consent.

Measurement of virus load

Fluorescent quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to determine the number of copies of viral RNA present in samples. Total RNA was extracted from 150 μL of various swab samples using an RNAiso Plus Kit (Takara, Dalian, China) followed the manufacturer's instructions. Next, total RNA was reverse transcribed with random hexamers using a Reverse Transcription Kit (Thermo Scientific). The cDNA was subjected to quantitative PCR in a 50-μL reaction mixture (Thermo Scientific DyNAmo SYBR Green qRT-PCR Kit) with primers EV71-S (5'-GTTCTTAACTCACATAGCA-3') and EV71-A (5'-TTGCAAAAACTGAGGGTT-3') for EV71 VP1, and the conditions consisted of a denaturation step at 95 °C for 15 min and 40 cycles of thermal cycling at 95 °C for 10 s and 60 °C for 60 s. A series of dilutions containing 1×10^7, 1×10^6, 1×10^5, and 1×10^4 copies/μL of a DNA fragment derived from EV71 was used to make a standard curve for calculating the copy numbers of virus RNA in various samples. Quantitative real-time RT-PCR was performed using an MxproMx3000P system.

Analysis of genetic polymorphism

Genomic DNA specimens were prepared from 200 μL of peripheral blood, using a commercial kit and following the manufacturer's instructions (Omega, USA). Genomic DNA was tested by electrophoresis on a 1% agarose gel and diluted to a working concentration of 5-10 ng/μl. Peripheral blood samples were obtained from all of the participants. An improved multiplex ligation detection reaction (iMLDR) method was used to detect the TLR3 rs3775290 polymorphism, which is located in exon 4 of TLR3 on chromosome 4q35, and the product size was 176 bp. The specific primers for TLR3 were as follows: forward, 5'-

ACTCACAGGCCAGGAATGGAGA-3'; reverse, 5'-GAG GCTGGAATGGTGAAGGAGA-3'. The PCR cycling conditions were 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 65 °C-0.5 °C/cycle for 40 s, and 72 °C for 150 s); 24 cycles of 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 150 s; 72 °C for 2 min; and then holding at 4 °C. The PCR products were purified by digestion with 1 U of shrimp alkaline phosphatase at 37 °C for 1 h and at 75 °C for 15 min. The ligation reaction contained 2 μL of 10× ligase buffer, 0.2 μL of Taq DNA ligase, 1 μL of probe mixture and 3 μL of purified PCR product mixture. In a double connection reaction, each site contains two 5' ends of allele-specific probes and an adjacent 3' end of an allele-specific probe. Each allele-specific connection product was distinguished by its fluorescence, while different loci were distinguished by the different lengths added to the 3' end of the allele-specific probe. The following probes were used: rs3775290FC, TCTCTCGGGTCAATTCGTCCTTGCCA GGAATGGAGAGGTCTAGAAAATATTGTC; FS37752 90RT, TGTTCGTGGGCCGGATTAGTGCCAGGAATG GAGAGGTCTAGAAAATATTGTT; FS3775290RP, GA AATCTATCTTTCCTACAACAAG TRCCTGCTTTTTTT TTTT.

The ligation cycling program consisted of 35 cycles of 94 °C for 1 min and 56 °C for 4 min; and then holding at 4 °C. Ligation products (0.5 μL) were analyzed using an ABI 3130 × 1 instrument, and the raw data were analyzed by GeneMapper 4.0 (Applied Biosystems, USA).

Estimation of cytokine production

Plasma concentrations of IFN-γ and IL-4 were measured using ELISA kits (R&D Systems) for human IFN-γ and IL-4 according to the manufacturer's protocols. The minimal detectable concentration was 8 pg/ml for IFN-γ and 0.22 pg/ml for IL-4. All samples were measured in duplicate, and the values were within the linear portion of the standard curve. To evaluate the effects of the TLR3c.1377C/T gene polymorphism on the balance between the Th1 and Th2 immune responses, we calculated the ratio of IFN-γ to IL-4 for each sample [17, 18].

Statistical analysis

Allelic genotypes were tested for Hardy-Weinberg equilibrium (HWE) using the chi-square test. The frequencies of genotypes and alleles were compared between the EV71-infected mild cases and severe cases by using the chi-square test. The association between the TLR3c.1377C/T polymorphism and the risk of severe disease was evaluated by calculating odds ratios (OR) and 95% confidence intervals (95% CI) using logistic regression. The effects of the TLR3 genotype on EV71 infection were evaluated by using the Mann-Whitney U test for non-normally distributed parameters, and the data are presented as median values (25th-75th percentile values). Normally distributed parametric data were expressed as mean ± SD. The levels of IFN-γ and IL-4 were analyzed by one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test or Student's *t*-test. Pearson's correlation was used to analyze the relationship between the virus load and the levels of IFN-γ and IL-4 and the ratio of IFN-γ to IL-4. All statistical analysis was carried out using the Statistical Package for Social Science (IBM SPSS software, USA), version17.0, and a *P*-value less than 0.05 was considered significant.

Results

Study population

We examined a total of 177 EV71-infected HFMD patients (112 males) aged from 1.0 to 6.7 years. There were 99 mild cases and 78 severe cases. One patient with severe illness died.

Distribution of genotypes and alleles of TLR3

The genotype frequencies of each group conformed to the Hardy-Weinberg equilibrium ($p > 0.05$). The genotype distributions and allele frequencies are shown in Table 1. The genotype distribution in the control group was 12.00% (27/225) for TT, 36.89% (83/225) for CT, and 51.11% (115/225) for CC, which was similar to that of EV71-infected patients: 12.99% (23/177) for TT, 44.63% (79/177) for CT, and 42.37% (75/177) for CC. The frequencies of the T and C alleles were 30.44% (137/450) and 69.56% (313/450) in the control group, compared to 35.31% (125/354), and 64.69% (229/354), respectively, in the EV71-infected patients ($p = 0.144$). However, the severe cases had a significantly higher frequency of TLR3 TT genotype compared to mild cases (17.95% vs. 9.09%, $p = 0.030$). Similarly, the frequency of the TLR3 T allele in the severe cases was significantly higher than in mild cases (42.95% vs. 29.29%, OR = 1.8, 95% CI = 1.2-2.8, $p = 0.01$) (Table 2).

As shown in Table 3, significant differences were found in EV71-infected patients with different genotypes in duration of fever, white blood cell (WBC) count, C-reactive protein (CRP) level, and blood glucose (BG) concentration ($p = 0.035$, $p = 0.029$, $p = 0.015$, $p = 0.041$). However, no significant differences were observed with respect to gender, age, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase-myocardial isozyme (CK-MB), and virus load.

Table 1 Genotypic and allelic frequencies of TLR3c.1377C/T polymorphism in EV71-infected patients and controls

TLR3c.1377C/T SNP	EV-71-infected patients (n = 177)	Controls (n = 225)	*p*-value	OR (95% CI)
Genotype				
C/C	75 (42.37%)	115 (51.11%)		
C/T	79 (44.63%)	83 (36.89%)		
T/T	23 (12.99%)	27 (12.00%)	0.207a	
Allele				
C	229 (64.69%)	313 (69.56%)		
T	125 (35.31%)	137 (30.44%)	0.144b	1.2 (0.9-1.7)

OR, odds ratio; CI, confidence interval

[a] Controls vs. EV71-infected patients using the chi square-test with a 3 × 2 contingency table

[b] Controls vs. EV71-infected patients using the chi square-test with a 2 × 2 contingency table

Table 2 Genotype frequencies and allele frequencies in mild cases and severe cases

TLR3c.1377C/T SNP	EV-71-infected cases (n = 177)	Mild cases (n = 99)	Severe cases (n = 78)	*p*-value	OR (95% CI)
Genotype					
C/C	75	50 (50.51%)	25 (32.05%)		
C/T	79	40 (40.40%)	39 (50.00%)		
T/T	23	9 (9.09%)	14 (17.95%)	0.030	
Allele					
C		140 (70.71%)	89 (57.05%)		
T		58 (29.29%)	67 (42.95%)	0.010	1.8a (1.2-2.8)

OR, odds ratio; CI, confidence interval

Table 3 Effects of the TLR3c.1377C/T genotype on EV71 infection

	C/C (n = 75)	C/T (n = 79)	T/T (n = 23)	*p*-value
Gender (male/female)	41/34	55/23	16/17	0.125a
Age (years)	3.1 (2.3-4.2)	2.8 (2.0-3.4)	2.8 (2.3-3.9)	0.141b
Duration of fever (days)	2.0 (1.5-3.5)	2.5 (2.0-3.5)	3.0 (2.0-4.0)	0.035b
WBC ($\times 10^9$/L)	8.2 (5.8-12.4)	9.9 (6.6-14.1)	12.6 (5.9-14.7)	0.029b
CRP (mg/L)	6.9 ± 0.3	7.6 ± 0.3	8.7 ± 0.7	0.015c
BG (mmol/L)	7.4 (5.7-10.2)	8.7 (5.8-11.3)	10.5 (6.2-13.1)	0.041b
ALT (U/L)	20.0 (17.0-25.0)	18.0 (15.0-25.0)	21.0 (18.0-28.0)	0.431b
AST (U/L)	24.0 (20.0-32.0)	26.0 (20.0-37.0)	26.0 (21.0-40.0)	0.435b
CK-MB (U/L)	14.0 (8.0-21.0)	15.0 (9.0-20.0)	17.5 (17.8-25.3)	0.234b
EV71 load ($\log_{10}$ copies/μl)	3.9 ± 0.4	4.0 ± 0.3	4.1 ± 0.3	0.163c

WBC, white blood cell count; CRP, C-reactive protein; BG, blood glucose; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK-MB, creatine kinase-myocardial isozyme

a Groups compared using chi-square test

b Values expressed as mean ± SD

c Values expressed as median (25th -75th percentile values)

Effects of TLR3c.1377C/T polymorphism on cytokines levels

Plasma levels of IFN-γ and IL-4 increased significantly in EV71-infected patients (117.2 ± 32.3 pg/mL, $p < 0.001$ and 6.4 ± 1.6 pg/mL, $p < 0.001$) compared to healthy controls (26.4 ± 8.0 pg/mL and 1.8 ± 0.4 pg/mL) (Fig. 1d and e). In EV71-infected patients, we observed that the plasma levels of IFN-γ in patients with genotype CC (135.5 ± 36.8 pg/mL) were significantly elevated compared to those with CT (114.1 ± 26.2 pg/mL, $p < 0.05$) and TT (102.0 ± 24.2 pg/mL, $p < 0.01$)

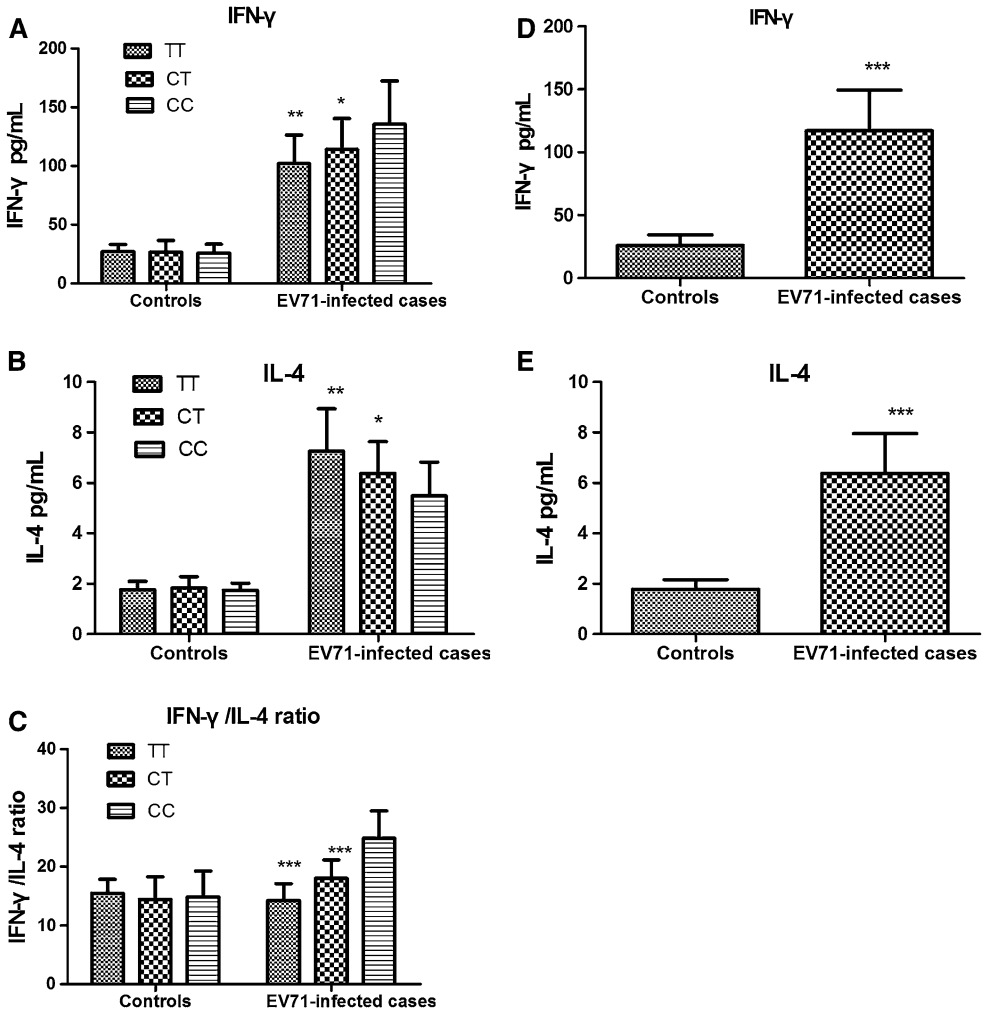

Fig. 1 Plasma levels of IFN-γ and the IL-4 and IFN-γ/IL-4 ratio were measured in 20 EV71-infected patients (8 mild cases and 12 severe cases) for each genotype and 12 controls for each genotype. Values are expressed as mean ± SD. **a.** IFN-γ levels in TT and CT were significantly lower than in CC in EV71-infected cases (**, $p < 0.01$, *, $p < 0.05$), but there was no difference in controls. **b.** In EV71-infected cases, IL-4 levels in TT and CT were clearly higher than in CC (**, $p < 0.01$, *, $p < 0.05$), but there was no difference in controls. **c.** The IFN-γ/IL-4 ratios in TT and CT were significantly higher than in CC in EV71-infected cases (***, $p < 0.001$), but there was no difference in controls. **d.** The serum levels of IFN-γ in EV71-infected patients were significantly higher than in controls (***, $p < 0.001$). **e.** Serum IL-4 concentrations were higher in EV71-infected cases than in controls (***, $p < 0.001$)

(Fig. 1a). In contrast, the IL-4 levels in EV71-infected patients were significantly higher in those with TT (7.3 ± 1.7 pg/mL, $p < 0.01$) and CT (6.4 ± 1.3 pg/mL, $p < 0.05$) compared to those with CC (5.5 ± 1.3 pg/mL) (Fig. 1b). Furthermore, our results showed that the ratio of IFN-γ to IL-4 was clearly lower in patients with TT (14.2 ± 2.8, $p < 0.001$) and CT (18.0 ± 3.1, $p < 0.001$) than in those with CC (24.9 ± 4.7) (Fig. 1c). No significant correlation was found between cytokine levels and genotypes in the healthy controls. There was no significant correlation between virus load and the plasma levels of IFN-γ, IL-4 or the ratio of IFN-γ to IL-4 in EV71-infected patients (Table 4).

Table 4 Correlation of EV71 load and cytokine levels in EV-71 infected cases

Genotype	Parameter	EV71 load Pearson correlation	Significance (two-tailed)
C/C	IFN-γ (pg/mL)	0.326	0.161
	IL-4 (pg/mL)	0.201	0.396
	IFN-γ /IL-4 ratio	0.193	0.415
C/T	IFN-γ (pg/mL)	0.045	0.850
	IL-4 (pg/mL)	0.095	0.690
	IFN-γ /IL-4 ratio	-0.069	0.772
T/T	IFN-γ (pg/mL)	0.404	0.078
	IL-4 (pg/mL)	0.394	0.086
	IFN-γ /IL-4 ratio	-0.009	0.970

Discussion

There is accumulating evidence that there is a significant relationship between genetic factors and the immune response to EV71 infection. Chang et al. reported that HLA-A33, which is a common phenotype in Asian populations but is rare in white populations, is significantly associated with EV71 infection [21]. Similarly, the TLR3c.1377T allele was found in the HapMap project to occur with different frequency in Chinese populations (40%) and European populations (21.7%) [11]. In our study, the TLR3c.1377T allele frequency was 32.6%, and we found no difference in the frequency of the TLR3c.1377C/T genotype and allele between EV71-infected patients and controls, but we found a significantly higher frequency of the TT genotype in severe cases compared to mild cases. The frequency of the TLR3c.1377T allele showed a similar trend. Our results indicate that the TLR3c.1377T allele is associated with an elevated risk of developing severe EV71 infection, although the reason for this effect is not yet known.

As one of the nucleic acid receptors, TLR3 represents the first line of immune defense against viral infection. Activation of the TLR3 signaling pathway could induce the production of antiviral cytokines and trigger a cascade of immune reactions. TLR3 deficiency has been reported to be associated with progressively worse clinical outcomes with significantly reduced production of IFN-γ and a shift in the protective antiviral Th1 response to a Th2-type immune response [10]. Huang et al. proposed that the polymorphism of TLR3c.1377C/T might regulate the expression of TLR3 and activation of TLR3 pathway [11]. In our study, the plasma levels of IFN-γ and IL-4 were significantly higher in EV71-infected patients than in healthy controls. In EV71-infected patients, the plasma levels of IFN-γ and the ratio of IFN-γ to IL-4 in patients with the TT genotype were significantly lower, whereas the plasma IL-4 levels in patients with the TT genotype were higher in the EV71-infected group. These significant differences were not found in healthy controls. IFN-γ is an effective downstream effector of the TLR3 signaling pathway and is known to reduce viral infection by inhibiting viral replication [22, 23]. Moreover, IFN-γ is a Th1 cytokine that upregulates Th1 immunity and favors cell-mediated immunity, which promotes clearance of the virus [24]. Decreased production of IFN-γ causing skewing to a Th2-type response, which is associated with poor prognosis in cases of EV71-related disease [25, 26]. In this study, a relationship between virus loads and the plasma levels of IFN-γ or the IFN-γ/IL-4 ratio was not found, but this might be due to the limited sample size.

When clinical manifestations were considered, we found that subjects with the TT genotype suffered from prolonged fever, which is one key symptom of severe HFMD [5, 15]. In addition, we found that the individuals with the TT genotype had elevated CRP levels, WBC counts and BG concentrations, which are considered key indicators for identifying severe EV71 infection [15]. Overall, our data suggest that the TLR3c.1377C/T polymorphism is involved in the inflammatory process in EV71 infection. However, the mechanisms underlying these associations have not been determined.

Some limitations of our study should be considered. The study was performed only in one ethnic group, and the sample size was relatively small. We investigated only one polymorphism in the TLR3 gene. Furthermore, we only measured the plasma levels of IFN-γ and IL-4. The expression and function of TLR3 should be examined further.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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