A Allele of ICAM-1K469E May Reduce the Incidence of Enterovirus71 Infection in Hand, Foot and Mouth Disease*

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ABSTRACT Objective: To investigate the relationship between ICAM-1, MCP-1 plasma level and gene polymorphisms with the susceptibility of enterovirus71 infection in hand, foot and mouth disease. Methods: By reverse transcription polymerase chain reaction (RT-PCR), EV71 specific primer was used to detect enterovirus71 in the serum of Hand, Foot and Mouth Disease (HFMD) children. PCR-RFLP techniques were performed to determine the genotypes of 1CAM-1 K469Eand MCP-1 A-2518G gene in HFMD group and in control group. The plasma levels of sICAM-1 and MCP-1 were detected by enzyme-linked immunosorbent assay. Results: Compared with that in the control cases, the plasma levels of sICAM-1 and MCP-1 of the HFMD patients were significantly increased ($P< 0.01$). In HFMD group, A allele of ICAM-1 K469E were less common than that in control group ($x^2=6.897$, $P< 0.01$). There were no significant differences in the distribution of frequency of MCP-1A-2518G genotype and allele frequency between the HFMD group and the control group ($P> 0.05$). Conclusion: Plasma level of sICAM-1 expression and the polymorphism of ICAM-1 K469E single nucleotide had relationship with EV71 infection of HFMD. A allele of ICAM-1K469E may reduce the incidence of enterovirus71 infection in HFMD. Plasma level of MCP-1 expression may had relationship with EV71 infection, butthere was no association between A / G polymorphism in MCP-1A-2518G gene and EV71 infection.

Key words: Enterovirus71; ICAM-1; MCP-1; Polymorphisms

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Introduction

Enterovirus 71 (EV71) is one of the main pathogen in Hand, Foot and Mouth Disease (HFMD) and central nervous system infection of children.Recent years,the epidemic of EV71 infected HFMD has uptrend in the Asia Pacific region [1,2]. EV71 has a strong neurotropic nature comparing to other family members of enterovirus, which is more likely to cause central nervous system damage and develop pulmonary edema, pulmonary hemorrhage or death furthermore [3]. Currently, predisposing factors, pathogenesis and immune characteristics of EV1 have not been clear, but the susceptibility relationship between the host genome and the EV71 has been aroused more attention. Soluble intercellular adhesion molecule 1 (sICAM-1) involves in a series of important physiological and pathological processes including cell signaling and activation, cell stretching and movement, cell growth and differentiation, inflammation, thrombosis and so on. Monocyte chemoattractant protein-l (monocyte chemoattractant protein-1, MCP-1) is a chemokine of CC-type cells and has specific chemotactic activation on monocyte / macrophage which could promot the inflammatory response. Recent studies have shown that the role of I-CAM-1, MCP-1 in viral encephalitis, mumps, viral myocarditis and other disease [4-8] have been paid more attention, and their immune response mechanism has also been extensively studied. This study was to explore the the possible role and significance of I-CAM-1 and MCP -1 EV71 HFMD through analysising the sites of the ICAM-1K469E in the EV71 infected children and normal children firstly and surveying the relationship between single nucleotide polymorphism in MCP-1A2518G site and the serum levels of sICAM-l and MCP-1.

1 Material and Methods

1.1 Clinical date

From May 2009 to September 2011, 153 cases, who were diagnosed to have hand-foot-and-mouth disease in Qingdao Women and Children Medical Healthcare Center and Binzhou People Hospital, were collected. 83 were boys while 70 were girls from 11 months years old to 6 years old with the average age was 2.5 years old. They were all accorded with the diagnosis standard in"Hand-foot-and-mouth Disease diagnosis and treatment Guidelines (2010 version) " which was worked out by the Ministry of Health [9]. In addition, their feces and their cerebrospinal fluid examined by RT-PCR qualitative method to detect EV71 nucleic acid are all positive [10]. Within 24 hours after they were hospitalized, 2ml venous blood was harvested in EDTA-anticoagulated vials in each patient and the blood was centrifugalized with the speed of 2000r/min for 10 minutes. And then took the supernatant and stored it in the fridge of - 20℃ in reserve. There were 126

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children in normal control of which 75 were boys while 51 were girls whose ages were from 2.6 to 7.5 years old; these children took health examine in the Affiliated Hospital of Qingdao University Medical College in the same period.They had no clinical infection symptom and their routine blood tests and biochemical indicator were all in normal reference check range.

1.2 Methods

1.2.1 Expression Level Test of sICAM-1and MCP-1in Plasma Chose part of the sample copies by random and detected the slCAM-1 and MCP-1 levels of the blood by enzyme-linked immunosorbent assay. The kit was supplied by Shenzhen Jingmei Kit Biotechnology Limited Company and the test was carried on strictly according to the operating rules in the specification.

1.2.2 ICAM-1K468E Site and MCP-1A518G Site Genetic Polymorphism Test After the centrifugation,genomic DNA was extracted from the blood by Phenol/Chloroform method,with the use of the kit which is the production from TIANGEN Company. The DNA samples were stored by cryopreservation in -20℃ after concentration and purity were tested. The primer were designed by Primer 3 supplied on the internet by American whitehead Biomedical Research Institute Center for Genome Research. The forward primer of K469E was 5'-GGAACCCATTGCCCGAGC-3' and the reverse primer was 5'-GGTGAGGATTGCATTAGGTC. The primers were estimated by BLAST and the length of the specificity expansion band was 223bp. The primers were synthesized by Shanghai Shenggong Biological Engineering Limited Company. The PCR was carried out in 10μl of buffer solution: 3μl ultra pure water, 5 μl TaKaRa mix, 0.5 μl each primer and 1 μl DNA sample. PCR was performed with the following steps:an initial denaturation at 95℃ for 5min, 35 cycles of 30 s at 95℃, 30 s at58.5℃and 20s at 72℃, and a final extension period of 10 min at 72℃. The amplification products were electrophoresed on a 2% the agarose gel and the remaining products were stored in -20℃. After amplification,the PCR products was digested with restriction endonuclease BstUI (supplied by NEB Company)at 60℃ for 4h. The reaction system was 10μl which contained 6μl PCR products, 1μl 10× buffer, 2.7μl ultra pure water, 0.3μl enzyme. The digestion products were electrophoresed on 2% agarose gel and were photographed to be analyzed with DL500 bp Marker for reference. The forward primer was TCTCTCACGCCAGCACTGACC and the reverse primer was GAGTGTTCACATAGGCTTCTG. The PCR reaction system was the same as the previous site. PCR was performed with the following steps: 95℃ for 5min, and then 35 cycles of 95℃ for 30s, 61℃ for 30s and 72℃ for 20s, and at 72℃ for 10 min. After amplification,the PCR products was digested with restriction endonuclease PvuII(supplied by NEB Company) at 37.5℃ for 4 hours and electrophoresed on 2% agarose gel.

1.3 Statistical Analysis

The statistical analysis software SPSS 17.0 was used for data processing and statistical analysis .t test was used in the comparison among sICAM-1 and MCP-1 . x^2 test was used in the comparison of genotype and allele frequency distribution .

2 Results

2.1 Determinations of expression levels of sICAM-1 and MCP-1

50 cases with HFMD and 48 cases of control group children were randomly selected to measure the expression levels of sICAM-1 and MCP-1. The results were shown in the following table. The expression level of sICAM-1 in plasma in HFMD group was significantly higher than the control group ($t = 3.264$, $P<0.01$). The expression level of MCP-1 in plasma in HFMD group was significantly higher than the control group ($t = 2.961$, $P<0.01$).

Table 1 Expression levels of sICAM-1 and MCP-1 in EV71 infection group and the control group

Group	Cases	sICAM-1 (μg / L)	MCP-1 (ng / L)
HFMD group	50	332.43± 60.98	134.75± 40.25
Control group	48	221.78± 45.32	90.36± 30.12

2.2 Polymorphism of sICAM-1 K469E

PCR product fragment of ICAM-1 was 223bp, where was 469 codon polymorphism at exon 6th. According to the restriction enzyme BstUI fragment, there were three genotypes, AA-type in 1 band (223bp), AG-type in 3 bands (223bp, 136bp, 87bp), GG-type in 2 bands (136bp, 87bp), bacause the replacement of T and C made amino acid lysine into glutamine (Fig 1).

HFMD groups and control groups were genetic equilibrium groups tested by the Hardy-Weinberg (P>0.05). Table 2 showed that ICAM-1 gene polymorphism exist in 153 cases of children with HFMD group and 126 cases of normal control children.

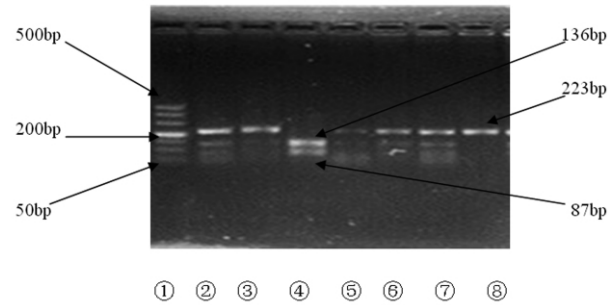

Figure 1 Electrophoresis results of AA,AG,GG three genotypes of ICAM-1 K469E

①500bpMarker②③⑤⑥⑦AGgenotype,④GGgenotype,⑧AAgenotype

Table 2 Gene polymorphism and allele frequency distribution of ICAM-1K469E

	Genotype			x^2	P	Allele		x^2	P
	AA	AG	GG			A	G		
HFMD group	63	76	14	13.549	0.001	202	104	6.897	0.009
Control group	78	36	12			192	60		

There were significant differences in genotype distribution between HFMD groups and control groups (x^2 = 13.549, P <0.01); Frequency of G allele was significantly higher than the control children (x^2 = 6.897, P <0.01). The results of comparison among AA , AG, GG, were shown in Table 3. The AA genotype in control group was significantly higher than HFMD groups, which had lower AG genotype, while GG genotype was no significant difference. According to the results above, ICAM-1 gene K469E polymorphism had significant correlation to EV71 infected HFMD and A allele could be one of the protect factors of the occurrence of HFMD.

Table 3 Genotype analysis of ICAM-1 K469E

Genotype / Group	HFMD groups	Control groups	x^2	P
AA	63	78	11.877	0.01
AG+GG	90	48		
AG	76	36	12.805	0.000
AA+GG	77	90		
GG	14	12	0.011	0.915
AA+AG	139	114		

2.3 Polymorphism of MCP−1A−2518G

PCR product fragment size of MCP-1 was 234bp and 2518 nucleotides in gene promoter region present A-G mutation. According to restriction endonuclease digestion fragments of the situation, there are three genotypes wild-type homozygote AA (1 band of 234bp), heterozygous AG (3 band of 234bp, 159bp, 75bp) and mutant homozygous GG (2 band of 159bp, 75bp) which were shown in Fig 2.

HFMD Group and control group were genetic equilibrium groups tested by the Hardy-Weinberg (P> 0.05). Table 4 showed that 153 cases of children with HFMD group and 126 cases of normal control children present MCP-1 gene polymorphism .AG was the most common in the genotype distribution of MCP-1A2518G and the allele G was more often than allele A. The distribution of genotype had no significant difference between HFMD group and control group (x^2 = 0.192, P> 0.05). The results of comparison of genotype frequencies between the two groups respectively were not statistically significant. Allele frequencies were not statistically significant (x^2 = 0.168, P> 0.05) stated that polymorphism of MCP-1A-2518G have no correlation to HFMD infection.

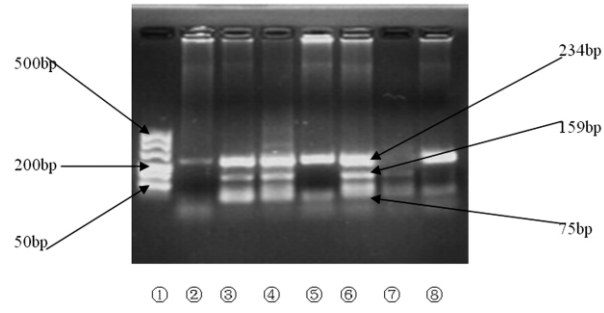

Figure 2 Electrophoresis results of AA,AG,GG three genotypes of MCP-1 A-2518G

①500bpMarker②⑤⑧AAgenotype,③④⑥AGgenotype,⑦GGgenotype

Table 4 Gene polymorphism and allele frequency distribution of MCP-1A-2518G

	Genotype			x^2	P	Allelomorph		x^2	P
	AA	AG	GG			A	G		
HFMD group	28	67	58	0.192	0.909	123	183	0.168	0.682
Control group	22	53	51			97	155		

3 Discussion

Enterovirus 71 is a main pathogen causing hand, foot and mouth disease or herpangina. The condition of the baby infects EV71 is mostly light, and the course is self-limited . However, serious ones may present the brain stem encephalitis, the nerve

source pulmonary edema or the cardiopulmonary failure. The pathogenesis of EV71 infection was not still clear, and the immunologic function disorder resulted of the viral pyemia may participate in its morbidity process besides the neurotropic nature of the EV71 infection[11-15].

ICAM-1 is a member of immunoglobulin superfamily and the main ligand of LFA-1, a member of integrins superfamily [16,17]. Normally, only small amounts of ICAM-1 are expressed in vascular endothelial cell [18]. In the present study, the plasma levels of sICAM-1 was significantly higher in patient group (P<0.01), which suggested that ICAM-1 may be involved in the pathological process of hand-foot-mouth disease (HFMD). The possible mechanism is that endothelial cells are activated by a large amount of inflammatory mediators (IL-l, TNF-α, INF-γ, et al.) after the EV71 infection, which leads to the increase of sICAM-1 levels. This process finally induces T cell proliferation and local immune reactions aiming to self-antigens.Moreover, the synthesis amount of ICAM-1 is controlled by genes and varies between individuals, which may be caused by the polymorphism of regulatory or promoter region of cytokine gene. The results of this study showed that there was significant difference of K469E gene between case group and control group and the frequency of A allele of the control group was significantly higher than that in HFMD group (P<0.01), suggesting a negative correlation between the K469E polymorphism of ICAM-1 gene and the risk of HFMD. Allele A may influence the occurrence of EV71 HFMD and play the protective role of reducing the risk of HFMD. A large-volume and multi-regional study is needed to determine the role of allele G in the risk of HFMD.

Monocyte Chemoattractant Protein-1 (MCP-1) is a potent chemotactic factor for Monocytes and T lymphocytes, inducing chemotaxis and activation of Monocytes, which may play an important role in defensive activities, rheumatic diseases and immunological diseases through enhancing inflammatory responses[19,20]. This study found that plasma MCP-1 levels were significantly higher in HFMD patients than that in healthy controls (P<0.01), which indicated MCP-1 was involved the pathology of HFMD, possibly promoting leukocyte infiltration. This study compared the MCP-1 genotype and alle distribution frequency in EV71 infection groups and that in control groups, but there was no significant difference in these two groups, which suggested that MCP-1 level in HFMD was not associated with its genetic polymorphism A-2518G. There are several possible explanations for this result: 1. Interaction or linkage disequilibrium between other known or unknown genes and MCP-1 gene may exist, which may affect incidence of HFMD. 2. HFMD may be associated with other single-nucleotide polymorphism of MCP-1 gene. 3. The sample size and sample collection region were limited in this study, so the result may be influenced by their genetic background and environment. To get more conclusive validation for whether MCP-1 A-2518G polymorphism is associated with HFMD, further case-control studies and prospective studies with larger cohorts of patients are required.

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ICAM-1K469E 位点 A 等位基因可降低 EV71 手足口病发生率 *

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摘要 **目的**:研究 ICAM-1 基因 K469E 位点、MCP-1A2518G 位点基因多态性及 sICAM-1、MCP-1 在血清中表达水平与 EV71 手足口病的关系,探讨 EV71 型手足口病的遗传易感因素。**方法**:运用限制性片段长度多态性—聚合酶链反应(PCR-RFLP)检测急性期 EV71 感染阳性的手足口病患儿和正常儿童中 ICAM-1K469E 位点及 MCP-1A2518G 位点碱基变异情况,同时采用双夹心抗体法(ELISA)检测血清 sICAM-1 和 MCP-1 水平。**结果**:EV71 手足口病组患儿血清中 sICAM-1 和 MCP-1 水平均显著高于正常对照组(P 均 <0.01)。EV71 手足口病组 ICAM-1K469E 位点中,A 等位基因的频率显著低于对照组(x^2=6.897,P<0.01)。EV71 手足口病组患儿 MCP-1 基因型分布、等位基因频率与对照组比较均无统计学意义(P>0.05)。**结论**:sICAM-1 表达水平和其基因 K469E 位点多态性与 EV71 手足口病有关,A 等位基因可降低 EV71 手足口病发生率。MCP-1 表达水平与 EV71 手足口病感染有关,但 MCP-1A-2518G 位点基因多态性与 EV71 手足口病感染无关。

关键词:肠道病毒 71 型;细胞间粘附分子 1;单核细胞趋化因子 1;基因多态性

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