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Association of Chemotactic Chemokine Ligand 5 Polymorphisms with the Risk of Developing Severe Enterovirus 71 Infection

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Abstract. Respiratory damage is a main manifestation of severe Enterovirus 71 (EV71) infection. Polymorphisms of -403G/A (rs2107538), -28C/G (rs2280788), and In1.1T/C (rs2280789) in chemotactic chemokine ligand 5 (*CCL5*) have linked with many respiratory diseases. In this study, we explored the possible correlation of *CCL5* polymorphisms with severe EV71 infection. Blood samples were obtained from 87 children hospitalized for EV71 infection. Fifty-seven healthy children were enrolled as asymptomatic controls. Genotype and allele frequencies were analyzed by logistic regression analysis. There were statistically significant differences in polymorphisms of *CCL5* -403G/A and In1.1T/C for dominant model ($P = 0.016$; $P = 0.027$) and additive model ($P = 0.010$; $P = 0.019$) between patients with severe EV71 infection and asymptomatic controls. With ordinal logistic regression model analysis, statistically significant differences were found between polymorphisms of *CCL5* (-403G/A) ($P = 0.034$) with the severity of EV71 infection after adjusting for age. The frequency of A-C-C haplotype was significantly higher in EV71 infection patients than controls ($P = 0.032$). These results suggest that *CCL5* -403G/A and In1.1T/C polymorphisms may contribute to severe EV71 infection and individuals with haplotype of A-C-C may exhibit higher risk of developing severe EV71 infection. These findings may provide insights into pathogenic and protective mechanisms of severe EV71 infection.

INTRODUCTION

Enterovirus 71 (EV71) that belongs to human enterovirus species A in the genus *Enterovirus*, family *Picornaviridae* is the main pathogen in hand, foot, and mouth disease (HFMD), a common illness in young children. EV71 epidemics have been reported worldwide.[1–3] In China, after the poliovirus has been nearly eradicated, the HFMD disease has become a big health problem caused by picornavirus and has provoked the national concern.[4] In 2013, the Ministry of Health of the People's Republic of China announced 1,855,559 HFMD cases, including 260 fatal cases.

EV71-infected disease exhibits diverse manifestations such as a mild hand, foot, and mouth syndrome to aseptic meningitis, encephalitis, respiratory damage, and myocarditis.[5] Neurogenic pulmonary edema (PE) is thought to be the main pathogenic process in Asia.[1,4,6] The exact mechanism for PE in EV71 infection is unclear. That the excessive release of cytokines causes the increased pulmonary vascular permeability may be a potential contributor to PE.[7] Shin-Min Wang and his colleagues reported a significant elevation of plasma interleukin (IL)-10, IL-13, and interferon (IFN)-γ levels observed in patients with PE. In our previous study, we showed that IL-1, IL-6, tumor necrosis factor (TNF)-α, IL-8, IFN-γ-induced protein (IP)-10, and regulated on chemotactic chemokine ligand 5 (CCL5) are induced in human primary monocyte-derived macrophages (MDMs) by EV71.[8]

CCL5, which is also called regulated on activation, normal T cell expressed and secreted (RANTES), is a member of the pro-inflammatory cytokine family known as "chemokines" and is highly produced by $CD8^+$ T-lymphocytes, macrophages, platelets, and epithelial cells. There is an accumulating evidence supporting the association of CCL5 activity with respiratory diseases such as asthma and chronic obstructive pulmonary disease.[9] Two single-nucleotide polymorphisms (SNPs) (-403G/A, rs2107538; -28C/G, rs2280788) in the promoter region and an SNP in the first intron (In1.1T/C, rs2280789) of the *CCL5* gene have been shown to interfere with CCL5 expression. Polymorphisms of *CCL5* (-403G/A, -28C/G, and In1.1T/C) have reportedly been associated with asthma, tuberculosis, respiratory syncytial virus (RSV) infection, and so on.[10–14]

Human genetic variation is a major determinant of susceptibility to many infectious diseases in humans. In our previous study using the same study cohort, we explored the association of variations of pattern-recognition receptors (PRRs) with EV71 infection which is the frontier of innate immunity. We found the polymorphism of *MDA5* (rs1990760) may be a risk factor for EV71 infection.[15] It is likely that susceptibility to most pathogens is determined by a large number of polymorphic genes. Association of EV71 infection with cytotoxic T lymphocyte-associated antigen 4 (*CTLA-4*), human leukocyte antigen (*HLA*)-*A33*, *IL-10*, *IFN*-γ, *MxA*, and *eNOS* gene polymorphisms has already been reported.[16–20] In this study, we explored the possible correlation of the polymorphisms of -403G/A (rs2107538), -28C/G (rs2280788), and In1.1T/C (rs2280789) in the *CCL5* gene with the severe EV71 infection. We hope that our results provide insights into protective and pathogenic mechanisms in EV71 infection.

MATERIAL AND METHODS

Study cohort. At Nanjing Children's Hospital (Jiangsu Province, China), we recruited 87 inpatients with confirmed EV71

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illness by EV71-specific IgM enzyme-linked immunosorbent assays (ELISAs) during the period of August 2010 through August 2011. We collected peripheral ethylenediaminetetraacetic acid (EDTA)-blood samples from these patients. According to the handbook of treatment of HFMD (2010) from the Ministry of Health of the People's Republic of China, these patients were diagnosed as severe cases of HFMD.

The control group consisted of 57 healthy children under 6 years old who went to Nanjing Children's Hospital for health examination during November 2010 and August 2011. The levels of EV71-neutralizing antibody were measured. All of the control children were EV71-IgM negative but EV71-IgG positive. After retrospective survey, these 57 children confirmed by their parents had no history of HFMD and herpangina.

The study was approved by the Ethical Committee of the National Institute for Viral Disease Prevention and Control, China Centers for Disease Control and Prevention and Nanjing Children's Hospital. Informed consents were obtained from the parents of each of the enrolled children.

DNA extraction and genotyping. According to the manufacturer's instructions, genomic DNA was isolated from peripheral EDTA-blood samples using a QIAamp DNA blood kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed to amplify genomic DNA. The following primers were used: forward, AATGCCCAGCTCAGATCAACTG; reverse, GCTGACAGGCATGAGTCAGAC. The cycling conditions were 94°C for 2 minutes followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C; and finally 1 cycle of 5 minutes at 72°C. After PCR, genotypes were determined by sequencing.

Statistical analysis. Each polymorphism was checked with Hardy–Weinberg equilibrium. The demographic data and polymorphism frequencies between control and cases groups were analyzed with χ^2 tests and Student's t test. Binary logistic regression adjusted for age was used to evaluate the associations between the alleles, genotypes, and severity of EV71 infection by calculating odds ratios (ORs) and 95% confidence intervals (CIs) separately. The association between polymorphisms of candidate genes and severity of EV71 infection was analyzed with ordinal logistic regression analysis separately. The haplotype frequencies were assessed using the implementation of the expectation–maximization algorithm analysis.[21] In all groups, two-tailed test was performed and P values less than 0.05 were considered to be statistically significant. The above analyses were performed using SNPstats software (http://bioinfo.iconcologia.net/snpstats/start.htm) and SPSS software, version 17.0 (SPSS Inc., Chicago, IL).

RESULTS

Demographic data, clinical characteristics, and Hardy–Weinberg equilibrium analysis. We enrolled 87 patients with EV71 infection and 57 healthy children for the study. On the basis of clinical manifestation, 87 patients were divided into two groups: 39 patients with encephalitis only (44.8%) and 48 encephalitis patients with other complications (55.2%) including five received diagnoses of encephalomyelitis, nine of myocardial damage, 20 of severe brainstem encephalitis (BE), four of PE, one of encephalomyelitis and myocardial damage, two of encephalomyelitis and BE, one of myocardial damage and BE, three of encephalomyelitis and PE, 2 of BE and PE and 1 complicated with encephalomyelitis, BE and PE.

In asymptomatic control group, there were 35 (61.4%) males and 22 (38.6%) females. Among 87 EV71-infected cases, 65 (74.7%) were male and 22 (25.3%) were female. There were no significant sex differences between case and control subjects. The median age of asymptomatic control group was 3.8 years (range: 0.3–5.8 years), while that of patients was 1.7 years (range: 0.1–4.6 years) ($P < 0.05$). The genotypic distributions of candidate genes between patients and controls agreed with Hardy–Weinberg equilibrium (data not shown).

Distribution of CCL5 alleles and genotypes among cases with severe EV71 disease and asymptomatic control subjects. Binary logistic regression analysis was used to assess the association of the allelic variants and genotypes of *CCL5* with the EV71 infection separately using the SNPstats software. Comparing the severe patients with EV71 infection and asymptomatic controls, significant differences were observed in the frequencies of genotypes, including *CCL5* -403G/A and In1.1T/C under dominant model (OR = 2.78, 95% CI = 1.21–6.40, $P = 0.014$ and OR = 2.55, 95% CI = 1.11–5.86, $P = 0.023$), additive model (OR = 2.42, 95% CI = 1.23–4.78, $P = 0.0072$ and OR = 2.26, 95% CI = 1.14–4.47, $P = 0.014$), after adjusting for age. For codominant model, the P value of -403G/A was 0.027, while the P value of In1.1T/C was 0.049 which may need more samples to verify the result. The -28G was extremely rare and there was no -28G/G genotype in our sample (Table 1).

The association between polymorphisms of candidate genes and the severity of EV71 infection. Three levels of severity status were treated as ordinal including 57 asymptomatic controls, 39 encephalitis patients and 48 encephalitis patients with other complications (low to high). Ordinal logistic regression models were used to analyze the association between candidate SNPs and severity of EV71 infection. Statistically significant differences were found between polymorphisms of *CCL5* (-403G/A) ($P = 0.034$) with the severity of EV71 infection after adjusting for age (Table 2).

The association of CCL5 haplotypes with severe EV71 disease. Strong linkage disequilibrium was found between all three polymorphisms allowing the construction of four haplotypes in agreement with previous studies.[12] To assess the probable association between the haplotypes with EV71 infection, we used SNPstats software to analyze the frequencies of *CCL5* haplotypes in patients with EV71 infection and asymptomatic controls. Using the G-C-T as reference, we found the statistical differences in haplotype of A-C-C between severe EV71 patients and controls (OR = 2.36, 95% CI = 1.08–5.13, $P = 0.032$) (Table 3).

DISCUSSION

EV71 infection commonly manifests as HFMD or herpangina. In a few patients, fulminant EV71 infection may lead to severe respiratory complications such as acute PE. The pathogenesis of PE due to EV71 infection remains unclear and controversial. Wu and others[22] noted that the brain stem lesion or system inflammatory response causes the increased pulmonary vascular permeability which is the most likely cause of PE. Although, Huang and others[23] suggested that left ventricular dysfunction resulting from EV71 BE is a more likely

TABLE 1
Differential distribution of *CCL5* alleles and genotypes among cases with severe EV71 disease and asymptomatic control subjects

SNPs	Models	Genotypes	Case (%), $N = 87$	Control (%), $N = 57$	OR (95% CI)*	*P* value*
CCL5 -403	Codominant	G/G	36 (41.4)	33 (57.9)	1.000 (ref.)	**0.027**
		G/A	41 (47.1)	21 (36.8)	**2.43 (1.02–5.77)**	
		A/A	10 (11.5)	3 (5.3)	**5.83 (1.07–31.73)**	
–	Dominant	G/G	36 (41.4)	33 (57.9)	1.000 (ref.)	**0.014**
		G/A–A/A	51 (58.6)	24 (42.1)	**2.78 (1.21–6.40)**	
–	Log-additive	G	113 (64.9)	87 (76.3)	1.000 (ref.)	**0.0072**
		A	61 (35.1)	27 (23.7)	**2.42 (1.23–4.78)**	
CCL5 -28	–	C/C	66 (75.9)	48 (84.2)	1.000 (ref.)	0.26
		C/G	21 (24.1)	9 (15.8)	1.78 (0.64–4.93)	
CCL5 1.1	Codominant	T/T	38 (43.7)	33 (57.9)	1.000 (ref.)	**0.049**
		T/C	40 (46)	21 (36.8)	2.27 (0.96–5.38)	
		C/C	9 (10.3)	3 (5.3)	5.04 (0.90–28.26)	
–	Dominant	T/T	38 (43.7)	33 (57.9)	1.000 (ref.)	**0.023**
		T/C–C/C	49 (56.3)	24 (42.1)	**2.55 (1.11–5.86)**	
–	Log-additive	T	116 (66.7)	87 (76.3)	1.000 (ref.)	**0.014**
		C	58 (33.3)	27 (23.7)	**2.26 (1.14–4.47)**	

CCL5 = chemotactic chemokine ligand 5; CI = confidence interval; EV71 = Enterovirus 71; OR = odds ratio; SNPs = single-nucleotide polymorphisms.
For results shown in bold, $P < 0.05$.
*Age-adjusted logistic regression models using the SNPstats software.

cause. Clinical studies have provided clues about the relationship between EV71 pathogenesis and immune responses in patients. Several cytokines and chemokines, such as TNF-α, IL-1β, 6, 10, 8, 13, and IFN-γ, are associated with BE and PE caused by EV71 infection.[7,24–26] In our previous study, we found that EV71 infection significantly increased the release of IL-8, IP-10, and CCL5 at 12-hour or 24-hour point of infection in human primary MDMs.[8] While Wang and others[27] explored the acute chemokine response in the blood and cerebrospinal fluid of children with EV71-associated BE and suggested that IL-8, IP-10, MCP-1, and possibly MIG, but not CCL5, were synthesized in the brain in response to encephalitis.

CCL5, a chemokine produced by stimulated airway epithelial cells that attracts eosinophils, basophils, monocytes, and memory T lymphocytes, is implicated in a variety of diseases characterized by pulmonary arterial hypertension, lung eosinophilia, and inflammation. CCL5 is mainly produced by $CD8^+$ T cells, epithelial cells, fibroblasts, and platelets. In *CCL5*, three SNPs, including -403G/A, -28C/G, and In1.T/C, have been studied alongside several viral infections, including hepatitis C virus and RSV.[12,14,28] According to National Center for Biotechnology Information SNP database, the allele frequencies for the three SNPs in partial Chinese population are as follows: -40G/A (64.6%/35.4%), -28C/G (85.1%/14.9%), and In1.T/C (30.2%/69.8%). Our study revealed that polymorphisms in -403 and In1.1of *CCL5* (rs2107538 and rs2280789) are associated with severe disease caused by an EV71 infection. The promoter allele -403A has been reported to upregulate the transcriptional activity of *CCL5*, whereas An and others[14] demonstrated that the In1.1C allele was associated with reduced *CCL5* expression. On the basis of the results of An and others[14] and Tian and others,[29] the G-C-T haplotype are thought to be associated with higher promoter activity. In our study, the estimated frequency of G-C-T haplotype was low in the patients with EV71 infection. In the other hand, the haplotypes of A-C-C and A-G-C correspond to lower transcriptional activity. In this study, individuals with A-C-C haplotype exhibited a higher risk for developing severe EV71 infection. These results may contribute to the explanation that there was no reportedly high level of CCL5 in patients with severe EV71 infection clinically. Polymorphisms in *CCL5* have been associated with bronchial disease, implying an association with pulmonary diseases caused by EV71.[12] We hope that our findings might shed light on the pathogenesis of severe EV71 infection.

Several limitations of our study need to be addressed. One limitation of our study is the lack of mild cases without complications due to EV71. In the control group, to reduce bias we excluded children with negative EV71-IgG antibodies, which was different from previous studies, but decreased the size of control samples. The association warrants further replication because they do not have a replication cohort and the sample size is small. The small size of

TABLE 2
Association between SNPs of candidate genes and severity of EV71 infection

Candidate polymorphisms	Genotypes	Cases		Control ($N = 57$)	OR (95% CI)*	*P* value*
		Complication patients ($N = 48$)	Encephalitis patients ($N = 39$)			
CCL5 -403G/A	GG	19 (39.6)	17 (43.6)	33 (57.9)	1.000 (ref.)	–
–	GA + AA	29 (60.4)	22 (56.4)	24 (42.1)	**2.032 (1.054–3.918)**	**0.034**
CCL5 -28C/G	CC	38 (79.2)	28 (79.8)	48 (84.2)	1.000 (ref.)	–
–	CG + GG	10 (20.8)	11 (28.2)	9 (15.8)	1.198 (0.544–2.640)	0.653
CCL5 In1.1T/C	TT	21 (43.8)	17 (43.6)	33 (57.9)	1.000 (ref.)	–
–	TC + CC	27 (56.2)	22 (56.4)	24 (42.1)	1.781 (0.927–3.419)	0.083

CCL5 = chemotactic chemokine ligand 5; CI = confidence interval; EV 71 = Enterovirus 71; OR = odds ratio; SNPs = single-nucleotide polymorphisms.
For results shown in bold, $P < 0.05$. Dependent variables were categorized as encephalitis patients with complications, encephalitis patients and asymptomatic controls. Three models were fitted for the SNPs of candidate genes as independent variables separately.
*P values and ORs are adjusted for age by ordinal logistic regression analysis.

TABLE 3

Differential distribution of *CCL5* -403/-28/In1.1 haplotypes in patients with severe EV71 disease and asymptomatic control subjects

Haplotype	-403	-28	In1.1	Case (%)	Control (%)	OR (95% CI)*	*P* value*
h 1	G	C	T	113 (64.9)	87 (76.3)	1.000 (ref.)	–
h 2	A	C	C	37 (21.3)	18 (15.8)	**2.36 (1.08–5.13)**	**0.032**
h 3	A	G	C	21 (12.1)	9 (7.9)	2.31 (0.79–6.74)	0.13
h 4	A	C	T	3 (1.7)	0	–	–

CI = confidence interval; EV71 = Enterovirus 71; OR = odds ratio.
For results shown in bold, $P < 0.05$. Global haplotype association P value: 0.052.
*Age-adjusted logistic regression models using the SNPstats software.

sample and multiple testing might influence the results of the statistical analysis.

Our data show that *CCL5* -403G/A (rs2107538) and In1.1T/C (rs2280789) may play a role in the risk of developing severe EV71 infection and the individuals with A-C-C haplotype may exhibit higher risk of severe EV71 infection. The findings are preliminary. And larger cohort with more cases and exact molecular mechanism is needed to further explore the role of these polymorphisms in EV71 infection in the future. We hope our findings may lay the foundation for identifying at-risk infants and provide insights into the pathogenesis of EV71 severe infection.

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