ORIGINAL INVESTIGATION

Association of functional polymorphisms in the *MxA* gene with susceptibility to enterovirus 71 infection

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Abstract Myxovirus resistance A (MxA) is an antiviral protein induced by type I interferons α and β (IFN-α and IFN-β) that can inhibit virus replication. We examined whether the *MxA* polymorphisms were related to the risk and severity of enterovirus 71 (EV71) infection in Chinese populations. The *MxA* C-123A and G-88T polymorphisms were genotyped in two independent case–control populations in China by polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) analysis. Multivariate logistic regression analysis was used to calculate adjusted odds ratios (ORs) and 95 % confidence intervals (95 % CIs). *MxA* messenger RNA was quantified by real-time quantitative PCR in peripheral blood mononuclear cells (PBMCs) from 45 healthy children and 19 patients with EV71 infection. Significantly decreased susceptibility to EV71 infection was observed for the -123A allele and -88T allele carriers, with ORs (95 % CIs) estimated as 0.56 (0.39–0.81) and 0.64 (0.47–0.88), respectively, in the northern population. This association was confirmed in the southern population, with ORs (95 % CIs) estimated as 0.58 (0.38–0.89) and 0.67(0.47–0.95), respectively. The $A_{-123}T_{-88}$ haplotype was also significantly associated with lower risk of EV71 infection in both the northern (OR = 0.62; 95 % CI = 0.44–0.85) and the southern population (OR = 0.63; 95 % CI = 0.43–0.92). Furthermore, we observed higher *MxA* messenger RNA levels in IFNβ1a-stimulated PBMCs from the -123A or -88T allele carriers compared with that from nocarriers. Our findings suggest that polymorphisms in the *MxA* promoter may play a role in mediating the susceptibility to EV71 infection in Chinese population.

Introduction

Enterovirus 71 (EV71) is positive-stranded RNA virus belonging to the enterovirus genus of the Picornauiridae

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family. EV71 has been regarded as the most important neurotropic enterovirus after the primary eradication of the poliovirus. There is no specific antiviral therapy or vaccine for EV71. Since its discovery in 1969, EV71 outbreaks have been reported periodically throughout the world. The size and frequency of EV71 outbreaks have greatly increased over the past decade in the Asia–Pacific region, especially in southeast Asia (Solomon et al. 2010). In mainland China, EV71 infection has become more and more severe and led to 509 and 569 deaths in 2011 and 2012, respectively, according to the report of Ministry of Health, China.

Individual susceptibility to EV71 infection seems to be variable (Chang et al. 2008; Yang et al. 2001, 2012). Patients with EV71 infection displayed wide clinical spectrum, ranging from asymptomatic or mild hand-foot-mouth disease (HFMD) to severe neurological disease. Similar to other infectious diseases, EV71 infection may also be the result of complex interactions between pathogen, environmental factors and host genes. The identification of susceptibility genes contributing to EV71 infection would assist in predicting individual and population risks of EV71 infection, as well as clarifying pathogenesis of this infectious disease.

Type I interferons α and β (IFN-α and IFN-β) system is a key component of the innate immune response and the first line of defense against viral infection including EV71 infection (Liu et al. 2005; Yi et al. 2011). The Myxovirus resistance (Mx) proteins are dynamin-like GTPases that are induced by IFN-α and IFN-β and have antiviral activity against RNA viruses (Sadler and Williams 2008). In humans, two distinct Mx (MxA and MxB) are encoded on chromosome 21, while only MxA has exhibited antiviral activity (Sadler and Williams 2008). Transfected cells (Chieux et al. 2001; Kochs and Haller 1999) and transgenic mice (Pavlovic et al. 1995) overexpressing MxA acquire a high degree of antiviral resistance against several RNA viruses. In humans, synthesis of MxA protein is induced during viral infections and may protect humans from infection (Roers et al. 1994; von Wussow et al. 1990). As for EV71, it has been shown that type I IFN subtypes can inhibit EV71 replication and the antiviral effect was caused by a strong induction of the downstream antiviral effectors especially MxA (Yi et al. 2011). On the basis of the functional role of MxA in the pathogenesis of viral infections in vivo and in vitro, we hypothesize that *MxA* might be a potential candidate susceptibility gene for EV71 infection.

Among the validated polymorphisms in *MxA* gene, two functional single nucleotide polymorphisms (SNPs) (C-123A, rs17000900; G-88T, rs2071430) in promoter region have been extensively studied. The electrophoretic mobility shift assays shown that these two polymorphisms could influence the binding of nuclear protein(s) (Ching et al. 2010). Furthermore, by luciferase reporter assay, the basal promoter activity was demonstrated to be higher in promoter constructs containing the minor alleles of the two polymorphisms (Furuyama et al. 2006; Hijikata et al. 2001; Torisu et al. 2004). We and other investigators have reported the association between the aforementioned *MxA* functional SNPs and risk of infections of viruses, including severe acute respiratory syndrome-coronavirus (SARS-CoV) (Ching et al. 2010; Hamano et al. 2005; He et al. 2006), hepatitis B virus (HBV) (Cao et al. 2009), hepatitis C virus (HCV) (Hijikata et al. 2000) and measles virus (Torisu et al. 2004). The role of the *MxA* polymorphisms in EV71 infection, however, has never been investigated. In the present study, we examined whether polymorphisms in the *MxA* gene were related to the risk and severity of EV71 infection among Chinese populations.

Patients and methods

Study population

Patients were recruited between April and November 2010 from 7 hospitals located in either northern or southern China. All the hospitals were surveillance sentinels which were set by China CDC. HFMD was clinically defined as a patient having oral ulcers and vesicular rash on the hands, feet, knees, or buttocks. Complicated patients were defined as cases with central nervous system (CNS) involvement (including encephalitis, poliomyelitis-like syndrome, and encephalomyelitis), myocarditis or pulmonary edema/hemorrhage. In each hospital, all patients who met the clinical definition and willing to participate into the study were recruited.

Stool samples were collected for EV71 RNA detection by real-time PCR using previously described primers (Verstrepen et al. 2002). Viral isolation was performed as described previously (Chen et al. 2010). Serum samples were collected to test the IgM antibody against EV71 by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Beijing Wantai Biological Pharmacy Enterprise Co, Ltd, China). Confirmation of EV71 infections was recognized by one of the following criteria: isolation of EV71 from stool specimens; positive EV71 viral RNA quantitation or positive EV71 IgM results during acute phase of infection.

The controls were randomly selected from healthy children who underwent routine physical examination in the same region during the same period that the cases were recruited. After interviewing their guardians, healthy children with no self-reported or medical-recorded history of HFMD or herpangina were recruited. Their sera samples were collected for EV71-specific IgG antibody test by the
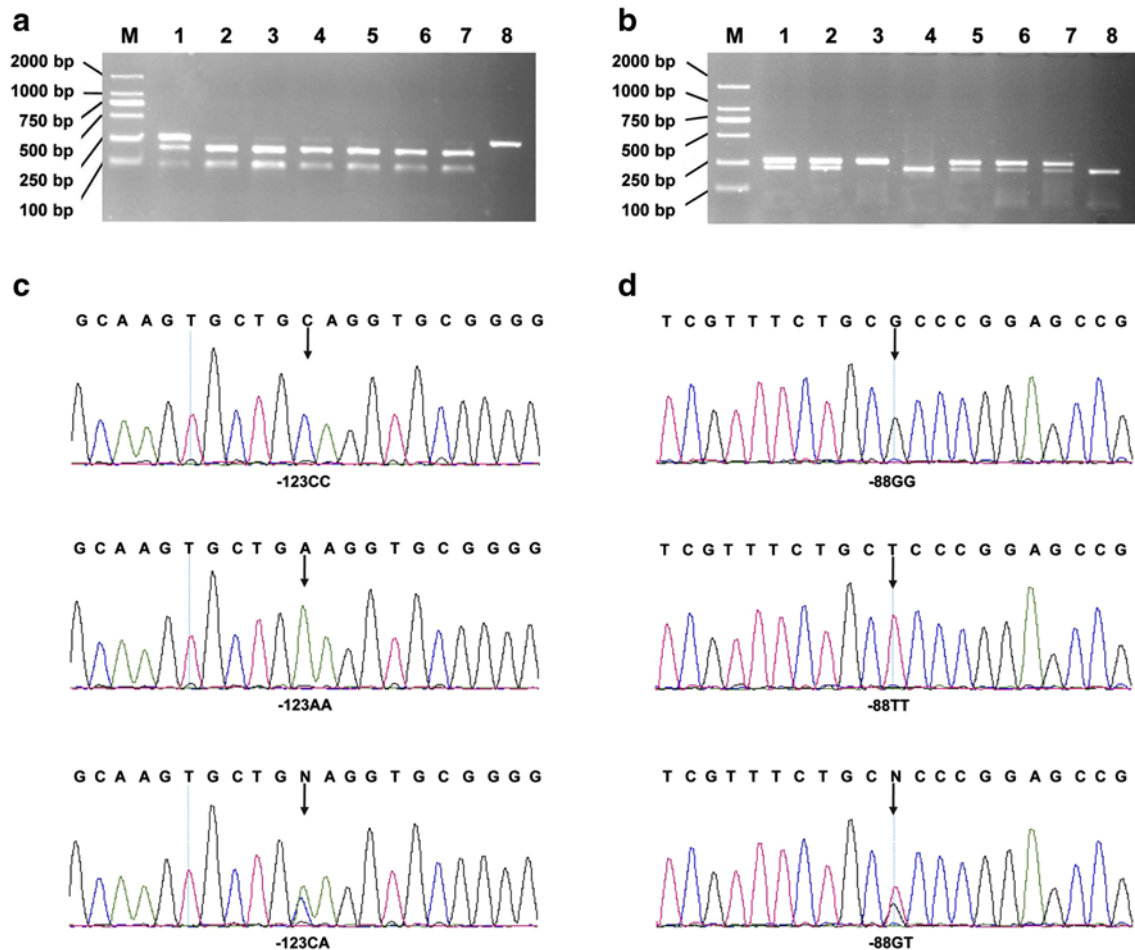
Fig. 1 Analysis of the *MxA* C-123A and G-88T polymorphisms. **a** PCR-based RFLP analysis for the C-123A polymorphism. Lanes 2–7 were CC genotypes; lane 1 was CA genotype; lane 8 was AA genotype; M was DL2000 marker. **b** PCR-based RFLP analysis for the G-88T polymorphism. Lanes 4 and 8 were GG genotypes; lanes 1, 2, and 5–7 were GT genotypes; lane 3 was TT genotype; M was DL2000 marker. **c** Sequencing analysis for genotypes of the C-123A polymorphism. The 3 charts represent the CC, CA and AA genotypes, respectively. **d** Sequencing analysis for genotypes of the G-88T polymorphism. The 3 charts represent the GG, GT and TT genotypes, respectively. The *arrows* localize the base changes at the nucleotide positions

Diagnostic Kit for IgG Antibody to Human EV71 (Beijing Beier Bioengineering Co, Ltd, China). Only those with negative EV71 IgG results were recruited as eligible controls. By checking the medical records or by interviewing the participants' guardians, we determined that all cases and controls were genetically unrelated Han Chinese.

For *MxA* expression assay, blood samples were obtained from a group of 20 healthy children (13 males and 7 females) aged 26–50 months (mean ± SD, 39.6 ± 6.6) from the northern population, 25 healthy children (16 males and 9 females) aged 24–52 months (mean ± SD, 38.2 ± 8.4) from southern population, and 19 patients with EV71 infection (12 males and 7 females) aged 24–50 months (mean ± SD, 37.8 ± 7.8). At recruitment, informed consent was obtained from all participants' guardians, and personal information on demographic factors and medical

history were collected via structured questionnaire. This study was performed with the approval of the Ethical Committee of Beijing Institute of Microbiology and Epidemiology and conducted according to the principles expressed in the Declaration of Helsinki.

Polymorphism genotyping

Genomic DNA was extracted from peripheral blood by Gene Relax DNA extraction kit (TianGen, Beijing, China) according to the manufacturer's instructions. The polymorphisms were genotyped using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) analysis. The primers 5′-GGGAAGGACAT GCCTAGGTTC-3′ and 5′-CCGCGAAGAAATGAAACT CAC-3′ were used for amplifying the target region. The

Table 1 Characteristics of patients with EV71 infection and control subjects

Variables	Cases No. (%)	Controls No. (%)	P value
All	(n = 441)	(n = 958)	
Northern population	(n = 253)	(n = 550)	
Age (months), mean (SD)	25.3 (13.4)	27.2 (18.6)	0.15[a]
Male, no. (%)	164 (64.8)	341 (62.0)	0.44[b]
Clinical diagnosis, no. (%)			
Uncomplicated HFMD	103 (40.7)		
Complicated cases	150 (59.3)		
Southern population	(n = 188)	(n = 408)	
Age (months), mean (SD)	33.0 (15.3)	34.4 (11.2)	0.19[a]
Male, no. (%)	118 (62.8)	238 (58.3)	0.31[b]
Clinical diagnosis, no. (%)			
Uncomplicated HFMD	37 (19.7)		
Complicated cases	151 (80.3)		

SD standard deviation

[a] *t* test, two-sided

[b] χ^2 test, two-sided

PCR conditions were performed as described previously except for an annealing temperature of 57.5 °C (Zhang et al. 2012). The reaction yielded a 263-base pair amplicon. PCR products of 3 µL were then digested with 4U of *Pst*I and 4U *Hha*I (Takara BioTech, Dalian, China) for the C-123A and G-88T polymorphisms, respectively. Genotyping was done by staff blinded to the subjects' case/control status. The accuracy of genotyping data obtained from PCR-RFLP analyses was validated by direct sequencing of a 15 % masked, randomly selected sample of cases and controls, and the results were 100 % concordant (Fig. 1).

Real-time analysis of *MxA* mRNA

Total RNA of peripheral blood mononuclear cells (PBMCs) from patients with EV71 infection and IFNβ1a (1,000 U/mL, R&D Systems) stimulated PBMCs from healthy children were extracted using Trizol reagent (Invitrogen, USA). Reverse transcription was done using reverse transcriptase system (Promega, USA) according to the manufacturer's instructions. Quantitative real-time PCR for *MxA*, with *GAPDH* as an internal reference gene, was performed using the Roche Light Cycler 2.0 (Roche Applied Science, USA) based on the SYBR-Green method. The primers used for *MxA* (Fernandez-Arcas et al. 2004) were 5′-ACAGCAAATCAAGGCACTGGA-3′ and 5′-CGGATCAGCTTCTCACCTTCTC-3′and for *GAPDH* (Xie et al. 2011) were 5′-GGGAAGGTGAAGGTCGG AGT-3′ and 5′-TTGAGGTCAATGAAGGGGTCA-3′. The PCR reaction mixture consisted of 0.1 µmol/L of each

primer, 1 X SYBR Green Master mix (Takara), and 50 ng complementary DNA to a final volume of 20 µL. Cycling conditions were 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 57 °C for 15 s. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All analyses were performed in a blinded fashion with the laboratory persons unaware of genotyping data.

Statistical analysis

Genotype and allele frequencies for each polymorphism were determined by gene counting. The fitness to Hardy–Weinberg equilibrium was tested using the random-permutation procedure implemented in the Arlequin package (http://lgb.unige.ch/arlequin). Associations between polymorphisms and risk of EV71 infection were estimated by use of logistic regression analyses. Odds ratios (ORs) and 95 % confidence intervals (CIs) were used to measure the strength of association. The Mantel–Haenszel (MH) test was performed for the combined analysis of the data from both populations. In view of the multiple comparisons, the correction factor $n (m − 1)$ (n loci with m alleles each) was applied to correct the significance level. These analyses were performed using SPSS software (version 15.0). The pairwise linkage disequilibrium (LD) index (Lewontin's D' and r^2) calculation was performed using the Arlequin package. The haplotype analysis was performed using PLINK software (version 1.07) (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al. 2007).

Results

Population characteristics

A total of 441 laboratory-confirmed patients with EV71 infection and 958 controls were included in the study, including 253 cases and 550 controls from northern China, and 188 cases and 408 controls from southern China. In both populations, controls were comparable with cases with regard to mean age and sex distribution. A total of 150 (59.3 %) patients of northern origin and 151 (80.3 %) cases of southern origin were determined as complicated patients. The characteristics of selected subjects are shown in Table 1.

Individual polymorphism and risk of EV71 infection

The genotyping results were presented in Table 2. The observed genotype frequencies of the C-123A and G-88T polymorphisms were in Hardy–Weinberg equilibrium (all $P > 0.05$, data not shown). The -123A-positive genotypes were significantly associated with decreased susceptibility to EV71 infection either in the northern population (OR,

Table 2 The genotype frequencies of *MxA* polymorphisms in patients with EV71 infection and control subjects

SNPs and genotypes	Controls No. (%)	Cases No. (%)	Crude[a] OR (95 % CI)	P value	Adjusted[b] OR (95 % CI)	P value
Northern population	(n = 550)	(n = 253)				
C-123A						
CC	379 (68.9)	197 (79.8)	Reference		Reference	
CA	161 (29.3)	46 (18.6)	0.55 (0.38–0.80)	0.001	0.55 (0.38–0.80)	0.002
AA	10 (1.8)	4 (1.6)	0.77 (0.24–2.49)	0.78	0.79 (0.24–2.55)	0.69
CA + AA	171 (31.1)	50 (20.2)	0.56 (0.40–0.81)	0.002	0.56 (0.39–0.81)	0.002
G-88T						
GG	280 (50.9)	153 (61.9)	Reference		Reference	
GT	231 (42.0)	78 (31.6)	0.62 (0.45–0.85)	0.003	0.62 (0.46–0.86)	0.004
TT	39 (7.1)	16 (6.5)	0.75 (0.41–1.39)	0.36	0.76 (0.41–1.41)	0.39
GT + TT	270 (49.1)	94 (38.1)	0.64 (0.47–0.87)	0.004	0.64 (0.47–0.88)	0.005
Southern population	(n = 408)	(n = 188)				
C-123A						
CC	286 (70.6)	151 (80.3)	Reference		Reference	
CA	109 (26.9)	34 (18.1)	0.59(0.39–0.90)	0.02	0.58 (0.38–0.90)	0.02
AA	10 (2.5)	3 (1.6)	0.57 (0.15–2.10)	0.56	0.57 (0.15–2.10)	0.40
CA + AA	119 (29.4)	37 (19.7)	0.59 (0.39–0.90)	0.01	0.58 (0.38–0.89)	0.01
G-88T						
GG	194 (47.9)	109 (58.0)	Reference		Reference	
GT	183 (45.2)	74 (39.4)	0.72 (0.50–1.03)	0.07	0.72 (0.50–1.03)	0.07
TT	28 (6.9)	5 (2.7)	0.32 (0.12–0.85)	0.02	0.33 (0.12–0.87)	0.03
GT + TT	211 (52.1)	79 (42.0)	0.67 (0.47–0.95)	0.02	0.67 (0.47–0.95)	0.02
Combined	(n = 958)	(n = 441)				
C-123A						
CC	665 (69.6)	348 (80.0)	Reference		Reference	
CA	270 (28.3)	80 (18.4)	0.57 (0.43–0.75)[c]	<0.001[c]	0.56 (0.43–0.75)	<0.001
AA	20 (2.1)	7 (1.6)	0.67 (0.28–1.60)[c]	0.37[c]	0.68 (0.29–1.63)	0.39
CA + AA	290 (30.4)	87 (20.0)	0.57 (0.44–0.75)[c]	<0.001[c]	0.57 (0.44–0.75)	<0.001
G-88T						
GG	474 (49.6)	262 (60.2)	Reference		Reference	
GT	414 (43.4)	152 (34.9)	0.66(0.52–0.84)[c]	0.001[c]	0.67 (0.53–0.85)	0.001
TT	67 (7.0)	21 (4.8)	0.57 (0.34–0.95)[c]	0.03[c]	0.58 (0.34–0.96)	0.04
GT + TT	481 (50.4)	173 (39.8)	0.65 (0.52–0.82)[c]	<0.001[c]	0.66 (0.52–0.83)	<0.001

The number of samples genotyped varies because of genotyping failure for some individuals

OR odds ratio, *CI* confidence interval

[a] ORs and P values were calculated by logistic regression analysis

[b] ORs and P values were calculated by multivariate logistic regression, adjusted for age (months) and sex

[c] ORs and P values were calculated by Mantel–Haenszel (MH) test

0.56; 95 % CI, 0.40–0.81; $P = 0.002$) or in the southern population (OR, 0.59; 95 % CI, 0.39–0.90; $P = 0.01$) (Table 2). Similarly, the -88T-positive genotypes were also significantly associated with decreased susceptibility to EV71 infection with an OR of 0.64 (95 % CI, 0.47–0.87; $P = 0.004$) in the northern population and 0.67 (95 % CI, 0.47–0.95; $P = 0.02$) in the southern population (Table 2).

Then multivariate logistic regression analyses were performed by adjusting for age and sex. For the C-123A polymorphism, subjects bearing at least one -123A allele had a decreased susceptibility to EV71 infection compared with those without -123A allele in both independent populations (in the northern population: adjusted OR, 0.56; 95 % CI,

0.39–0.81; $P = 0.002$; in the southern population: adjusted OR, 0.58; 95 % CI, 0.38–0.89; $P = 0.01$) (Table 2). For the G-88T polymorphism, subjects bearing at least one -88T allele had a decreased susceptibility to EV71 infection compared with those without -88T allele in both independent populations (in the northern population: adjusted OR, 0.64; 95 % CI, 0.47–0.88; $P = 0.005$; in the southern population: adjusted OR, 0.67; 95 % CI, 0.47–0.95; $P = 0.02$) (Table 2). The associations remained significant after correction for multiple comparisons.

When northern and southern population were combined, both the -123A-positive and -88T-positive genotypes were significantly associated with decreased susceptibility to EV71 infection, for the -123A-positive

Table 3 The genotype frequencies of *MxA* polymorphisms in uncomplicated patients and complicated patients

SNPs and genotypes	Uncomplicated cases	Complicated cases	Crude[a]		Adjusted[b]	
	No. (%)	No. (%)	OR (95 % CI)	P value	OR (95 % CI)	P value
Northern population	(n = 103)	(n = 150)				
C-123A						
CC	72 (71.3)	125 (85.6)	Reference		Reference	
CA	26 (25.7)	20 (13.7)	0.44 (0.23–0.858)	0.01	0.36 (0.17–0.78)	0.01
AA	3 (3.0)	1 (0.7)	0.19 (0.02–1.88)	0.15	0.28 (0.02–4.37)	0.37
CA + AA	29 (28.7)	21 (14.4)	0.42 (0.22–0.79)	0.006	0.36 (0.17–0.76)	0.007
G-88T						
GG	56 (55.5)	97 (66.4)	Reference		Reference	
GT	35 (34.6)	43 (29.4)	0.71 (0.41–1.24)	0.22	0.63 (0.33–1.22)	0.17
TT	10 (9.9)	6 (4.1)	0.35 (0.12–1.00)	0.06	0.51 (0.15–1.75)	0.29
GT + TT	45 (44.5)	49 (33.6)	0.63 (0.37–1.06)	0.08	0.61 (0.33–1.13)	0.12
Southern population	(n = 37)	(n = 151)				
C-123A						
CC	33 (89.2)	118 (78.2)	Reference		Reference	
CA	4 (10.8)	30 (19.9)	2.10 (0.70–6.38)	0.24	2.07 (0.68–6.31)	0.20
AA	0 (0)	3 (2.0)	NA	NA	NA	NA
CA + AA	4 (10.8)	33 (21.9)	2.31 (0.76–6.98)	0.17	2.30 (0.76–6.97)	0.14
G-88T						
GG	21 (56.8)	88 (58.3)	Reference		Reference	
GT	15 (40.5)	59 (39.1)	0.94 (0.45–1.97)	0.87	0.94 (0.45–1.97)	0.86
TT	1 (2.7)	4 (2.6)	0.96 (0.10–8.99)	1.00	1.03 (0.11–9.78)	0.98
GT + TT	16 (43.2)	63 (41.7)	0.94 (0.45–1.94)	0.88	0.94 (0.45–1.96)	0.87
Combined	(n = 140)	(n = 301)				
C-123A						
CC	105 (75.5)	243 (81.8)	Reference		Reference	
CA	31 (22.3)	50 (16.8)	0.71 (0.42–1.20)[c]	0.20[c]	0.70 (0.41–1.18)	0.19
AA	3 (2.2)	4 (1.4)	0.54 (0.11–2.72)[c]	0.45[c]	0.66 (0.14–3.09)	0.60
CA + AA	34 (24.5)	54 (18.2)	0.69 (0.42–1.15)[c]	0.16[c]	0.70 (0.42–1.15)	0.17
G-88T						
GG	77 (55.4)	185 (62.3)	Reference		Reference	
GT	51 (36.7)	102 (34.3)	0.79 (0.50–1.22)[c]	0.28[c]	0.83 (0.54–1.30)	0.43
TT	11 (7.9)	10 (3.4)	0.42 (0.17–1.06)[c]	0.07[c]	0.39 (0.16–0.96)	0.04
GT + TT	62 (44.6)	112 (37.7)	0.72 (0.47–1.10)[c]	0.13[c]	0.75 (0.49–1.15)	0.19

The number of samples genotyped varies because of genotyping failure for some individuals.

OR odds ratio, *CI* confidence interval, *NA* not applicable

[a] ORs and *P* values were calculated by logistic regression analysis

[b] ORs and *P* values were calculated by multivariate logistic regression, adjusted for age (months) and sex

[c] ORs and *P* values were calculated by Mantel–Haenszel (MH) test

genotype (MH population-weighted OR, 0.57; 95 % CI, 0.44–0.75; *P* < 0.001), for the -88T-positive genotype (MH population-weighted OR, 0.65; 95 % CI, 0.52–0.82; *P* < 0.001) (Table 2). There was no significant heterogeneity for both the -123A-positive and -88T-positive genotypes associated ORs among the study populations (Breslow-Day *P* = 0.87 for C-123A; Breslow-Day *P* = 0.85 for G-88T).

The effects of C-123A and G-88T genotypes on clinical severity of EV71 infection were also assessed. In both populations, there was no association between G-88T polymorphism and severity of EV71 infection. For C-123A polymorphism, no association was observed with severity of EV71 infection in the southern population. While in the northern population, subjects bearing at least one -123A allele had a decreased susceptibility to EV71 infection

Table 4 Haplotypes for *MxA* gene and their associations with risk to EV71 infection and disease severity

Haplotypes	Frequency		OR (95 % CI)	*P* value	Frequency		OR (95 % CI)	*P* value
	Control	Case			Uncomplicated case	Complicated case		
Northern population								
Global[a]				0.01				0.01
Haplotype-specific[b]								
$C_{-123}G_{-88}$	0.72	0.78	1.37 (1.06–1.75)	0.01	0.73	0.81	1.56 (1.03–2.36)	0.04
$A_{-123}T_{-88}$	0.16	0.11	0.62 (0.44–0.85)	0.003	0.16	0.08	0.44 (0.25–0.79)	0.005
$C_{-123}T_{-88}$	0.12	0.11	0.97 (0.71–1.34)	0.87	0.11	0.11	0.99 (0.61–1.62)	0.98
Southern population								
Global[a]				0.02				0.08
Haplotype-specific[b]								
$C_{-123}G_{-88}$	0.70	0.78	1.51 (1.12–2.05)	0.006	0.77	0.78	1.05 (0.55–2.02)	0.88
$A_{-123}T_{-88}$	0.16	0.10	0.63 (0.43–0.92)	0.01	0.05	0.12	2.31 (0.80–6.64)	0.09
$C_{-123}T_{-88}$	0.14	0.12	0.84 (0.58–1.23)	0.36	0.18	0.10	0.48 (0.22–1.04)	0.07
Combined								
Global[a]				<0.001				0.18
Haplotype-specific[b]								
$C_{-123}G_{-88}$	0.71	0.78	1.42 (1.17–1.73)	<0.001	0.74	0.79	1.37 (0.98–1.92)	0.07
$A_{-123}T_{-88}$	0.16	0.11	0.62 (0.48–0.80)	<0.001	0.13	0.10	0.73 (0.47–1.13)	0.16
$C_{-123}T_{-88}$	0.12	0.11	0.92 (0.72–1.17)	0.48	0.13	0.11	0.83 (0.55–1.24)	0.37

OR odds ratio, *CI* confidence interval

[a] The global *P* value indicates the overall significance of the association of *MxA* C-123A and G-88T haplotype, determined using the omnibus haplotype test of association

[b] In haplotype-specific analyses, the ORs with 95 % CI was determined by testing each haplotype against all others

compared with those without -123A allele, (adjusted OR = 0.36, 95 % CI = 0.17–0.76, *P* = 0.007) (Table 3).

Haplotypes and risk of EV71 infection

By LD analysis, the *D′* values for the *MxA* C-123A and G-88T polymorphisms in the northern and southern populations were both 1.0, whereas r^2 was 0.48 and 0.44, respectively. Three haplotypes (C_{-123}–G_{-88}, C_{-123}–T_{-88} and A_{-123}–T_{-88}) were observed based on these two polymorphisms. Consistent with the findings of published studies (Ching et al. 2010; Weinstock-Guttman et al. 2007), the A_{-123}–G_{-88} haplotype was absent in our two populations. The overall haplotype analysis gave a *P* value of 0.01 in the northern and 0.02 in the southern population (Table 4). Compared with all other haplotypes, the A_{-123}–T_{-88} was associated with a significantly lower risk to EV71 infection in the two independent populations (in the northern population: OR, 0.62; 95 % CI, 0.44–0.85; *P* = 0.003; in the southern population: OR, 0.63; 95 % CI, 0.43–0.92; *P* = 0.01) as well as in the combined population (OR, 0.62; 95 % CI, 0.48–0.80; *P* < 0.001) (Table 4). There was a significantly association between the *MxA* A_{-123}–T_{-88} haplotype and severity of EV71 infection in the northern

population (OR, 0.44; 95 % CI, 0.25–0.79; *P* = 0.005), which however failed to be detected in the southern population (Table 4).

Quantitative real-time RT-PCR for *MxA* expression

As shown in Fig. 2, the relative *MxA* mRNA level from individuals with -123A allele (AA + AC genotypes) was significantly higher than that from individuals with -123CC genotype in both northern and southern populations (*t* test; *P* < 0.05). With regard to the G-88T polymorphism, subjects with the -88T allele (TT + GT genotypes) appeared to express higher *MxA* mRNA than those with the -88GG genotype in both northern and southern populations (*t* test; *P* < 0.05). In addition, those subjects that are homozygous for the major C-123A and G-88T haplotype (CC/GG) had lower levels of *MxA* mRNA than subjects that are heterozygous (CA/GT) in both populations (*t* test; *P* < 0.05).

The relative *MxA* mRNA level was also compared between uncomplicated and complicated cases (Fig. 3). There were no significant differences in the relative *MxA* mRNA level between uncomplicated and complicated cases in both populations (*t* test; *P* > 0.05).
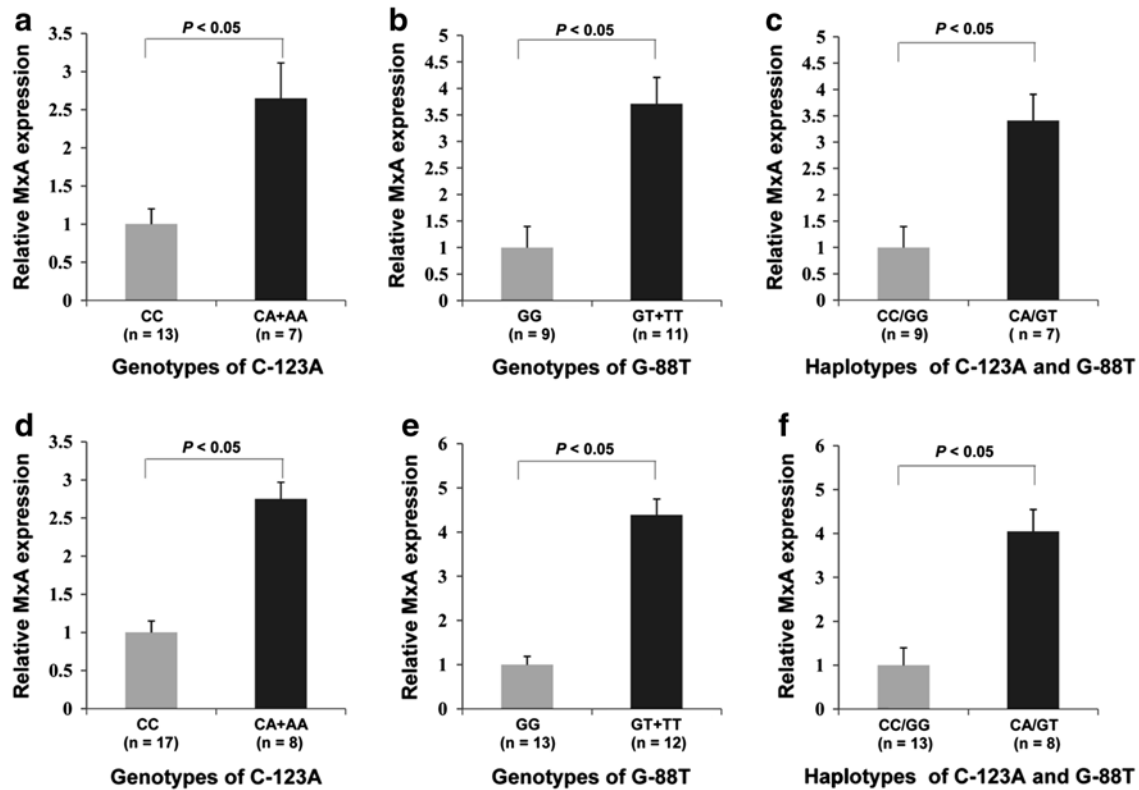
Fig. 2 Quantitative real-time RT-PCR for MxA in healthy children. Expression of *MxA* for three different genotypes of C-123A and G-88T was measured in RNA from 45 healthy children (20 from the northern population and 25 from the southern population) stimulated with IFNβ1a. Normalization for mRNA quantity was performed with human GAPDH control primers for each sample and final abundance figures adjusted to yield an arbitrary value of 1 for -123CC or -88GG genotype. Columns mean from triplicate measurements; the *vertical bars* indicate the standard deviation (SD). **a** Correlation of *MxA* mRNA expression with C-123A genotypes in the northern population. Compared to the CC carriers, the A allele carriers had a markedly elevated *MxA* transcription (*P* < 0.05; *t* test). **b** Correlation of *MxA* mRNA expression with G-88T genotypes in the northern population. Compared to the GG carriers, the T allele carriers had a markedly elevated *MxA* transcription (*P* < 0.05; *t* test). **c** Correlation of *MxA* mRNA expression with *MxA* haplotypes in the northern population. The subjects that are homozygous for the major C-123A and G-88T haplotype (CC/GG) had lower levels of *MxA* mRNA than subjects that are heterozygous (CA/GT) (*t* test; *P* < 0.05). **d** Correlation of *MxA* mRNA expression with C-123A genotypes in the southern population. Compared to the CC carriers, the A allele carriers had a markedly elevated *MxA* transcription (*P* < 0.05; *t* test). **e** Correlation of *MxA* mRNA expression with G-88T genotypes in the southern population. Compared to the GG carriers, the T allele carriers had a markedly elevated *MxA* transcription (*P* < 0.05; *t* test). **f** Correlation of *MxA* mRNA expression with *MxA* haplotypes in the southern population. The subjects that are homozygous for the major C-123A and G-88T haplotype (CC/GG) had lower levels of *MxA* mRNA than subjects that are heterozygous (CA/GT) (*t* test; *P* < 0.05)

Discussion

In this study, we found that two functional polymorphisms in the promoter of *MxA* gene, C-123A and G-88T, were significantly associated with decreased susceptibility to EV71 infection in two independent Chinese subpopulations. Haplotype A₋₁₂₃–T₋₈₈, derived from these two polymorphisms was also shown to be associated with the decreased risk of EV71 infection. Furthermore, consistent with the results of population-based association study, higher *MxA* messenger RNA levels in IFNβ1a-stimulated PBMCs from the -123A or -88T allele carriers were observed. To the best of our knowledge, this study is the first to report a genetic association between *MxA* and susceptibility to EV71 infection,

confirming the initial hypothesis that MxA may play a role in the pathogenesis of this disorder.

The genetic association between *MxA* polymorphism and EV71 infection is biologically plausible. MxA is an antiviral protein induced by type I IFNs that can inhibit viral replication. Following stimulation with type I IFNs, MxA protein accumulates to high levels in the cytoplasm on intracellular membranes. At the point of infection, MxA are released and bind viral nucleocapsids or other viral components, to trap and then degrade them (Sadler and Williams 2008). With regard to EV71, it has been shown that the superior anti-EV71 effect of type I IFN was caused by a strong induction of the downstream antiviral effectors, especially MxA (Sadler and Williams 2008). In addition,
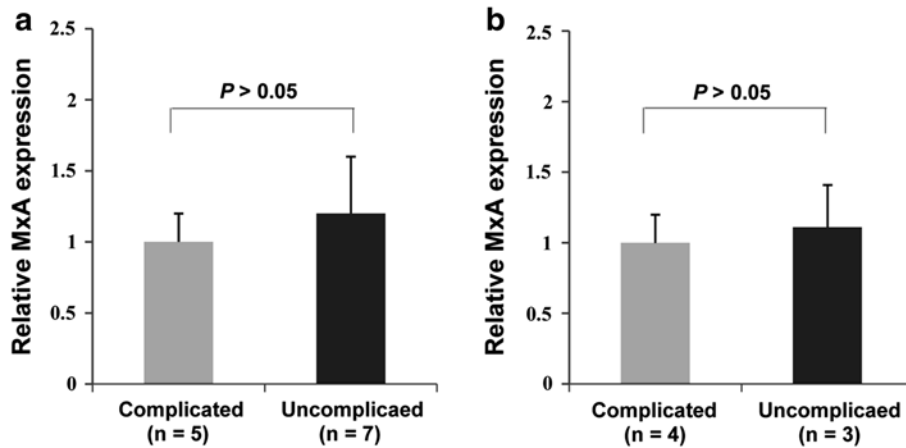
Fig. 3 Quantitative real-time RT-PCR for *MxA* in patients with EV71 infection. Expression of *MxA* was measured in RNA from 19 patients with EV71 infection (12 from the northern population and 7 from the southern population). Normalization for mRNA quantity was performed with human GAPDH control primers for each sample and final abundance figures adjusted to yield an arbitrary value of 1 for complicated cases. Columns mean from triplicate measurements; the *vertical bars* indicate the standard deviation (SD). **a** *MxA* mRNA expression in patients with EV71 infection in the northern population. Compared to the complicated cases, the uncomplicated cases had a comparable *MxA* transcription level ($P > 0.05$; *t* test). **b** *MxA* mRNA expression in patients with EV71 infection in the southern population. Compared to the complicated cases, the uncomplicated cases had a comparable *MxA* transcription level ($P > 0.05$; *t* test)

it has recently been reported that the antiviral activity of MxA extended to coxsackieviruses B virus, a member of the enterovirus family (Chieux et al. 2001). Thus, it is highly possible that MxA can bind to the components of EV71 and exert its role as an antivirus, although additional studies are needed to clarify this possibility.

The *MxA* C-123A and G-88T polymorphisms are both functional polymorphisms. Previous in vitro studies showed that -123A and -88T alleles provided stronger binding affinity to nuclear proteins than the wild-type alleles (Ching et al. 2010). Furthermore, the C-123A and G-88T polymorphisms are in strong LD and the A_{-123}–T_{-88} haplotype displays higher promoter activity compared with the C_{-123}–G_{-88} haplotype in luciferase reporter assay (Furuyama et al. 2006; Hijikata et al. 2001; Torisu et al. 2004). In the present study, we also detected higher *MxA* transcription in IFNβ1a-stimulated PBMCs from the -123A or -88T allele carriers. Given the well-characterized antiviral role of MxA, one might expect that individuals who carry the -123A and/or -88T alleles, and thus have increased expression of MxA, may be at a lower susceptibility to EV71 infection.

In this study, the association with risk of EV71 infection was stronger for the C-123A polymorphism than the G-88T polymorphism. This finding is biologically rational. Although administration of type I IFN can inhibit EV71 replication in vitro and in mouse model, a recent study found that EV71 inhibits the expression of IFN-β in infected cells (Lei et al. 2010), which suggests that the virus evades the antiviral activity of IFN-β. As the C-123A polymorphism affects basal MxA expression without IFN-α and IFN-β induction, whereas the G-88T polymorphism influenced MxA expression after IFN-β stimulation, the C-123A SNP probably exerts greater effect on basal MxA expression in innate immune response against EV71, in which endogenous IFN induction is suppressed. In addition, we found markedly elevated *MxA* transcription in response to IFNβ1a stimulation for genotypes of the G-88T polymorphism, while the response is not so marked for the C-123A polymorphism. Our results are also consistent with previous findings that the C-123A polymorphism plays a more important role in modifying basal MxA expression, and contributes more significantly to the innate immune response against SARS (Ching et al. 2010).

In this study, we observe associations between *MxA* C-123A polymorphism and A_{-123}–T_{-88} haplotype with disease severity only in northern population. This result may be due to the limited sample size of patients with uncomplicated cases ($n = 37$) in the southern population in our study. However, no association between C-123A polymorphism and severity of EV71 infection was observed when northern and southern populations were combined for analysis. Moreover, there were no significant differences in the relative *MxA* mRNA level between uncomplicated and complicated cases. Therefore, our results indicate that *MxA* C-123A polymorphism might not play a role in disease severity of EV71 infection. Still, this result warrants further confirmation in future studies.

To our knowledge, only three genetic association studies have addressed susceptibility genes that related to EV71 infection (Chang et al. 2008; Yang et al. 2001, 2012). Some of the results, however, could not be replicated in

subsequent studies in other populations. The inconsistent results could be attributable to the small sample size and/or inadequate adjustment for confounding factors such as age and sex. Age and sex are well-known confounding factors for EV71 infection, because individuals aged 1–4 years are associated with a higher risk (Chan et al. 2003; Mao et al. 2010), and the proportion of male was higher in patients with EV71 infection (Chen et al. 2007). This tendency was also identified in our case patients. In the present study, the association was derived from two different subpopulations of large sample size, with potential confounding factors (sex and age) adjusted. Our study, therefore, could probably pose as one of the largest case–control studies demonstrating valid and replicable association of EV71 infection.

Recently, several association studies have shown that the *MxA* gene was related to the susceptibility to various specific infections (Cao et al. 2009; Ching et al. 2010; Hamano et al. 2005; He et al. 2006; Hijikata et al. 2000). Some of the results, however, could not be replicated in subsequent studies because of multiple factors, such as the different ethnicities of study populations and/or different pathogenesis of the studied diseases. Although the highly significant association between *MxA* and susceptibility to EV71 derived from a biologically based a priori hypothesis, our findings should be independently verified in populations of different ancestry, such as white subjects and African subjects.

In conclusion, our study shows an association between the *MxA* polymorphisms and decreased susceptibility to EV71 infection in two Chinese subpopulations. These findings may provide support for the importance of MxA in the pathogenesis of EV71 infection. If confirmed by other studies, knowledge of genetic factors contributing to the pathogenesis of EV71 infection as presented here would be important for the assessment of host susceptibility to EV71 infection and other infectious diseases, especially those sharing a mode of action similar to that of EV71 infection.

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