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Original Article

Associations of IL-2 and IL-4 Expression and Polymorphisms With the Risks of *Mycoplasma pneumoniae* Infection and Asthma in Children☆

Rong-Shan Wang,[a] Hong-Xing Jin,[a] Shi-Qiang Shang,[b,*] Xi-Yong Liu,[a] Shu-Jun Chen,[a] Zhi-Biao Jin[a]

[a] *Department of Pediatrics, Yiwu Maternity and Child Care Hospital, Zhejiang, China*
[b] *Department of Pediatrics, Laboratory of the Children's Hospital of Zhejiang Province, Zhejiang, China*

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ABSTRACT

Introduction: Asthma is an inflammatory disorder of the airways and the symptoms of asthma could be exacerbated by *Mycoplasma pneumoniae* infection. Interleukin-2 and interleukin-4 have been implicated in immune and inflammatory reactions. We examined the associations of *IL2* and *IL4* polymorphisms and expression with the risks of asthma and *M. pneumoniae* infection in children.
Methods: A total of 392 asthmatic children and 849 controls were recruited into the study. Eight polymorphisms in *IL2* and *IL4* were genotyped with Sequenom MassARRAY platform. *M. pneumoniae* infection and copy number was determined with fluorescence PCR. IL-2 and IL-4 serum expression levels were determined by using ELISA.
Results: We found a significant association of *IL2* rs6534349 polymorphism with increased asthma risk (heterozygotes, *P*=.029; homozygous variants; *P*=.013) and of *IL4* rs2227284 polymorphism with reduced asthma risk (heterozygotes, *P*=.026; homozygous variants; *P*=.001). Besides, the association of other polymorphisms, except rs2070874 polymorphism, became apparent when the asthmatic children were grouped according to GINA classification of asthma control and severity. In addition, IL-2 and IL-4 serum expression levels were significantly higher in *M. pneumoniae* negative (*P*=.038) and positive (*P*=.011) subjects respectively. This observation holds true among asthmatic patients (*P*=.016 for IL-2 and *P*=.042 for IL-4), but only the IL-4 observation remained correct among non-asthmatic controls (*P*=.032). We also observed that the rs6534349 GG genotype was significantly associated with increased odds of getting high load *M. pneumoniae* infection (*P*=.0376).
Conclusions: *IL2* and *IL4* could be important biomarkers for estimating the risks of asthma and *M. pneumoniae* infection in children.

Relación entre la expresión de IL-2 e IL-4 y sus polimorfismos y los riesgos de padecer infección por *Mycoplasma pneumoniae* y asma en niños

Palabras clave:
Asma
Niños
Interleucina-2
Interleucina-4
Mycoplasma pneumoniae

RESUMEN

Introducción: El asma es una afección inflamatoria de las vías respiratorias. Las infecciones por *Mycoplasma pneumoniae* pueden exacerbar los síntomas del asma. Se ha demostrado que la interleucina 2 y la interleucina 4 participan en las reacciones inmunitarias e inflamatorias. Hemos estudiado la relación entre los polimorfismos de la *IL2* y la *IL4* y su expresión y el riesgo de padecer asma e infección por *M. pneumoniae* en niños.
Métodos: Se reclutó a 392 niños asmáticos y 849 controles para el estudio. Se genotiparon 8 polimorfismos en *IL2* e *IL4* con la plataforma MassARRAY de Sequenom. La infección por *M. pneumoniae* y el número de copias se establecieron mediante PCR fluorescente. Los niveles séricos de expresión de IL-2 e IL-4 se midieron con ELISA.

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* Corresponding author.
E-mail address: shiqiangshang945@gmail.com (S.-Q. Shang).

Resultados: Hallamos una relación significativa entre el polimorfismo rs6534349 de *IL2* y el aumento de riesgo de sufrir asma (heterocigóticos, p = 0,029; variantes homocigóticas, p = 0,013), así como entre el polimorfismo rs2227284 de *IL4* y una reducción del riesgo de padecer asma (heterocigóticos, p = 0,026; variantes homocigóticas, p = 0,001). Además, la relación con otros polimorfismos, excepto el rs2070874, se hizo evidente al agrupar a los niños asmáticos según la clasificación GINA de control y gravedad del asma. Asimismo, los niveles séricos de expresión de IL-2 e IL-4 fueron significativamente mayores en los sujetos no infectados (p = 0,038) e infectados (p = 0,011) por *M. pneumoniae*, respectivamente. Esta observación también se cumple entre los pacientes asmáticos (p = 0,016 para IL-2 y p = 0,042 para IL-4), pero en los controles no asmáticos solo se cumple en el caso de la IL-4 (p = 0,032). Del mismo modo, observamos que el genotipo GG rs6534349 estaba claramente relacionado con un aumento de las posibilidades de tener una infección con alta carga de *M. pneumoniae* (p = 0,0376).
Conclusiones: La *IL2* y la *IL4* podrían ser biomarcadores importantes para calcular el riesgo de padecer asma, así como infección por *M. pneumoniae*, en niños.

Introduction

Asthma is a complex chronic inflammatory disease of the airways characterized by airflow obstruction and hyperresponsiveness. In children, asthma arises principally from allergic inflammation, and can lead to varying degrees of airway obstruction, including but not limited to dyspnea, coughing, chest tightness, and recurrent episodes of wheezing. In China and the rest of the world, asthma in children represents a major public health concern in pediatric pulmonology, and the prevalence of childhood asthma increases every year.[1,2]

Mycoplasma pneumoniae is an important pathogen which has been primarily recognized as a causative agent of community-acquired pneumonia, especially among children.[3] The link between *M. pneumoniae* infection and asthma was first suspected several decades ago.[4] More recently, various lines of evidence have emerged and *M. pneumoniae* infection has been implicated in the onset and exacerbation of asthma.[5,6] *M. pneumoniae* infection damages respiratory epithelial cells, thus increasing airway reactivity and resulting in the activation of a large number of T cells.[7] T cells trigger the activation of B cells, which produce antibodies and release inflammatory cytokines, which in turn contribute to the development of asthma-related symptoms.

Despite the complexity of the mechanisms underlying the onset of asthma, interleukins (ILs), especially IL-2 and IL-4, are known to play a central role in this process.[8] IL-2 promotes the maturation of primitive T cells, while IL-4, through its complex interaction with IL-12, helps to determine whether T cells should differentiate into Th1 or Th2 cells, thus producing different types of cytokines.[9] These events cause major pathophysiological changes during asthma, and therefore play a key role in the development of the disease. In addition, the important role of IL-2 and IL-4 in the adaptive immune system suggests that cytokines could affect the ability of immune cells to fight off *M. pneumoniae* infection. We therefore hypothesized that variations in IL-2 and IL-4 levels and function could be associated with the risk of asthma and *M. pneumoniae* infection.[10,11] A potential factor which could influence the interindividual variation in IL-2 and IL-4 levels and function, and therefore the risk of asthma, is single nucleotide polymorphisms (SNPs) within the genes encoding the cytokines.[12,13] In this study, we aimed to establish how SNPs in IL-2 and IL-4 genes may be associated with the risk of asthma in children. In particular, we addressed the rs6822844, rs6534349, rs2069762 and rs3136534 polymorphisms of IL-2, and rs2243250, rs2070874, rs2227284 and rs2243290 polymorphisms of IL-4. We also aimed to investigate the presence of differential serum expression levels of IL-2 and IL-4 in *M. pneumoniae*-positive and negative subjects, and to examine the relationship between SNPs and the risk of high-load *M. pneumoniae* infection.

Methods

Subjects

The study was approved by the ethics committee of the Yiwu Maternity and Child Care Hospital, Zhejiang (Ref. No: 2009/PED/0215.045). A total of 392 children with asthma and 849 non-asthmatic controls aged between 4 and 15 years old were recruited from the Yiwu Maternity and Child Care Hospital and the Children's Hospital of Zhejiang Province between March 2009 and October 2013. Asthma in children was ascertained by routine diagnosis based on the Global Initiative for Asthma (GINA) guidelines. Controls were children without asthma or allergic symptoms who visited the same hospitals for other medical problems unrelated to asthma. Among the controls, a total of 83 had stridor, 89 had foreign body aspiration, 80 had persistent cough, 66 underwent diagnosis for pulmonary infections, and 531 were healthy children who attended the hospital for follow-up after diagnosis of a transient respiratory infection. Controls and cases were matched by frequency in terms of gender and age. All subjects were of Chinese Han ethnicity. Informed consent was obtained from the parents of the participating subjects before inclusion in the study.

Detection of Mycoplasma pneumoniae *Infection*

DNA was extracted from respiratory specimens (throat swab, No.=528; bronchoalveolar lavage, No.=432; sputum specimens, No.=204; bronchial aspirate, No.=77) obtained from the subjects. Bronchoalveolar lavage was performed on 318 controls (i.e. those who had stridor, foreign body aspiration, persistent cough and who underwent diagnosis for pulmonary infections) as part of their diagnosis or treatment, and on 114 asthmatic children for identification of infectious agents, cytokines and cellular profiles (either for diagnostic purposes or as part of another ongoing research study). A single bronchoalveolar lavage was performed on each subject. Detection of *Mycoplasma pneumoniae* infection among the subjects was performed with fluorescence PCR *Mycoplasma pneumoniae* Detection Kit (Acon Biotech, Hangzhou, China), according to the manufacturer's instructions.

ELISA Quantification of IL-2 and IL-4

Serum was isolated from the blood samples collected and diluted 1:4 in sample diluent before being used for ELISA quantification of IL-2 and IL-4. The serum IL-2 and IL-4 concentrations were determined with the Human Interleukin 2/IL-2 ELISA Kit and Human Interleukin 4/IL-4 ELISA Kit (Xinqidi Biological Technology Co. Ltd., Wuhan, China), which have a sensitivity of <0.40 pg/ml

and <0.20 pg/ml respectively, according to the manufacturer's protocols.

SNP Genotyping

DNA was isolated from subjects' blood samples using TIANamp blood DNA kit (Tiangen Biotech, Beijing, China). Polymorphisms were genotyped on the DNA isolated using the Sequenom MassARRAY platform (Sequenom, San Diego, USA), according to the manufacturer's instructions. Genotyping was repeated in 10% of the samples for confirmation purposes. The reproducibility rate of the genotypes was 100%.

Statistical Analysis

The distribution of genotypes in asthmatic children and non-asthmatic controls was compared using a χ^2 test, and the association between the polymorphisms and asthma risk was evaluated by logistic regression analysis. To examine the relationship between polymorphisms and the risk of predisposition to high-load *M. pneumoniae* infection, the genotype distribution was compared using the χ^2 test followed by logistic regression analysis. Expression of IL-2 and IL-4 in *M. pneumoniae*-positive and *M. pneumoniae*-negative patients was compared using a *t*-test to determine any significant difference in the expression between the two groups. For all analyses, $P<.05$ was considered significant.

Results

Subjects Characteristics and M. pneumoniae Infection

Cases and controls were frequency-matched in terms of gender and age. No significant differences were found for either gender ($P=.99$) or mean age ($P=.98$). Based on GINA classification, 177 cases were controlled asthma, 118 were partially controlled asthma and 97 were uncontrolled asthma. In addition, 102 were classified as severe, 80 moderate and 210 mild.

Detection of M. pneumoniae Infection

A total of 344 subjects tested positive for *M. pneumoniae* infection, of which 130 were children with asthma and 214 were non-asthmatic controls. Of the 897 *M. pneumoniae*-negative subjects, 262 were children with asthma and 635 were non-asthmatic controls. Table 1 shows the prevalence of *M. pneumoniae* detected in different types of specimens. The type of specimen used did not significantly affect the detection of *M. pneumoniae* ($P=.70$).

The number of bacterial copies in the *M. pneumoniae*-positive subjects ranged from 6.43×10^3 to 6.48×10^7 organisms ml^{-1}, with a median and mean of 3.96×10^5 and 1.67×10^7 organisms ml^{-1}, respectively. A total of 206 and 138 subjects had bacterial load lower and higher than mean, respectively.

Genotype Distribution in Asthmatic Children and Healthy Controls

Of the 8 polymorphisms studied, significant differences between cases and controls were observed in only 2, namely rs6534349 polymorphism of IL-2 ($P=.006$) and rs2227284 polymorphism of IL-4 ($P=.001$) (Table 2). No deviation from Hardy-Weinberg equilibrium was observed for any of the 8 polymorphisms ($P>.05$).

Association of the Polymorphisms and Asthma Risk

Significant risk association was only observed for the IL-2 rs6534349 and IL-4 rs2227284 polymorphisms (Table 2). For the IL-2 rs6534349 polymorphism, the heterozygous AG genotype and homozygous variant GG genotype resulted in a 1.374-fold (95% CI=1.033–1.828, $P=.029$) and a 2.554-fold (95% CI=1.216–5.361, $P=.013$) increase in risk, respectively. Heterozygous TG genotype of the IL-4 rs2227284 polymorphism, meanwhile, showed an odds ratio of 0.750 (95% CI=0.583–0.965, $P=.026$), while the homozygous GG genotype showed an odds ratio of 0.433 (95% CI=0.262–0.716, $P=.001$).

Asthmatic Risk Association Based on GINA Classification of Asthma Control

The genotype distribution and risk association of the polymorphisms based on GINA classification of asthma control are shown in Table 3. For controlled asthma, only the IL-2 rs2069762 polymorphism was found to be associated with risk of the disorder ($P=.047$). None of the polymorphisms in the IL-4 gene appeared to confer a risk or protect its carriers from asthma. In the partially controlled asthma group, however, 3 IL-2 polymorphisms (rs6822822, rs2069762, rs6534349) and 1 IL-4 polymorphism (rs2227284) showed a risk association with asthma ($P=.040$, .001, .020 and .032, respectively). In the uncontrolled group, 5 polymorphisms, i.e. IL-2 rs6534349 ($P=.001$), rs2069762 ($P=.038$), and rs3136543 ($P=.038$), as well as IL-4 rs2243250 ($P=.031$) and rs2227284 ($P=.004$), showed significant asthmatic risk association.

Asthmatic Risk Association Based on GINA Classification of Asthma Severity

The distribution of polymorphism genotypes in patients with different levels of asthma severity is shown in Table 4, along with the corresponding risk association. For individuals with severe asthma, significant associations were observed for rs2243290 ($P=.002$). For moderate asthma, only rs6534349 was significant ($P=.007$). Finally, rs2069762 and rs2227284 polymorphisms were associated with a risk of mild asthma ($P=.007$ and $P=.008$, respectively).

IL-2 and IL-4 Expression in M. pneumoniae-negative and Positive Subjects

Table 5 shows serum expression levels (in terms of concentration) of IL-2 and IL-4 in *M. pneumoniae*-negative and positive subjects. Mean IL-2 concentration in *M. pneumoniae*-negative

Table 1
Detection of *M. pneumoniae* Infection Using Different Samples.

Specimen	*M. pneumoniae* positive	*M. pneumoniae* negative	*P*-value
Throat swab (No.=528)	154 (29.17%)	374 (70.83%)	
Bronchoalveolar lavage (No.=432)	113 (26.16%)	319 (73.84%)	
Sputum specimens (No.=204)	54 (26.47%)	150 (73.53%)	.70
Bronchial aspirate (No.=77)	23 (29.87%)	54 (70.13%)	
Total	344 (27.72%)	897 (72.28%)	

Table 2
Genotype Distribution and Risk Association in Asthmatic Children and Healthy Controls.

SNP	Genotype	Cases (No.)	%	Controls (No.)	%	*P*	Odds ratio	*P*
rs6822844	GG	277	70.66	562	66.20	.595	Reference	–
	GT	104	26.53	249	29.33		0.847 (0.647–1.110)	.230
	TT	11	2.81	38	4.48		0.587 (0.296–1.167)	.129
rs6534349	**AA**	**279**	**71.17**	**665**	**78.33**	**.006**	Reference	–
	AG	**98**	**25.00**	**170**	**20.02**		**1.374 (1.033–1.828)**	**.029**
	GG	**15**	**3.83**	**14**	**1.65**		**2.554 (1.216–5.361)**	**.013**
rs2069762	GG	41	10.46	78	9.19	.545	Reference	–
	GT	153	39.03	357	42.05		0.815 (0.534–1.245)	.344
	TT	198	50.51	414	48.76		0.910 (0.601–1.377)	.655
rs3136534	AA	176	44.90	346	40.75	.223	Reference	–
	AC	180	45.92	402	47.35		0.880 (0.684–1.133)	.322
	CC	36	9.18	101	11.90		0.701 (0.460–1.068)	.098
rs2243250	TT	165	42.09	333	39.22	.536	Reference	–
	TC	177	45.15	412	48.53		0.867 (0.671–1.121)	.276
	CC	50	12.76	104	12.25		0.970 (0.660–1.427)	.878
rs2070874	TT	167	42.60	337	39.69	.531	Reference	–
	TC	176	44.90	410	48.29		0.866 (0.671–1.119)	.272
	CC	49	12.50	102	12.02		0.969 (0.658–1.429)	.875
rs2227284	**TT**	**225**	**57.40**	**408**	**48.06**	**.001**	Reference	–
	TG	**146**	**37.24**	**353**	**41.58**		**0.750 (0.583–0.965)**	**.026**
	GG	**21**	**5.36**	**88**	**10.37**		**0.433 (0.262–0.716)**	**.001**
rs2243290	AA	174	44.39	331	38.99	.190	Reference	–
	AC	173	44.13	416	49.00		0.791 (0.613–1.021)	.072
	CC	45	11.48	102	12.01		0.839 (0.565–1.247)	.386

subjects was significantly higher than in *M. pneumoniae*-positive subjects (*P*=.038). The opposite was observed for IL-4 (*P*=.011).

When the subjects were classified according to presence of asthma, a similar trend was observed for asthmatic patients (*P*=.016 for IL-2; *P*=.042 for IL-4). Among non-asthmatic controls, however, IL-2 levels in *M. pneumoniae*-positive and negative subjects did not differ significantly (*P*=.492). Nonetheless, as in asthmatic subjects, a significantly lower mean concentration of IL-4 was observed among *M. pneumoniae*-negative subjects compared to *M. pneumoniae*-positive subjects (*P*=.032).

Genotype Distribution in M. pneumoniae*-positive Subjects With Low- and High-load Infections*

Table 6 summarizes the distribution of genotypes of the 8 polymorphisms in *M. pneumoniae*-positive subjects with low and high bacterial loads. No significant difference was observed between the 2 study groups in terms of the genotypic distributions of all 8 polymorphisms (*P*>.05). None of the genotypic distributions deviated significantly from the Hardy-Weinberg equilibrium (*P*>.05).

Relationship of Polymorphisms With Risk of Predisposition to High-load M. pneumoniae *Infection*

The association between the 8 polymorphisms and the risk of predisposition to high-load *M. pneumoniae* infection is also shown in Table 6. Significant association was observed only in IL-4 rs2227284 polymorphism (*P*=.0376). No statistically significant association was observed for the other polymorphisms (*P*>.05).

Discussion

Asthma is a disorder of the airways which arises principally from chronic inflammation of the respiratory system, the symptoms of which are thought to be exacerbated by *M. pneumoniae* infection.[5,6] Eradication of *M. pneumoniae* infection and the development of asthma may be influenced by cytokines, key mediators of immune and inflammatory reactions. Two important cytokines linked to the development of asthma are IL-2 and IL-4. We hypothesized that polymorphisms within IL-2 and IL-4 genes could be associated with a risk of asthma and *M. pneumoniae* infection in children.

We investigated the association of 4 IL-2 polymorphisms and 4 IL-4 polymorphisms with risk of asthma in children. Our results showed that the IL-2 rs6534349 polymorphism and IL-4 rs2227284 polymorphism could significantly increase and decrease asthma risk, respectively. The association appeared to be dose-dependent. In other words, the risk and protective effects of the polymorphisms were stronger when the variant alleles were present in 2 copies (homozygous variant) than in 1 copy (heterozygous). We also analyzed the association of the polymorphisms with different levels of asthma control and severity based on GINA classification. We found that among controlled patients, only one polymorphism (rs2069762) was associated with asthma risk, while four polymorphisms (rs6822844, rs6534349, rs2069762 and rs2227284) were associated with asthma risk among the partially controlled group, and five polymorphisms (rs6534349, rs2069762, rs3136534, rs2243250 and rs2227284) were associated with asthma risk among uncontrolled group. The association of increasing numbers of polymorphisms with increasing loss of control suggests the involvement of an extensive network of genetic interactions and highlights the complexity of the disease.

The number of polymorphisms significantly associated with asthma risk, however, was similar in patients with different levels of asthma severity. Mild asthma patients demonstrated risk associated with two polymorphisms (rs2069762 and rs2227284), whereas moderate and severe patients each demonstrated risk associated with one polymorphism (rs6534349 for moderate, rs2243290 for severe). It is interesting to note that entirely different polymorphisms were involved in asthmatic patients with different degrees of severity, suggesting that each polymorphism could exert a unique effect which contributes to the development of asthma. Nonetheless, it should be pointed out that when the analysis was performed according to levels of asthma severity, the sample size became too low to assure reliable data interpretation.[14] This is one of the limitations of this study.

Table 3
Genotype Distribution and Risk Association in Asthmatic Children and Healthy Controls Based on GINA Classification of Asthma Control.

GINA classification of control	SNP	Genotype	Cases (No.)	Controls (No.)	Odds ratio	*P*
Controlled	rs6822844	GG	64	562	Reference	–
		GT	30	249	1.058 (0.668–1.673)	.809
		TT	3	38	0.693 (0.208–2.309)	.550
	rs6534349	AA	73	665	Reference	–
		AG	20	170	1.071 (0.635–1.807)	.795
		GG	4	14	2.602 (0.834–8.115)	.099
	rs2069762	GG	4	78	Reference	–
		GT	32	357	1.747 (0.600–5.085)	.305
		TT	**61**	**414**	**2.873 (1.101–8.130)**	**.047**
	rs3136534	AA	42	346	Reference	–
		AC	46	402	0.945 (0.607–1.470)	.802
		CC	9	101	0.734 (0.345–1.559)	.421
	rs2243250	TT	40	333	Reference	–
		TC	42	412	0.848 (0.537–1.339)	.481
		CC	15	104	1.200 (0.637–2.261)	.571
	rs2070874	TT	47	337	Reference	–
		TC	43	410	0.752 (0.485–1.165)	.202
		CC	7	102	0.492 (0.215–1.122)	.091
	rs2227284	TT	57	408	Reference	–
		TG	33	353	0.669 (0.425–1.051)	.081
		GG	7	88	0.569 (0.251–1.290)	.177
	rs2243290	AA	44	331	Reference	–
		AC	41	416	0.741 (0.473–1.161)	.191
		CC	12	102	0.885 (0.450–1.739)	.723
Partially controlled	rs6822844	GG	89	562	Reference	–
		GT	**24**	**249**	**0.608 (0.378–0.978)**	**.040**
		TT	5	38	0.830 (0.318–2.167)	.704
	rs6534349	AA	89	665	Reference	–
		AG	23	170	1.010 (0.620–1.647)	.965
		GG	**6**	**14**	**3.202 (1.199–8.546)**	**.020**
	rs2069762	GG	17	78	Reference	–
		GT	**27**	**357**	**0.347 (0.180–0.667)**	**.001**
		TT	74	414	0.820 (0.459–1.464)	.502
	rs3136534	AA	48	346	Reference	–
		AC	56	402	1.004 (0.665–1.515)	.984
		CC	14	101	0.999 (0.529–1.886)	.998
	rs2243250	TT	40	333	Reference	–
		TC	63	412	1.273 (0.835–1.940)	.262
		CC	15	104	1.200 (0.637–2.261)	.571
	rs2070874	TT	53	337	Reference	–
		TC	47	410	0.728 (0.479–1.107)	.138
		CC	18	102	1.122 (0.629–2.001)	.696
	rs2227284	TT	71	408	Reference	–
		TG	**39**	**353**	**0.634 (0.418–0.962)**	**.032**
		GG	8	88	0.522 (0.242–1.124)	.096
	rs2243290	AA	47	331	Reference	–
		AC	54	416	0.914 (0.602–1.386)	.673
		CC	17	102	1.173 (0.645–2.133)	.599
Uncontrolled	rs6822844	GG	124	562	Reference	–
		GT	50	249	0.910 (0.634–1.305)	.608
		TT	3	38	0.357 (0.108–1.177)	.090
	rs6534349	AA	117	665	Reference	–
		AG	**55**	**170**	**1.838 (1.280–2.641)**	**.001**
		GG	5	14	2.029 (0.717–5.742)	.182
	rs2069762	GG	20	78	Reference	–
		GT	**50**	**357**	**0.546 (0.307–0.969)**	**.038**
		TT	107	414	1.008 (0.590–1.721)	.976
	rs3136534	AA	86	346	Reference	–
		AC	78	402	0.780 (0.556–1.095)	.151
		CC	**13**	**101**	**0.517 (0.277–0.966)**	**.038**
	rs2243250	TT	85	333	Reference	–
		TC	**72**	**412**	**0.684 (0.484–0.967)**	**.031**
		CC	20	104	0.753 (0.441–1.285)	.299
	rs2070874	TT	67	337	Reference	–
		TC	86	410	1.055 (0.743–1.497)	.764
		CC	24	102	1.183 (0.706–1.983)	.522
	rs2227284	TT	97	408	Reference	–
		TG	74	353	0.881 (0.631–1.231)	.460
		GG	**6**	**88**	**0.286 (0.121–0.675)**	**.004**
	rs2243290	AA	83	331	Reference	–
		AC	78	416	0.747 (0.531–1.051)	.094
		CC	16	102	0.625 (0.350–1.116)	.112

Table 4
Genotype Distribution and Risk Association in Asthmatic Children and Healthy Controls Based on GINA Classification of Asthma Severity.

GINA classification of severity	SNP	Genotype	Cases (No.)	Controls (No.)	Odds ratio	*P*
Severe	rs6822844	GG	73	562	Reference	–
		GT	28	249	0.865 (0.546–1.372)	.539
		TT	1	38	0.202 (0.027–1.497)	.117
	rs6534349	AA	73	665	Reference	–
		AG	26	170	1.393 (0.863–2.247)	.174
		GG	3	14	1.952 (0.548–6.952)	.302
	rs2069762	GG	10	78	Reference	–
		GT	24	357	0.524 (0.241–1.140)	.103
		TT	68	414	1.281 (0.632–2.596)	.491
	rs3136534	AA	46	346	Reference	–
		AC	46	402	0.860 (0.558–1.327)	.497
		CC	10	101	0.744 (0.362–1.528)	.421
	rs2243250	TT	44	333	Reference	–
		TC	45	412	0.826 (0.532–1.283)	.396
		CC	13	104	0.946 (0.490–1.824)	.868
	rs2070874	TT	44	337	Reference	–
		TC	47	410	0.878 (0.567–1.357)	.558
		CC	11	102	0.826 (0.411–1.658)	.590
	rs2227284	TT	60	408	Reference	–
		TG	36	353	0.693 (0.447–1.073)	.100
		GG	6	88	0.463 (0.194–1.107)	.083
	rs2243290	AA	54	331	Reference	–
		AC	**34**	**416**	**0.501 (0.318–0.787)**	**.002**
		CC	14	102	0.841 (0.448–1.577)	.589
Moderate	rs6822844	GG	53	562	Reference	–
		GT	24	249	1.022 (0.616–1.693)	.932
		TT	3	38	0.837 (0.250–2.803)	.773
	rs6534349	AA	56	665	Reference	–
		AG	19	170	1.327 (0.768–2.293)	.310
		GG	**5**	**14**	**4.241 (1.473–12.208)**	**.007**
	rs2069762	GG	4	78	Reference	–
		GT	24	357	1.310 (0.442–3.885)	.625
		TT	52	414	2.449 (0.861–6.966)	.093
	rs3136534	AA	29	346	Reference	–
		AC	42	402	1.246 (0.760–2.044)	.382
		CC	9	101	1.063 (0.487–2.319)	.877
	rs2243250	TT	33	333	Reference	–
		TC	36	412	0.881 (0.538–1.445)	.617
		CC	11	104	1.067 (0.521–2.186)	.858
	rs2070874	TT	35	337	Reference	–
		TC	34	410	0.798 (0.487–1.307)	.371
		CC	11	102	1.038 (0.509–2.117)	.917
	rs2227284	TT	42	408	Reference	–
		TG	34	353	0.935 (0.582–1.503)	.783
		GG	4	88	0.441 (0.154–1.263)	.127
	rs2243290	AA	38	331	Reference	–
		AC	35	416	0.732 (0.452–1.185)	.205
		CC	7	102	0.597 (0.259–1.379)	.227
Mild	rs6822844	GG	151	562	Reference	–
		GT	52	249	0.777 (0.548–1.101)	.156
		TT	7	38	0.685 (0.300–1.565)	.370
	rs6534349	AA	150	665	Reference	–
		AG	53	170	1.382 (0.968–1.972)	.074
		GG	7	14	2.216 (0.879–5.587)	.091
	rs2069762	GG	27	78	Reference	–
		GT	**61**	**357**	**0.493 (0.294–0.826)**	**.007**
		TT	122	414	0.851 (0.525–1.378)	.512
	rs3136534	AA	101	346	Reference	–
		AC	92	402	0.784 (0.571–1.076)	.132
		CC	17	101	0.576 (0.329–1.009)	.053
	rs2243250	TT	88	333	Reference	–
		TC	96	412	0.881 (0.538–1.445)	.617
		CC	26	104	0.946 (0.579–1.543)	.824
	rs2070874	TT	88	337	Reference	–
		TC	95	410	0.887 (0.641–1.226)	.469
		CC	27	102	1.013 (0.624–1.646)	.956
	rs2227284	TT	123	408	Reference	–
		TG	76	353	0.714 (0.518–0.983)	.038
		GG	**11**	**88**	**0.414 (0.214–0.801)**	**.008**
	rs2243290	AA	82	331	Reference	–
		AC	104	416	1.009 (0.730–1.394)	.956
		CC	24	102	0.949 (0.572–1.575)	.841

Table 5
IL-2 and IL-4 Serum Expression Level in *M. pneumoniae*-negative and Positive Subjects.

Subjects	Gene	Expression	*M. pneumoniae* negative	*M. Pneumoniae* positive	*P*
Overall	IL-2	Range (pg/ml)	0.85–156.36	0.46–144.36	
		Mean±SD (pg/ml)	**80.23±45.98**	**74.55±41.98**	**.038**
	IL-4	Range (pg/ml)	0.22–15.34	0.83–15.89	
		Mean±SD (pg/ml)	**7.72±4.34**	**8.41±4.28**	**.011**
Asthmatics	IL-2	Range (pg/ml)	1.56–156.36	0.52–143.99	
		Mean±SD (pg/ml)	**81.74±45.69**	**74.58±41.48**	**.016**
	IL-4	Range (pg/ml)	0.46–15.34	1.13–15.89	
		Mean±SD (pg/ml)	**7.93±4.29**	**8.61±4.32**	**.042**
Control	IL-2	Range (pg/ml)	0.85–151.18	0.46–144.36	
		Mean±SD (pg/ml)	77.73±46.51	74.46±43.25	.492
	IL-4	Range (pg/ml)	0.22–11.42	0.83–13.97	
		Mean±SD (pg/ml)	**7.50±4.38**	**8.21±4.23**	**.032**

It may be that these polymorphisms could change the expression of protein products, thereby causing the risk modifications mentioned above.[15] Specifically, it seems that the variant alleles of rs6822844, rs2069762 and rs3136534 polymorphisms could decrease the expression of protein products, while those of rs6534349, rs2243250, rs2227284 and rs2243290 polymorphisms could increase the expression of protein products. Our hypothesis is based on the fact that IL-2 is a pro-inflammatory cytokine, so increased expression of IL-2 may produce a higher level of inflammation, facilitating the development of asthma, and vice versa.[16] In contrast, IL-4 is an anti-inflammatory protein, and increased IL-4 expression can lead to reduced inflammation, which in turn would protect the host from asthma, and vice versa. However, further research is needed to confirm this.

A lack of association was observed between rs2070874 polymorphism and the risk of asthma in all classifications of study subjects (i.e. overall, classification based on GINA control, and classification based on GINA severity), suggesting that this polymorphism is not involved in the development of asthma.

The difference in IL-2 and IL-4 serum expression levels was also compared between subjects with and without *M. pneumoniae* infection, who were seen to have significantly higher levels of IL-2 and IL-4 expression, respectively. This finding was not unexpected, as inflammation is one of the earliest responses of the immune system.[17] It seems likely that a higher IL-2 level, which triggers inflammation, plays a role in fighting off pathogenic infections.[18] Subjects with a higher level of IL-2 were therefore *M. pneumoniae*-negative. The relationship between IL-2 levels and *M. pneumoniae* infection appeared to be significant only among asthmatic patients, but not the controls. This observation concurs with the fact that IL-2 plays an important role in the pathogenesis of asthma.[19] In contrast, IL-4 suppresses inflammation, and a high level of IL-4 does not allow the host immune system to function in an optimum state. Patients with higher IL-4 were therefore *M. pneumoniae*-positive, regardless of whether they were asthmatic or non-asthmatic.

A higher *M. pneumoniae* load has been associated with worse clinical severity in respiratory diseases.[20] Therefore, we further compared the distribution of the 8 IL-2 and IL-4 polymorphisms

Table 6
Genotype Distribution and Risk Association in all Subjects With Low and High *M. pneumoniae* Loads.

SNP	Genotype	Low *M. pneumoniae* load (No.)	%	High *M. pneumoniae* load (No.)	%	*P*	Odds ratio	*P*
rs6822844	GG	147	71.35	92	66.67	.6427	Reference	–
	GT	52	25.24	41	29.71		1.2598 (0.7755–2.0467)	.3509
	TT	7	3.40	5	3.62		1.1413 (0.3518–3.7026)	.8258
rs6534349	AA	145	70.39	109	78.99	.1488	Reference	–
	AG	56	27.18	28	20.29		0.6651 (0.3965–1.1157)	.1223
	GG	5	2.43	1	0.72		0.2661 (0.0306–2.3102)	.2299
rs2069762	GG	23	11.17	14	10.14	.686	Reference	–
	GT	77	37.38	58	42.03		1.2375 (0.5865–2.6111)	.5760
	TT	106	51.46	66	47.83		1.0229 (0.4919–2.1269)	.9516
rs3136534	AA	89	43.20	57	41.30	.9356	Reference	–
	AC	96	46.60	66	47.83		1.0735 (0.6797–1.6952)	.7611
	CC	21	10.19	15	10.87		1.1153 (0.5314–2.3407)	.7730
rs2243250	TT	87	42.23	54	39.13	.8211	Reference	–
	TC	98	47.57	68	49.28		1.1179 (0.7062–1.7697)	.6343
	CC	21	10.19	16	11.59		1.2275 (0.5894–2.5567)	.5840
rs2070874	TT	85	41.26	52	37.68	.794	Reference	–
	TC	98	47.57	69	50.00		1.1509 (0.7247–1.8278)	.5515
	CC	23	11.17	17	12.32		1.2082 (0.5907–2.4712)	.6044
rs2227284	TT	109	52.91	66	47.83	.1019	Reference	–
	TG	86	41.75	56	40.58		1.0754 (0.6825–1.6945)	.7540
	GG	11	5.34	16	11.59		**2.4022 (1.0514–5.4887)**	**.0376**
rs2243290	AA	87	42.23	54	39.13	.844	Reference	–
	AC	97	47.09	68	49.28		1.1294 (0.7132–1.7887)	.6038
	CC	22	10.68	16	11.59		1.1717 (0.5658–2.4266)	.6696

in subjects with high-load and low-load *M. pneumoniae* to find out whether any of the polymorphisms is associated with low-load infection. No significant difference was observed in the distribution of the polymorphisms between the two groups, but significant risk association was observed for the homozygous variant genotype of rs2227284 polymorphism. This suggests that the homozygous variant genotype of rs2227284 polymorphism could predispose its carriers to high-load *M. pneumoniae* infection, and is in line with Wang et al.[21] who showed that the rs2227284 polymorphism could affect humoral response.

In fact, several studies have investigated the association of various polymorphisms within IL-2 and IL-4 genes with asthma risk.[22–24] However, the majority of these studies incorporated only one or two polymorphisms in their analysis. This may result in misleading interpretations, as the effect of a polymorphism could be compensated by the presence of the other polymorphisms within the same gene.[25] The strength of our study lies in the incorporation of a large number of polymorphisms within two closely-related genes.

Conclusions

In this study, we identified two polymorphisms which could serve as predictive biomarkers for estimating asthma risk in children. We also showed that serum IL-2 and IL-4 levels differed significantly between subjects with and without *M. pneumoniae* infection. Among *M. pneumoniae*-positive subjects, the rs2227284 GG genotype was significantly linked to increased likelihood of presenting high-load infection. However, we recognize that this study has several limitations, such as the small sample size, especially in terms of subjects with *M. pneumoniae* infection. Further studies with larger sample sizes are needed in this area.

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Authors' Contributions

R.S.W. and H.X.J. recruited the study subjects, extracted DNA from all the samples and performed detection of *Mycoplasma pneumoniae* infection. S.Q.S. genotyped the SNPs and drafted the manuscript. X.Y.L. and S.J.C. quantified the serum expression levels of IL-2 and IL-4 and performed statistical analysis. Z.B.J. was responsible for the study conception, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare they have no conflict of interest directly or indirectly related to the manuscript contents.

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