Association of a four-locus gene model including *IL13, IL4, FCER1B*, and *ADRB2* with the Asthma Predictive Index and atopy in Chinese Han children

Running title: a four-locus gene model in API and atopy

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ABSTRACT

Background: Asthma is a complex and heterogeneous disease. We found that gene-gene interactions among *IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108 in asthmatic children of Chinese Han nationality. This four-locus set constituted an optimal statistical interaction model.

Objective: This study examined associations of the four-gene model consisting of *IL13*, *IL4*, *FCER1B*, and *ADRB2* with the Asthma Predictive Index (API) and atopy in Chinese Han children.

Methods: Four single-nucleotide polymorphisms (SNPs) in the four genes were genotyped in 385 preschool children with wheezing symptoms using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Student's t test and χ^2 tests were used for this analysis.

Results: Significant correlations were found between the four-locus gene model and the stringent and loose API (both $P<0.0001$). Additionally, a high-risk asthma genotype was a risk factor for the positive API (stringent API: OR= 4.08, loose API: OR=2.36). We also found a statistically significant association of the four-locus gene model with atopy ($P<0.01$, OR= 2.09).

Conclusions: Our results indicated that the four-locus gene model consisting of *L13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713 and *FCER1B* rs569108 was associated with the API and atopy. These findings provide an evidence of the gene model for determining a high risk of developing asthma and atopy in Chinese Han children.

Key words: Asthma Predictive Index; atopy; gene model; single-nucleotide polymorphism.

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RESUMEN

Antecedentes: El asma es una enfermedad compleja y heterogénea. En este estudio, encontramos que las interacciones gen-gen entre IL13 rs20541, IL4 rs2243250, ADRB2 rs1042713 y FCER1B rs569108, en niños asmáticos de nacionalidad china Han, constituyen un modelo estadístico óptimo de interacción.

Objetivo: Este estudio examinó un modelo de las asociaciones de cuatro genes (IL13, IL4, FCER1B y ADRB2) con el Índice Predictivo de Asma (IPA) y la atopía en niños Han chinos.

Métodos: Se genotiparon cuatro polimorfismos de un solo nucleótido (SNP) en los cuatro genes, en 385 niños en edad preescolar con síntomas de sibilancias, utilizando espectrometría de masas con desorción/ionización mediante láser asistida por Matriz (MALDI). Para el análisis estadístico de utilizaron el test t de Student y el c2.

Resultados: Se encontraron correlaciones significativas entre el modelo génico de los cuatro locus y el valor de IPA estricto y laxo (ambos P <0,0001). Además, el genotipo de riesgo alto de asma fue un factor de riesgo para IPA positivo (IPA estricto: OR = 4.08, IPA laxo: OR = 2.36). También, encontramos una asociación estadísticamente significativa entre el modelo génico de los cuatro locus, con atopia (P <0.01, OR = 2.09).

Conclusiones: Nuestros resultados indicaron que el modelo génico de cuatro locus compuesto por L13 rs20541, IL4 rs2243250, ADRB2 rs1042713 y FCER1B rs569108 estaba asociado con IPA y atopía. Estos hallazgos proporcionan la evidencia de la utilidad de este modelo génico para determinar el riesgo alto de desarrollar asma y atopia en niños chinos Han.

Palabras clave: Índice Predictivo de Asma; atopia; modelo génico; Polimorfismo de nucleótido simple.

INTRODUCTION

Asthma is a most common respiratory disease in childhood and is characterized by chronic airway inflammation, airway hyper-responsiveness and reversible airflow obstruction [1, 2]. The pathogenesis of asthma is complex and involves multiple inflammatory cells and cytokines. Genes that encode these cytokines are decisive factors for the development of asthma. Our previous case-control study reported that *IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108, four single-nucleotide polymorphisms (SNPs) not only had significant individual effect on asthma (G allele of rs20541, T allele of rs2243250, A allele of rs1042713 and G allele of rs569108), but also interact to confer a higher risk of asthma in Chinese Han children. These SNPs were chosen to build a four-way gene-gene interaction model to determine asthma susceptibility using the multifactor-dimensionality reduction (MDR) method [3]. Furthermore, a numerous of SNPs in *ADRB2*, *IL-13*, *IL-4* and *FCER1B* are associated with elevated immunoglobulin E (IgE) levels and the development of both atopy and asthma [4-7]. However, it is unknown whether this four-locus gene model is correlated with atopy.

Most of wheezing symptoms occur in children of preschool age, and approximately 50% of children experience a wheezing illness during the first six years of life. However, only a fraction of children show asthma symptom later in life [8]. Therefore, this period in life is important for asthma development, and certain gene variants might be associated with asthma or wheezing phenotype. Although transient wheezing is often non-atopic, recurrent wheezing in young children who have the signs of atopy

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is a risk precursor of asthma [9, 10]. Currently, no specific diagnosis standard exists for asthma in children younger than 6 years old in China [11]. The Chinese guideline for childhood asthma 2016 recommends that the diagnosis of asthma in children < 6 years of age is based on the following criteria: (1) symptom patterns (recurrent wheezing, exercise-induced cough or wheezing, an intermittent nocturnal cough that is not caused by viral infection and continued wheezing after the age of 3 years) and (2) therapeutic response to an asthma controller and/or reliever treatment [12]. Therefore, identifying children who have the potential to develop persistent asthma and initiating treatment early is critical. The Asthma Predictive Index (API) serves as a clinically applicable tool for identifying preschool children with recurrent wheezing at high risk for subsequent asthma [13, 14]. Researchers have found that expression of inflammation genes *TLR4* and *TNF-α* combined with API contributes to correctly predicting asthma in preschool children with wheezing [15].

In this study, we genotyped the following four SNPs in candidate genes: *IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108. We attempted to determine whether potential correlations existed between the gene model and API or atopy. Additionally, we aimed to provide a scientific basis for early screening of children at high risk for asthma and improve primary disease prevention.

METHODS

Study population

A total of 385 preschool children with wheezing symptoms were recruited into this

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study. We obtained written informed consent from their guardians. All patients were from the asthma out-patient and ward of the Children's Respiratory Department at Xinhua Hospital. All the patients had at least one episode of wheezing symptoms (i.e., a continuous high-pitched sound with musical quality emitting from the chest during expiration), and the interval between two wheezing episodes was at least 7 days without respiratory symptoms [16]. The study exclusion criteria consisted of the following: congenital pulmonary disease, congenital cardiac disease, congenital vascular malformation, congenital immune defects, foreign body aspiration, and tuberculosis of the bronchial lymph nodes. 250 of the 385 patients had complete allergen testing results. The study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Ethics Approval Number: XHEC-D-2016-393) and was conducted according to the principles in the Declaration of Helsinki.

Evaluation of API and atopy

We developed two indices to classify children as potentially at risk for asthma at school age, which were the stringent API and the loose API. The stringent API included frequent wheezing during the first 3 years of life and either one major risk factor (parental history of asthma or eczema) or two of three minor risk factors (eosinophilia, wheezing without colds, and allergic rhinitis). The loose API included any wheezing during the first 3 years of life and the same combination of risk factors described previously (Table 1) [14].

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The plasma specific IgE antibodies levels for a panel of locally relevant aeroallergens (including *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, cockroach, cat or dog hair and ragweed) and food allergens (including milk, egg, peanut, and seafood) were measured by the fluorescent enzyme immunoassay. Specific IgE concentration ≥0.35 kIU/L was considered positive, and children with at least one positive allergen-specific IgE test response were classified as atopic [17, 18].

Genotyping and grouping

Genomic DNA was isolated from an oral mucosal swab using magnetic genomic DNA extraction Kits (EmerTher, Shang Hai, China). Multiplex polymerase chain reaction (PCR) was used to detect the genotypes of the four loci (*IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108). The following PCR cycling conditions were used: 95°C for 2 min; 45 cycles at 95°C for 30 sec, at 56°C for 30 sec, and 72°C for 60 sec; and finally 72°C for 5 min. The conditions used for SAP enzyme digestion reaction were 37°C for 40 min and then termination at 85°C for 5 min. The following conditions were used for iPLEX : 95°C for 30 sec; 5 inner cycles at 52°C for 5 sec and at 85°C for 5 sec; and 40 outside cycles at 94°C for 5 sec, 52°C for 5 sec and 85°C for 5 sec. After completing the multiplex PCR, we used matrix-assisted laser desorption ionization time-of-flight mass spectrometry to discriminate the four SNPs. The genotyping results were validated by blind retesting of 10% of the total samples, and the concordance rate reached 99.5%. The call rate for each SNP was greater than 99%.

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In the present analysis, the different risk genotypes for asthma were defined according to the number of risk allele homozygotes, and the subjects without any risk homozygotes individuals (i.e. *IL13* rs20541 AA or GA, *IL4* rs2243250 CC or TC, *ADRB2* rs1042713 GG or AG, and *FCER1B* rs569108 AA or AG) were the reference group. Those with any different genotype combinations who had a significantly higher risk of asthma (if $P<0.05$ and $OR>1$) compared with the reference group were defined as the children at higher genetic risk for asthma. The others were defined as the children at lower genetic risk for asthma. The grouping results of different genotypes combination were shown in table 2.

Statistical analysis

A total of 385 preschool children with wheezing symptoms were divided into two groups according to the grouping method. The results were expressed as proportions or mean and standard deviations (SD). Patient characteristics were compared using the Student's t-test or ANOVA for numerical data. Chi-squared or Fisher's exact test were used to compare categorical variables. All the SNPs were examined for Hardy-Weinberg equilibrium using the χ^2 test. The allele frequencies were estimated by using the gene-counting method. Associations of API and atopy with different groups were assessed with the Pearson χ^2 test or the Fisher exact test as appropriate. All comparisons were made two-tailed, and P values less than 0.05 were considered statistically significant. The statistical analyses were conducted using the SPSS package version 20.0 (IBM Corporation, USA).

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RESULTS

Study population

385 preschool children with wheezing symptoms were enrolled in this study. The grouping method indicated that 174 subjects were at high genetic risk of asthma, and the others were at low genetic risk of asthma. No significant difference was observed between the two groups in terms of age. However, more male children exhibited wheezing in these two groups than female children. The percent of males in the high-risk genotype group was lower than that in the low-risk genotype group, but this difference was not statistically significant. Moreover, significant difference existed with respect to the age of wheezing onset ($P<0.01$). More children in the high-risk genotype group began to wheeze in infancy than those in the low-risk group. There were no difference in the positive history of personal allergies and family atopy between the two groups (Table 3).

Associations between the loose and stringent API and different risk genotype groups

All four SNPs examined were in Hardy-Weinberg equilibrium ($P>0.05$). As shown in Table 4, the percentage of subjects with the positive API was significantly higher in high-risk genotype group than that in low-risk genotype group regardless of loose API or stringent API (both $P<0.0001$). Furthermore, high genetic risk of asthma was a risk factor of the positive loose and stringent API (loose API: OR= 2.36; stringent API: OR= 4.08) (Table 4).

Association of different groups with atopy

More atopy positive subjects were found in the high-risk genotype group than those in the low-risk genotype group, and this difference was statistically significant (P<0.01, OR=2.09) (Table 5).

DISSCUSSION

This is the first study to report the associations between a four-locus gene model consisting of *IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108 and both the API and atopy. Furthermore, it is also the first study of using a gene model to examine atopic wheezing in Chinese Han preschool children.

Epidemiologic studies indicate that the development of asthma and allergic sensitization is determined early in life [19]. During this period, wheezing illness is heterogeneous, and only 30% of preschool children with recurrent wheezing still have asthma at the age of 6 years [20]. However, it is difficult to discriminate young children with wheezing symptoms who will have asthma at school age. The API was first proposed by the Tucson Children's Respiratory Study team in the year of 2000 [14], which is based on factors that were found during the first 3 years of life and it was found to predict continued wheezing at school age. The API is attractive for use in clinical practice because it is simple to assess and requires no additional equipment or expertise [21]. One validation study about API reported that a subject with the positive loose API had 4-fold increase in the probability of developing asthma

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compared with a negative subject. Additionally, compared to a negative subject, the positive stringent API led to 8-fold increased probability of developing asthma [22]. In our study, we found that more children with positive API in the high-risk genotype group than that in the low-risk genotype group independent of the loose API or stringent API. Furthermore, the correlation between stringent API and the four-locus gene model (stringent API: P<0.0001, OR=4.08; loose API: P<0.0001, OR=2.36) was stronger. These findings suggested that children with high-risk genotypes for asthma are susceptible to developing asthma. In addition, more male children exhibited wheezing than female children in the two groups, which is consistent with the phenomenon that boys are more susceptible to wheezing than girls before the age of 6 years [23]. Interestingly, a significantly higher proportion of children in high-risk genotype group began to wheeze at a younger age, especially during their first year of age. This may show an evidence for association of the gene model with the age of wheezing onset.

Asthma is generally considered a multifactorial disease involving immunologic, genetic, environmental, and other factors. Its chronic airway inflammation mechanism and type have been widely demonstrated. Atopy is present in most of the childhood asthma patients and is more common in severe asthma [24]. Central to the process of allergic-dependent asthma is an enhanced Th2 response, which produces increased interleukin (IL)-5, IL-4, and IL-13 secretion. IL-4 drives B-cell isotype class switching and IgE synthesis, and IgE binds to mast cell high-affinity IgE receptors and leads to mast cell activation following allergen-mediated IgE cross-linking [25].

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Our previous study reported that IL13 R130Q was associated with childhood asthma and it could promote the production of IgE [26]. The results of gene-gene interactions between *IL13* and *IL4RA* demonstrated a significant association with plasma total IgE [27]. Moreover, β2 adrenoceptor agonists are used to relieve bronchoconstriction by modulating airway smooth muscle. A study from Korea reported that four-locus gene-gene interactions among *B2ADR* 46A>G, *CCR3-520T*>G, *CysLTR1*-634C>T, and *FCER1B*-109T>C had substantial effects on aspirin intolerant asthma [28]. Our results revealed that the four-locus gene model was significantly associated with atopy and the high risk genotype was a risk factor of atopy ($P<0.01$, OR=2.09).

This study demonstrated the predictive role of the four-locus gene model for asthma in young children and showed that it was associated with atopy in Chinese Han children for the first time. We hypothesized that the four-locus gene model consisting of *IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108 could be used to discriminate atopic asthma and non-atopic asthma. Our results were consistent with the data shown in a five-locus gene model from Filipino [29].

However, there were several limitations in our study. First, all patients with wheezing symptoms were from the asthma out-patient and ward of Children's Respiratory Department, and this may have resulted in data bias. Second, the absence of control group was another limitation. In our future studies, healthy children will be recruited and genetic differences will be compared between wheezing children and the healthy controls. Third was the method used to clarify different risk genotype groups.

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The method didn't fully consider the role of single risk allele, and the use of only homozygous risk alleles may weaken the role of individual risk allele for the disease. Therefore, it is of great necessity to use some other approaches such as genetic risk score (GRS) in our future study. The GRS is an emerging method for exploring correlations between SNPs and clinical phenotypes of complex diseases. The approach integrates weak effects of multiple SNPs and dramatically enhances the predictability of complex diseases by gene polymorphisms [30]. The GRS has been widely applied in genetic studies of common complex diseases, such as diabetes, coronary heart disease, asthma and others [31-33]. Results of the Wheezing Illnesses Study Leidsche Rijn (WHISTLER) cohort illustrated that an adult-derived GRS for allergy predicted the risk of developing allergies during childhood [33].

In our future studies, we will conduct a series of validation tests for the gene model in different ethnic groups, birth cohorts, lung function groups and different asthma phenotypes. We believe that only this four-locus model is extensively validated, and we can consider its use in clinical practice.

In conclusion, the results of this study suggested that the four-locus gene model comprising of *IL13* rs20541, *IL4* rs2243250, *ADRB2* rs1042713, and *FCER1B* rs569108 was significant associated with the API and atopy. Our findings provide an evidence of the model for determining a high risk genotype of developing asthma and atopy. The gene model may become a useful tool for objectively predicting the risk of asthma in Chinese Han preschool children after comprehensive validation studies.

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Table 1. Asthma Predictive Index*

Major Criteria	Minor Criteria
1. Parental MD asthma†	1. MD allergic rhinitis §
2. MD eczema‡	2. Wheezing apart from cold
	3. Eosinophilia (> 4%)

* Loose index for the prediction of asthma: Early wheezing plus at least one of two major criteria or two of three minor criteria. Stringent index for the predication of asthma: early frequent wheezing plus at least one of two major criteria or two of three minor criteria.

† History of a physician diagnosis of asthma.

‡ Physician diagnosis of atopic dermatitis as reported in questionnaires at ages 2 or 3.

§ Physician diagnosis of allergic rhinitis as reported in questionnaires at ages 2 or 3.

Table 2. High and low risk genotype groups of asthma with different genotypes

Groups	Genotype			
	IL13 rs20541	IL4 rs2243250	ADRB2 rs1042713	FCER1B rs569108
Low-risk genotype	AA or GA	CC or TC	GG or AG	AA or AG
	AA or GA	CC or TC	GG or AG	GG
	AA or GA	CC or TC	AA	AA or AG
	AA or GA	TT	GG or AG	AA or AG
	GG	CC or TC	GG or AG	AA or AG
	AA or GA	CC or TC	AA	GG
	GG	CC or TC	GG or AG	GG
High-risk genotype	AA or GA	TT	GG or AG	GG
	AA or GA	TT	AA	AA or AG
	GG	CC or TC	AA	AA or AG
	GG	TT	GG or AG	AA or AG
	AA or GA	TT	AA	GG
	GG	CC or TC	AA	GG
	GG	TT	GG or AG	GG
	GG	TT	AA	AA or AG
	GG	TT	AA	GG

Table 3. Baseline demographic characteristics in preschoolers with high-risk and low-risk genotype groups

variables	High-risk group	Low-risk group	P value
Number	174	211	NA
Age (m) (mean±SD)	42.3±21.37	44.2±21.40	0.39
Males (%)	113 (64.9%)	148 (70.1%)	0.28
Age of wheezing onset (%)			
<1 year old	58 (58%)	42 (42%)	
1-3 years old	70 (46.4%)	81 (53.6%)	0.001
> 3 years old	46 (34.3%)	88 (65.7%)	
Positive personal history of allergies * (%)	159 (91.4%)	184 (87.2%)	0.191
Positive family history of atopy [&] (%)	110 (63.2%)	125 (59.2%)	0.426

NA, not available; SD, standard deviation.

*Personal history of allergies was defined as a positive history of rhinitis, eczema and food allergy.

[&] Family atopy was defined as a positive history of rhinitis, asthma and eczema in at least one of the family members [34].

Table 4. Association between loose and stringent API and different risk of genotype groups

Group	Loose API		Stringent API	
	Positive No. (%)	Negative No. (%)	Positive No. (%)	Negative No. (%)
High-risk group	119 (68.4%)	55 (31.6%)	95 (54.6%)	79 (45.4%)
Low-risk group	101 (47.9%)	110 (52.1%)	48 (22.7%)	16 (77.3%)
P value	5.12E-5		1.22E-10	
OR (95%CI)	2.36 (1.55-3.58)		4.08 (2.63-6.33)	

API, asthma predictive index; OR, odds ratio; CI, confidence interval.

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Table 5. Association of different groups with atopy

Group	Positive No. (%)	Negative No. (%)	P value	OR (95%CI)
High-risk group	79 (71.8%）	31 (28.2%)	0.006	2.09 (1.22-3.55)
Low-risk group	77 (55%)	63 (45%)		

OR, odds ratio; CI, confidence interval.