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Polymorphisms in Toll-Like Receptor 4 Gene Are Associated With Asthma Severity but not Susceptibility in a Chinese Han Population

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Abstract

Background and Objectives: The toll-like receptor 4 (*TLR4*) gene links human innate immunity and adaptive immunity via bacterial endotoxin recognition, and plays a considerable role in the pathogenesis of asthma. The effects of the genetic variants of *TLR4* on asthma are still largely unknown. This study aimed to evaluate the effects of *TLR4* polymorphisms on asthma risk and asthma-related phenotypes in a Chinese Han population.
Methods: We consecutively recruited 318 unrelated adult asthmatic patients and 352 healthy volunteers. Four tagging single nucleotide polymorphisms (SNPs) in the *TLR4* gene were detected using GenomeLab SNPstream or TaqMans Genotyping. We conducted case-control and case-only studies to investigate the association between the selected tagging SNPs in *TLR4* and asthma and asthma-related phenotypes.
Results: We found no evidence to support a significant association between *TLR4* SNPs and asthma susceptibility. However, our results revealed that the TT homozygote of rs1927914 was associated with lower forced expiratory volume in the first second (percent predicted) in asthmatic patients. An evidently positive association was found between asthma severity and both the TT genotype of rs1927914 and the GG genotype of rs10983755 and rs1927907 (P=.024, P=.009, and P=.013, respectively), indicating that the C allele of rs1927914 and the A allele of rs10983755 and rs1927907 have a protective effect on asthma severity.
Conclusion: TLR4 polymorphisms do not contribute to asthma susceptibility but they may influence the severity of asthma.

Key words: Asthma. Atopy. Phenotype. Polymorphism. Toll-like receptor 4.

Resumen

Antecedentes y objetivos: El gen del receptor tipo Toll 4 (*TLR4*) interrelaciona la inmunidad innata y la inmunidad adaptativa humanas por medio del reconocimiento de endotoxinas bacterianas y desempeña un papel importante en la patogenia del asma. Todavía se sabe muy poco de los efectos de las variantes del gen *TLR4* sobre el asma. El objetivo de este estudio fue evaluar los efectos de los polimorfismos de *TLR4* sobre el riesgo de asma y los fenotipos relacionados con el asma en una población china Han.
Métodos: Se incluyó de forma consecutiva a 318 pacientes asmáticos adultos no relacionados y a 352 voluntarios sanos. Mediante el sistema GenomeLab SNPstream o TaqMans Genotyping se detectaron cuatro marcadores de polimorfismos de un solo nucleótido (SNP) en el gen *TLR4*. Se llevaron a cabo estudios de casos y controles y de solo casos para estudiar la asociación entre los marcadores de SNP seleccionados en el *TLR4* y el asma y los fenotipos relacionados con el asma.
Resultados: No se obtuvieron datos que permitan respaldar una asociación significativa entre los SNP del *TLR4* y la predisposición al asma. No obstante, los resultados revelaron que el homocigoto TT del polimorfismo rs1927914 estaba asociado a un menor volumen espiratorio

forzado en el primer segundo (porcentaje predicho) en pacientes asmáticos. Se observó una asociación positiva evidente entre la gravedad del asma y el genotipo TT del polimorfismo rs1927914 y el genotipo GG de los polimorfismos rs10983755 y rs1927907 (p = 0,024, p = 0,009 y p = 0,013, respectivamente), lo que significa que el alelo C del polimorfismo rs1927914 y el alelo A de los polimorfismos rs10983755 y rs1927907 tienen un efecto protector sobre la gravedad del asma.
Conclusión: Los polimorfismos del gen *TLR4* no contribuyen a la predisposición al asma aunque pueden influir en la gravedad del asma.

Palabras clave: Asma. Atopia. Fenotipo. Polimorfismo. Receptor tipo Toll 4.

Introduction

Asthma is a chronic inflammatory respiratory disorder characterized by overzealous type 2 T-helper (T_H2)-biased immune responses and influenced by genetic and environmental factors [1]. According to the hygiene hypothesis, frequent exposure to microbes and infections early in childhood is postulated to protect against the development of asthma and atopy [2]. The concepts that a missing immune deviation of allergen-specific responses from the T_H2 to the T_H1 profile and impaired regulatory T cell (Treg) activity have been established [3].

Toll-like receptors (TLRs) are pattern-recognition receptors that play an essential role in the activation of the innate and adaptive immune system [4,5]. Following binding to diverse conserved motifs in pathogens termed pathogen-associated molecular patterns, TLRs initiate intracellular signaling pathways, and in turn, activate antigen-presenting cells (APCs), influence T-cell polarization and development [6], and modulate Treg function [7]. These are all key events in the pathogenesis of asthma. TLR4 is the most extensively studied member of the TLR family, and it recognizes lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria. It is well recognized that LPS modulates allergic airway inflammation. However, there are contradictory findings, reported by studies suggesting protective roles for LPS through the attenuation of T_H2 responses and studies showing exacerbating effects of LPS on asthma [8,9].

The *TLR4* gene (gene ID 7099) is located at chromosome 9q33.1. With the emergence of the hygiene hypothesis, many genetic association studies were performed in diverse populations to examine the role of *TLR4* single-nucleotide polymorphisms (SNPs) in asthma and atopy [10-17]. However, results have been contradictory, and data on the simultaneous action of several polymorphisms are not well understood. Small effects of genotypes on complex traits have proven difficult to detect, and tagging SNPs and haplotype structures should be taken into consideration.

Up to now, no studies have been performed on the association between *TLR4* SNPs and asthma in Chinese populations. Thus, the aim of this study was to investigate the association between haplotype-tagging SNPs, which capture all the essential genetic information about the *TLR4* gene locus, and asthma as well as asthma-related phenotypes in an ethnic Chinese community.

Materials and Methods

Participants

We enrolled 318 asthmatic patients (aged 14 to 75 years) and 352 nonasthmatic controls (aged 16 to 74 years) from March 2006 to May 2007 (Table 1). All the participants were unrelated Han Chinese residing in Nanjing and surrounding regions. The patients were consecutively recruited from the outpatient department at the First Affiliated Hospital of Nanjing Medical University. Asthma diagnosis and severity were verified by an experienced pulmonary specialist according to the Global Initiative for Asthma (GINA) guidelines [18]. The patients were categorized into 4 groups based on the clinical features of their disease (stage 1 group, intermittent; stage 2 group, mild-persistent; stage 3 group, moderate-persistent; and stage 4 group, severe-persistent). Each patient underwent a detailed workup, including medical history, family history, smoking habit, occupation, general physical examination, medication, skin prick tests (SPTs), complete blood count, and extended laboratory tests. The healthy volunteers were recruited from among spouses of the patients and the general population, and had to meet the following criteria: *a)* good health status and matched with the cases for age, sex, and area of residence; *b)* no positive SPT results; and *c)* normal levels of total serum immunoglobulin (Ig) E. All the participants provided written informed consent for the study procedures. The study protocol was approved by the institutional ethics committee.

Assessment of Clinical Data

Atopy was defined by at least 1 positive response to SPTs performed with 13 common aeroallergens, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Felis domesticus*, *Canis familiaris*, cockroach, pollen, ragweed, mugwort, mold (*Cladosporium* and *Alternaria*) and animal allergens (cat, dog, and horse). Smoking habit was measured in pack-years (number of packs of cigarettes smoked per day multiplied by the number of smoking years). Participants with a smoking history of less than 5 pack-years were defined as nonsmokers; otherwise, they were considered smokers [19]. SPTs, total serum IgE levels, serum levels of high-sensitivity C reactive protein (hsCRP) and pulmonary function assessments have been described in detail in a previous study by our group [20].

Table 1. Demographic Characteristics of Patients With Asthma and Healthy Controls

	Controls (n=352)	Patients (n=318)	*P* Value[a]
Mean (SD) age, y	38.26 (13.31)	39.80 (14.23)	NS
Sex, No. (%)			
Male	152 (43.18)	135 (42.45)	NS
Female	200 (56.82)	183 (57.55)	
Smoking, No. (%)			
Non-smokers	242 (82.88)	280 (88.05)	NS
Smokers	50 (17.12)	38 (11.95)	
Mean (SD) hsCRP, mg/L	2.16 (7.37)	2.50 (5.79)	NS
Mean (SD) EOS, $\times 10^6$/mL	0.14 (0.11)	0.46 (0.71)	<.001
Mean (SD) $\log_{10}$ IgE, IU/mL	1.19 (0.60)	1.82 (0.50)	<.001
Mean (SD) FEV_1%	94.75 (18.07)	74.11 (23.66)	<.001
FEV_1/FVC, %	88.66 (9.43)	76.18 (12.78)	<.001
Atopy, No. (%)	0	237 (75.00)	–
Asthma severity, No. (%)			
Stage 1 (intermittent)	–	70 (22.01)	–
Stage 2 (mild persistent)	–	50 (15.72)	–
Stage 3 (moderate persistent)	–	87 (27.36)	–
Stage 4 (severe persistent)	–	111 (34.91)	–
ICS treatment, No (%)	–	175 (55.03)	–

Abbreviations: EOS, eosinophil count; FEV_1%, percent of predicted forced expiratory volume in the first second; FEV_1/FVC: FEV_1 as percentage of forced vital capacity; hsCRP, high-sensitivity C reactive protein; ICS, inhaled corticosteroid; $\log_{10}$IgE, $\log_{10}$-transformed immunoglobulin E levels; NS, nonsignificant.
[a]Analyzed by *t* test or χ^2 test as appropriate.

Table 2. Tagging Single Nucleotide Polymorphisms (SNPs) Selected from HapMap

Tagging SNPs (NCBI rs Number)	Base Change	HWE	Genenotyped Call Rate, %	MAF, %	
				Controls	Cases
rs1927914	T>C	0.03	99.7	40.71	41.35
rs10983755	G>A	0.14	99.9	26.92	27.67
rs11536879	A>G	0.57	97.3	13.36	13.32
rs1927907	G>A	0.36	96.7	25.15	25.64

Abbreviations: HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency.

SNP Selection and Genotyping

Haplotype-tagging SNPs were selected with the HapMap database (http://www.hapmap.org) and the Tagger algorithm using a pairwise tagging approach. Based on the largest number of SNPs with a minor allele frequency (MAF) of less than 0.05 in the Chinese Han population in Beijing and linkage disequilibrium of r^2>0.8, 4 haplotype-tagging SNPs capturing the genetic information of all common SNPs at the locus were selected for genotyping (Table 2). These were rs1927914 and rs10983755, located in the 5' flanking region of the *TLR4* gene, while rs11536879 and rs1927907 are located in the intron. Genomic DNA was extracted from EDTA-anticoagulated peripheral blood with the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We genotyped rs1927914 and rs10983755 using GenomeLab SNPstream genotyping platform (Beckman Coulter, Fullerton, California, USA), and rs11536879 and rs1927907 using TaqMans SNP Genotyping Assays (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems, Foster City, California, USA) at the Chinese National Human Genome Center in Shanghai, China. The polymerase chain reaction primers and extension

Table 3. Polymerase Chain Reaction Primers and Extension Probes

SNP	Primer	Sequence 5'-3'
rs1927914	Forward	ATTGGAAGTGCTTGGAGGA
	Reverse	TTGTAAAGCTTTTAGGACAGTGTCT
	Probe	GCGGTAGGTTCCCGACATATAGTAGAACTATCTAGGACTTAGCAT
rs10983755	Forward	CCCAGTCCACCACAAAAT
	Reverse	TAGATAGTTCTACTGTAATATCCTCCAAGC
	Probe	GGATGGCGTTCCGTCCTATTTCCCTCACAGCTTGGTTTTTGACAC
rs11536879	TaqMans	
rs1927907	TaqMans	

Abbreviation: SNP, single nucleotide polymorphism.

probes with the tag sequence were designed using the web-based Autoprimer design tool (http://www.autoprimer.com), and synthesized by SBSgene (SBS Genetech Technology, Shanghai, China), or purchased from Applied Biosystems (Table 3). SNPstream genotyping and TaqMans SNP genotyping were performed as previously described [21,22]. We duplicated 10% of the samples to confirm the concordance and accuracy of genotyping. A sample call rate of over 96% was observed with 100% matching for quality control samples and blind replicates.

Statistical Analysis

Differences in the distribution of demographic characteristics, clinical data, and genotypes of the TLR4 variants between cases and controls were evaluated using the *t* test, the χ^2 test, or the Fisher exact test as appropriate. The Hardy-Weinberg equilibrium (HWE) was tested using the χ^2 goodness-of-fit test to compare the observed genotype frequencies with the expected frequencies among the controls. Logistic regression was used to estimate crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs), adjusted for age, sex, and smoking status, as a measure of association with the risk of asthma. Linear regression and ordinal logistic regression, adjusted for age, sex, smoking status, inhaled corticosteroid treatment, and atopy, were applied in the case-only study. The EM algorithm in SAS 9.1.3 PROC HAPLOTYPE was used to infer haplotype frequencies based on the observed genotypes. A 2-sided *P* value of less than .05 was considered significant and all calculations were carried out with SAS 9.1.3 (SAS Institute, Cary, North Carolina, USA).

Results

Demographic and Clinical Characteristics

The demographic and clinical characteristics of the study population are summarized in Table 1. No significant differences were observed between the cases and controls for age, sex, smoking habit, or serum hsCRP. However, significant differences in peripheral eosinophil counts, serum IgE, percent

Table 4. Genotype Frequencies of *TLR4* Single Nucleotide Polymorphisms (SNPs) in Patient with Asthma and Controls

SNP	Genotype	Controls, No (%)[a]	Patients, No (%)[a]	Adjusted Odds Ratio (95% CI)[b]	*P* Value
rs1927914	TT	113 (32.29)	109 (34.28)	1	
	TC	189 (54.00)	155 (48.74)	0.78 (0.55-1.11)	NS
	CC	48 (13.71)	54 (16.98)	1.16 (0.72-1.89)	NS
rs10983755	GG	182 (51.85)	170 (53.46)	1	
	GA	149 (42.45)	120 (37.74)	0.85 (0.61-1.18)	NS
	AA	20 (5.70)	28 (8.81)	1.45 (0.77-2.72)	NS
rs11536879	AA	260 (74.71)	225 (74.01)	1	
	AG	83 (23.85)	77 (25.33)	1.06 (0.73-1.53)	NS
	GG	5 (1.44)	2 (0.66)	0.48 (0.09-2.64)	NS
rs1927907	GG	184 (55.09)	177 (56.37)	1	
	GA	132 (39.52)	113 (35.99)	0.87 (0.62-1.22)	NS
	AA	18 (5.39)	24 (7.64)	1.33 (0.68-2.58)	NS

Abbreviations: CI, confidence interval; NS, nonsignificant.
[a]Because of genotyping failure, the total numbers of case and controls for each SNP may be less than 318 and 352, respectively.
[b]Analyzed by logistic regression adjusted for age, sex, and smoking.

of predicted forced expiratory volume in the first second (FEV_1%) and FEV_1 as a percentage of forced vital capacity (FEV_1/FVC) were pronounced between both groups (P<.001 in all cases).

TLR4 Gene Polymorphisms Allele/Genotype Distributions

The distribution of each of the genetic variants met the conditions of the HWE (Table 2). There seemed to be similar allele distributions for each SNP between cases and controls (P>.05, Table 2). The genotype distributions of the polymorphisms in the cases and controls are shown in Table 4. We compared the frequencies of each SNP between the cases and controls by using recessive, co-dominant, and dominant genetic models. Regrettably, no significant differences in genotype distributions were observed (P>.05, Table 4, with only the co-dominant model shown). Also, when the asthmatic patients were stratified by atopy, no positive results were found (data not shown).

Table 5. Association between *TLR4* Single Nucleotide Polymorphisms (SNPs) and Asthma-Related Phenotypes

SNP	Eos×106/mL	FEV_1, %	FEV_1/FVC, %	hsCRP mg/L	log_{10} IgE IU/mL	Asthma Severity, No. (%)			
						Stage 1	Stage 2	Stage 3	Stage 4
rs1927914									
TT	0.57 (1.12)	67.58 (25.00)	66.72 (14.34)	2.67 (6.87)	1.87 (0.51)	19 (17.43)	14 (12.84)	33 (30.28)	43 (39.45)
TC	0.41 (0.33)	71.54 (25.01)	69.07 (14.50)	2.70 (5.81)	1.78 (0.51)	36 (23.23)	25 (16.13)	41 (26.45)	53 (34.19)
CC	0.37 (0.29)	71.13 (28.83)	68.55 (16.44)	1.55 (2.43)	1.86 (0.46)	15 (27.78)	11 (20.37)	13 (24.07)	15 (27.78)
P value	.157[a]	.043[a]	.109[a]	.370[a]	.724[a]	.024[b]			
rs10983755									
GG	0.50 (0.92)	68.38 (25.28)	67.55 (14.74)	2.52 (6.09)	1.85 (0.50)	31 (18.24)	21 (12.35)	50 (29.41)	68 (40.00)
GA	0.41 (0.33)	72.52 (26.65)	68.82 (15.27)	2.71 (5.93)	1.80 (0.52)	32 (26.67)	23 (19.17)	30 (25.00)	35 (29.17)
AA	0.41 (0.26)	70.50 (23.63)	69.27 (13.13)	1.42 (2.36)	1.76 (0.44)	7 (25.00)	6 (21.43)	7 (25.00)	8 (28.57)
P value	.398[a]	.297[a]	.542[a]	.606[a]	.629[a]	.009[b]			
rs11536879									
AA	0.45 (0.59)	69.73 (24.59)	67.99 (14.32)	2.74 (6.40)	1.82 (0.52)	49 (21.78)	36 (16.00)	62 (27.56)	78 (34.67)
AG	0.37 (0.30)	72.48 (28.16)	69.14 (16.56)	1.92 (4.08)	1.82 (0.47)	18 (23.38)	13 (16.88)	21 (27.27)	25 (32.47)
GG	0.35 (0.49)	40.00 (21.21)	50.85 (0.35)	5.57 (6.55)	1.69 (0.41)	0 (0.00)	0 (0.00)	0 (0.00)	2 (100.00)
P value	.570[a]	.197[a]	.265[a]	.547[a]	.624[a]	.858[b]			
rs1927907									
GG	0.50 (0.90)	68.85 (25.21)	67.78 (14.60)	2.66 (6.12)	1.84 (0.50)	33 (18.64)	23 (12.99)	51 (28.81)	70 (39.55)
GA	0.42 (0.33)	71.82 (26.82)	68.30 (15.55)	2.03 (4.74)	1.81 (0.52)	30 (26.55)	22 (19.47)	29 (25.66)	32 (28.32)
AA	0.39 (0.30)	72.54 (24.52)	70.67 (12.86)	1.78 (2.55)	1.78 (0.45)	7 (29.17)	5 (20.83)	5 (20.83)	7 (29.17)
P value	.536[a]	.441[a]	.497[a]	.410[a]	.792[a]	.013[b]			

Abbreviations: Eos, eosinophils; FEV_1%, percent of predicted forced expiratory volume in 1 second; FEV_1/FVC: FEV_1 as percentage of forced vital capacity; hsCRP, high-sensitivity C reactive protein; log_{10}IgE, log_{10}-transformed immunoglobulin E levels; SNPs, single nucleotide polymorphisms. Stage 1: intermittent; Stage 2: mild-persistent; Stage 3: moderate-persistent; Stage 4: severe-persistent.
[a]Linear regression adjusted for age, sex, smoking status, inhaled corticosteroid treatment, and atopy.
[b]Ordinal logistic regression adjusted for age, sex, smoking status, inhaled corticosteroid treatment, and atopy.

Haplotype Analysis

We analyzed the distributions of haplotypes in *TLR4* and their effects on asthma. Similarly, none of the haplotypes showed a significant association with asthma (data not shown).

TLR4 Genotypes and Asthma-Related Phenotypes

Subsequently, we focused on the associations between genotypes and asthma-related phenotypes in a case-only study. As shown in Table 5, patients who carried the TT homozygote of rs1927914 had a significantly lower FEV_1% than those who carried the TC/CC genotype (*P*=.043, adjusted for age, sex, smoking status, inhaled corticosteroid treatment, and atopy). Besides, the overrepresentation of the TT genotype in patients with moderate- and severe-persistent asthma revealed a positive association between this genotype and asthma severity (*P*=.024). These associations were more evident for the GG genotype of rs10983755 and rs1927907 than for the GA/AA genotype (*P*=.009 and *P*=.013, respectively). These results indicate that the C allele of rs1927914 and the A allele of rs10983755 and rs1927907 exert a protective effect on asthma severity. However, no associations were found between these SNPs and other asthma-related phenotypes, such as eosinophil count, FEV_1/FVC, serum hsCRP, and total serum IgE levels.

Discussion

This study provides the first evaluation of the role of *TLR4* polymorphisms in asthma and asthma-related phenotypes in a Chinese ethnic population. Using haplotype-tagging SNPs capturing all the essential genetic information of the *TLR4* gene locus, we found no evidence to support a significant association between *TLR4* polymorphisms and asthma susceptibility. However, our results indicate that the TT homozygote of rs1927914 was associated with lower FEV_1% in asthmatic patients. An evidently positive association was found between asthma severity and the TT genotype of rs1927914 and the GG genotype of rs10983755 and rs1927907.

TLR4 plays a crucial role in host defense by triggering robust innate immune activation followed by protective adaptive immunity against infectious bacterial diseases. However, inappropriate TLR4 responses contribute to acute and chronic inflammation [23]. Our group recently discovered that compared to other $CD4^+$ T cells, TLR4 was preferentially expressed in human Tregs, which are key regulators of the T_H1/T_H2 balance [24]. The activation of TLR4 may directly or indirectly modulate the function of Tregs, underlining the important role of TLR4 in asthma [25,26].

Previous studies by our group have revealed the role of gene polymorphisms in a Chinese population with asthma [27,28]. Although the potential role of TLR4 as a regulator of immune responses has received considerable attention in recent years, the results of published association studies on *TLR4* SNPs and asthma risk are inconsistent. Fagerås et al [17] were the first to report that the *TLR4* SNP Asp299Gly was associated with a 4-fold higher prevalence of asthma and a 7-fold higher prevalence of atopic asthma in Swedish children. More recently, Kerkhof et al [29] showed that variant alleles of *TLR4* influenced susceptibility to adverse effects of traffic-related air pollution on the development of asthma in childhood. However, most, but not all, studies have indicated a lack of association between *TLR4* polymorphisms and asthma [10-15].

Interestingly, our results underscore that the presence of *TLR4* polymorphisms may influence lung function and confer some protection against severe asthma, strongly supporting findings from a Turkish study of children with asthma [30]. Animal experiments have shown *TLR4* to be a candidate gene contributing to the response to ozone-induced lung permeability and inflammation [31], and *TLR4*-deficient mice have been seen to be protected against ozone-induced airway hyperresponsiveness [32]. Moreover, in another study, increasing ozone exposure has been found to predict lower lung function and increased biomarkers of respiratory and systemic inflammation in adults with asthma [33]. Thus, we hypothesize that genetic variants of *TLR4* may be associated with a decreased ozone response, consequently affecting lung function and asthma severity. In addition, since endotoxin may exacerbate already existing asthma, genetic variants associated with a blunted response to LPS may also be expected to be associated with less severe disease. However, the functional relevance of the SNPs genotyped in the present study needs to be investigated in more detail.

In our study, asthma severity and asthma-related phenotypes were classified following restrictive criteria, which, in theory, should limit possible confounding effects. Additionally, we selected haplotype-tagging SNPs from the HapMap database, thus capturing all the essential genetic information of the *TLR4* gene locus and excluding the possibility of missing other potentially functional polymorphisms. However, our study had several limitations typically associated with case-control studies. First, the sample size was relatively small for some genotype distributions, which may be prone to false negatives due to low statistical power or fortuitous false-positive results. Second, environmental factors other than smoking (eg, occupational exposure, pets, allergen exposure, and domestic endotoxin levels) might interact with the *TLR4* genotype or act as potential confounders in the analysis. Third, rigid adherence to an empirical significance level of *P*<.05 may be too conservative.

In summary, our study suggests that *TLR4* polymorphisms are associated with asthma severity but not susceptibility in the study population. Further functional and larger, better-designed prospective studies are warranted to explore the exact biological mechanism of *TLR4* genotypes and haplotypes and their effects on asthma development in diverse populations.

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