ORIGINAL ARTICLE

Polymorphisms in the *Toll-like Receptor 2* Subfamily and Risk of Asthma: A Case-control Analysis in a Chinese Population

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■ Abstract

Background and objective: Cell activation through toll-like receptors (TLRs) has robust bipolar effects on host immunity and the pathogenesis of asthma. The TLR2 subfamily is a pivotal member of the TLR family. We sought to determine whether mutations in *TLR2* subfamily genes affect the risk of asthma.
Methods: A total of 318 asthmatic patients and 352 nonasthmatic controls were recruited. Eight single-nucleotide polymorphisms in *TLR2* subfamily genes were detected using GenomeLab SNPstream (Beckman Coulter, Fullerton, California, USA).
Results: We found that patients with the *TLR2*/rs7656411 TT variant homozygote had a significantly reduced risk of asthma when compared with those with the GG wild-type homozygote (adjusted odds ratio [OR], 0.63; 95% confidence interval [CI], 0.41-0.98; *P*=.036). Furthermore, a positive association was observed between the T allele of rs2381289 in *TLR6* and allergic rhinitis in asthma (OR, 1.79; 95% CI, 1.10-2.91; *P*=.025), while the A allele of rs11466651 in *TLR10* was negatively associated with allergic rhinitis (OR, 0.49; 95% CI, 0.26-0.95; *P*=.046).
Conclusion: Our results indicate that a genetic variant in the *TLR2* subfamily may play a role in susceptibility to asthma.

Key words: Asthma. Allergic rhinitis. Single-nucleotide polymorphism. SNPstream. Toll-like receptors.

■ Resumen

Antecedentes y objetivo: La activación celular mediante receptores tipo Toll (TLR, por sus siglas en inglés) tiene importantes efectos bipolares sobre la inmunidad del huésped y la patogenia del asma. La subfamilia TLR2 es un miembro fundamental de la familia TLR. El objetivo era determinar si las alteraciones genéticas en genes de la subfamilia TLR2 influyen en el riesgo de asma.
Métodos: Se incluyó a un total de 318 pacientes asmáticos y 352 controles no asmáticos. Se detectaron ocho polimorfismos de un solo nucleótido en los genes TLR2 mediante el sistema GenomeLab SNPstream.
Resultados: Se observó que los pacientes homocigotos para la variante TT de TLR2/rs7656411 presentaban un riesgo significativamente menor de padecer asma en comparación con los pacientes homocigotos para la forma GG natural (oportunidad relativa [OR] ajustada = 0,63; intervalo de confianza [IC] del 95% = 0,41-0,98; *p* = 0,036). Asimismo, se observó una relación positiva entre el alelo T de rs2381289 en TLR6 y la rinitis alérgica en el asma (OR = 1,79; IC del 95% = 1,10-2,91; *p* = 0,025), mientras que el alelo A de rs11466651 en TLR10 se asoció negativamente con la rinitis alérgica (OR = 0,49; IC del 95% = 0,26-0,95; *p* = 0,046).
Conclusión: Los resultados indican que una variante genética en la subfamilia TLR2 puede influir en la predisposición al asma.

Palabras clave: Asma. Rinitis alérgica. Polimorfismo de un solo nucleótido. SNPstream. Receptores tipo Toll.

Introduction

Asthma is one of the most common chronic inflammatory respiratory disorders caused by dysregulated immune responses. It has been speculated that overzealous helper T (T_H) 2–biased immune responses might result in the development of asthma [1]. As a bridge linking innate and adaptive immunity, toll-like receptors (TLRs) can skew specific immune responses in opposing directions [2].

TLRs are a class of pattern recognition molecules with similar structural and functional properties. They initiate intracellular signaling pathways, bind to downstream protein kinases, induce activation of transcription factors, and initiate transcription of inflammatory cytokines and other host response elements [3]. TLRs also influence T-cell polarization and development [4], both of which are key events in the induction and perpetuation of asthma and atopy [5].

To date, 12 members of the TLR family have been identified in mammals. TLRs can be divided into 6 subfamilies [6]. The TLR2 subfamily comprises TLR1, TLR2, TLR6, and TLR10 [7]. The *TLR2* gene is located at 4q31.3-q32 [8]. *TLR1, TLR6*, and *TLR10* are located within a 54-kb region on chromosome 4p14 and encode proteins with highly homologous amino acid sequences [9]. TLR2 forms heterodimers with TLR1 and TLR6. In addition, human TLR10 is thought to heterodimerize with TLR2 and TLR1, although ligands for these heterodimers have not yet been discovered [10].

Genetic variability may play a role in asthma [11]. A previous study by our group revealed the role of gene polymorphisms in a Chinese population with asthma [12]. Several authors have examined the association between individual polymorphisms in the *TLR2* subfamily and asthma; however, the results are contradictory, and data on the simultaneous action of several polymorphisms are not well understood [13-20]. Small effects of genotypes on complex traits have proven difficult to detect, and haplotype structures should be taken into consideration. This study explores the role of the *TLR2* subfamily loci in asthma in an ethnic Chinese community.

Materials and Methods

Study Population

We enrolled 318 asthmatic patients (age, 14 to 75 y) and 352 nonasthmatic controls (age, 16 to 74 y) from March 2006 to May 2007 (Table 1). All participants were unrelated Han Chinese residing in Nanjing city and the surrounding regions. The case group was consecutively recruited from the First Affiliated Hospital, Nanjing Medical University, Nanjing, China. All participants underwent a detailed workup, including medical history, family history, smoking habit, occupation, general physical examination, medication, skin prick testing (SPT), complete blood count, and extended laboratory tests. Asthma was diagnosed and severity classified according to the Global

Table 1. Demographic Characteristics of Asthmatic Patients and Controls[a]

	Controls (n=352)	Cases (n=318)	P
Age, y	38.26 (13.31)	39.80 (14.23)	NS
Gender, n (%)			
Female	200 (56.82)	183 (57.55)	NS
Male	152 (43.18)	135 (42.45)	
Smoking, n (%)			
Nonsmoker	242 (82.88)	280 (88.05)	NS
Smoker	60 (17.12)	38 (11.95)	
Atopy, n (%)	0	237 (75.00)	–
Eosinophils, ×10^9/L	0.14 (0.11)	0.46 (0.71)	<.0001
Log$_{10}$ IgE, IU/mL[b]	1.19 (0.60)	1.82 (0.50)	<.0001
FEV$_1$, %	94.75 (18.07)	74.11 (23.66)	<.0001
FEV$_1$/FVC, %	88.66 (9.43)	76.18 (12.78)	<.0001
AR, n (%)			
No	0	137 (43.08)	–
Yes	0	181 (56.92)	–

Abbreviations: AR, allergic rhinitis; FEV$_1$, forced expiratory volume in 1 second; FVC, forced vital capacity; NS, nonsignificant.
[a]Data are presented as mean (SD), unless otherwise indicated. Differences between groups were evaluated using the *t* test or χ^2 test as appropriate.
[b]Log$_{10}$ IgE, log$_{10}$-transformed immunoglobulin E levels.

Initiative for Asthma (GINA) guidelines [21]. The asthmatic patients were subdivided into 4 groups based on their clinical features (stage 1, intermittent; stage 2, mild-persistent; stage 3, moderate-persistent; and stage 4, severe-persistent).

The healthy volunteers were recruited from the spouses of the patients and the general population. Controls had to meet the following criteria: *(a)* good health status and matched with the cases for age, gender, and area of residence; *(b)* no positive result for SPT; and *(c)* normal levels of total serum immunoglobulin (Ig) E. The institutional ethics committee approved the study protocol and all participants gave their written informed consent.

Assessment of Clinical Data

Smoking habit was measured in pack-years, namely, the number of packs of cigarettes smoked per day multiplied by the number of smoking years. Participants who had smoked <5 pack-years were defined as nonsmokers; otherwise, they were considered as smokers [22]. Atopy was determined by SPT as at least 1 positive result to 13 common aeroallergens, including *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Felis domesticus, Canis familiaris*, cockroach, pollen, ragweed, mugwort, moulds (*Cladosporium* and *Alternaria*) and animal allergens (cat, dog, and horse). Allergic rhinitis was defined as rhinitis appearing at least twice after exposure to a particular allergen and not related to infection. SPT, total serum IgE levels, and lung function assessments have been described in detail elsewhere [23].

Genotyping

DNA was extracted from EDTA-anticoagulated peripheral blood leukocytes with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. As a preliminary study, we selected and genotyped 8 SNPs (Table 2). Genotyping was performed using the GenomeLab SNPstream genotyping platform (Beckman Coulter, Fullerton, California, USA) and its accompanying SNPstream software suite at the Chinese National Human Genome Center in Shanghai, China. The polymerase chain reaction (PCR) primers and extension 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). SNPstream genotyping was performed as previously described [24,25].

Each sample was run blind, and PCR-negative controls were included in each 384-well plate. We duplicated 10% of samples to confirm the concordance and accuracy of genotyping. A sample call rate >99% was observed with 100% matching in the replicates.

Statistical Analysis

Differences in the distribution of demographic characteristics, selected variables, and genotypes of the TLR2 subfamily variants between cases and controls were evaluated using the *t* test or the Fisher exact test as appropriate. The Hardy–Weinberg equilibrium was tested using the $\chi 2$ goodness-of-fit test to compare the observed genotype frequencies with the expected frequencies among the controls. The associations between genotypes and risk of asthma were estimated by computing the odds ratio (OR) and its 95% confidence interval (CI) using logistic regression analysis for crude ORs and adjusted ORs when adjusting for age, gender, and smoking status. Statistical significance was set at a *P* value <.05. The EM algorithm in SAS 9.1.3 PROC HAPLOTYPE (SAS Institute, Cary, North Carolina, USA) was used to infer haplotype frequencies based on the observed genotypes. All statistical analyses were carried out using SAS 9.1.3. A 2-sided *P* value <.05 was considered significant.

Results

Demographic and Clinical Characteristics

Table 1 summarizes the demographic and clinical characteristics of the study population. There were no significant differences between the cases and the controls for age, gender, or smoking habit. However, differences in peripheral eosinophil counts, serum IgE, forced expiratory volume in 1 second (FEV_1)

Table 2. Candidate Genes and Variants

Gene (Accession No.) and Locus	NCBI rs No.	Base Change	Minor Allele Frequencies, %		Hardy-Weinberg Equilibrium	Genotyped, %
			Control	Case		
TLR1 4p14	rs4833095	C/T	36.4	37.7	0.59	99.9
TLR2 4q31.3	rs7656411	G/T	48.9	43.1	0.96	99.7
TLR6 4p14	rs5743808	T/C	5.3	3.9	0.30	99.9
	rs5743831	A/G	22.4	22.2	0.82	100
	rs2381289	C/T	41.2	47.6	0.49	100
TLR10 4p14	rs11466651	G/A	8.5	6.8	0.70	100
	rs11466655	G/A	21.9	21.7	0.97	99.9
	rs4504265	C/A	48.3	47.5	0.32	100

and FEV$_1$/forced vital capacity were more pronounced in the cases than in the controls (*P*<0.0001 for all comparisons, Table 1).

TLR2 Subfamily Gene Polymorphism Allele/ Genotype Distributions

The observed genotype frequencies for the 8 SNPs were all in Hardy–Weinberg equilibrium in the controls (*P*≥.30, Table 2). The allele distributions of the SNPs did not differ significantly cases and controls (*P*≥.11, Table 2). The allele and genotype distributions of the polymorphisms in the cases

and controls are shown in Table 3. The frequencies of the GG, GT, and TT genotypes of rs7656411 were 26.2%, 49.9%, and 23.9% in the controls, and 32.5%, 48.9%, and 18.6% in the cases. Logistic regression analysis revealed that the rs7656411TT homozygote was associated with a significantly reduced risk of asthma (crude OR, 0.63; 95% CI, 0.41-0.97, *P*=.036), compared with the rs7656411GG wild-type homozygote. Furthermore, the multivariate analysis of the genotypes adjusted for age, gender, and smoking confirmed this association. However, there were no associations between the genotypes of other SNPs and risk of asthma.

Table 3. Logistic Regression Analyses of Associations Between Polymorphisms and Risk of Asthma

	Controls[a]		Patients[a]		Adjusted OR[b]	*P*
	n	%	n	%	(95% CI)	
TLR1-rs4833095						
CC	144	41.0	133	41.8	1	
TC	158	45.0	130	40.9	0.94 (0.67-1.33)	NS
TT	49	14.0	55	17.3	1.26 (0.79-2.01)	NS
TLR2-rs7656411						
GG	92	26.2	103	32.5	1	
GT	175	49.9	155	48.9	0.84 (0.58-1.21)	NS
TT	84	23.9	59	18.6	0.63 (0.41-0.98)	.037
TLR6-rs5743831						
AA	211	60.0	191	60.1	1	
GA	124	35.2	113	35.5	0.95 (0.68-1.32)	NS
GG	17	4.8	14	4.40	0.96 (0.45-2.06)	NS
TLR6-rs5743808						
TT	314	89.5	293	92.1	1	
TC	37	10.5	25	7.9	0.80 (0.46-1.37)	NS
TC+CC	37	10.5	25	7.9	0.79 (0.46-1.37)	NS
TLR6-rs2381289						
CC	109	31.0	94	29.6		
TC	168	47.7	145	45.6	1.02 (0.71-1.47)	NS
TT	75	21.3	79	24.8	1.15 (0.74-1.77)	NS
TLR10-rs11466651						
GG	294	83.5	275	86.5	1	
GA	56	15.9	43	13.5	0.89 (0.57-1.39)	NS
GA+AA	58	16.5	43	13.5	0.86 (0.55-1.33)	NS
TLR10-rs11466655						
AA	214	61.0	195	61.3	1	
GA	120	34.2	108	34.0	1.02 (0.73-1.42)	NS
GG	17	4.8	15	4.7	1.02 (0.48-2.17)	NS
TLR10-rs4504265						
CC	98	28.1	93	29.3	1	
CA	165	47.3	147	46.4	0.98 (0.67-1.41)	NS
AA	86	24.6	77	24.3	0.95 (0.62-1.46)	NS

Abbreviations: CI, confidence interval; NS, nonsignificant; OR, odds ratio.
[a]Because of genotyping failure, the total case and control numbers for each SNP may be less than 318 and 352 respectively.
[b]Age, gender, and smoking were included in the model to adjust for potential confounders.

Table 4. Allergic Rhinitis in Asthmatic Patients With Respect to the Genotypes of rs2381289 and rs11466651

	Allergic Rhinitis, No. (%)	Nonallergic Rhinitis, No. (%)	OR (95% CI)	P
STLR6-rs2381289				
CC	44 (46.81)	50 (53.19)	1	
TC+TT	137 (61.16)	87 (38.84)	1.79 (1.10-2.91)	.025
TLR10-rs11466651				
GG	163 (59.27)	112 (40.73)	1	
GA+AA	18 (41.86)	25 (58.14)	0.49 (0.26-0.95)	.046

Abbreviations: CI, confidence interval; OR, odds ratio.

Gene–Gene Interaction and Haplotype Analysis

No significant gene-gene interactions were found between SNPs of the *TLR2* subfamily (data not shown). Haplotype analysis was performed in a further step in *TLR6, TLR10*, and *TLR1-6-10*. None of the haplotypes showed a significant association with asthma (data not shown).

Genotypes of the TLR2 Subfamily and Allergic Rhinitis

More patients harboring the rare T allele of rs2381289 had allergic rhinitis (*P*=.025; OR, 1.79; 95% CI, 1.10-2.91) than those who did not carry either allele. The inverse situation was observed when the effect of the rare A allele of rs11466651 on allergic rhinitis was measured (*P*=.046; OR, 0.49; 95% CI, 0.26-0.95) (Table 4). These investigations were performed in patients with asthma (case-only association study).

Discussion

We investigated the associations between 8 SNPs of the *TLR2* subfamily and risk of asthma in a Chinese ethnic population. Our results showed that the TT variant genotype of the homozygous polymorphism rs7656411 in *TLR2* was associated with a significantly decreased risk of asthma. We also found that the T allele of the rs2381289 SNP in *TLR6* was positively associated with allergic rhinitis in asthma, while the A allele of rs11466651 SNP in *TLR10* was negatively associated with allergic rhinitis.

As an important member of the TLR family, TLR2 has several unique attributes. First, it can bind to a wide variety of exogenous ligands [6]. Second, it is expressed in many different cell types (eg, immune effector cells and nonimmune cells such as fibroblasts and epithelial cells) [6], which are upregulated by different inflammatory stimuli and stress hormones and downregulated by vigorous exercise [26]. Third, TLR2 is also unusual in that it partners with other TLRs to form heterodimers, as the functional elements achieved specificity and inclusiveness of recognition with the low number of receptors [27]. Activation of TLR2 has recently been reported to introduce a T_H2 immune response in experimental asthma, thus supporting the hypothesis that TLR2 might play a critical role in asthma [28].

Although the potential role of TLR2 as a regulator of immune responses has received considerable attention in recent years, the results of published association studies on *TLR2* SNPs and risk of asthma are contradictory. The few published studies that have investigated the role of *TLR2* SNPs in the etiology of asthma have not done so in Chinese populations. For example, a European study has shown that farmers' children harboring the T allele in *TLR2*/–16934 (rs4696480) were less likely to have asthma and current asthma symptoms [15]. Noguchi et al [18] reported no associations between either polymorphisms or haplotype of *TLR2* and the risk of asthma, although in in vitro studies they did find that a polymorphism in the promoter region of *TLR2*—whether an insertion or a deletion of the DNA sequence—alters the expression of the *TLR2* gene. More recently, Kormann et al [20] found that functionally relevant *TLR1* and *TLR6* variants have protective effects on atopic asthma.

TLR activation in several types of cells is associated with release of effector molecules, such as cytokines and chemokines [29], which can influence the adaptive immune responses and the pathogenesis of allergic disease. It seems likely that the rs7656411 TT SNP may be in linkage disequilibrium with other untyped disease-causing SNPs. Consequently, the rs7656411 TT alteration could modify the effect on these protein-DNA interactions, thus contributing to the onset of a number of inflammatory diseases, including asthma. However, the functional relevance of rs7656411 SNP should be investigated in more detail.

Allergic rhinitis and asthma are inflammatory conditions of the airway that often occur concomitantly in the same patient. Allergic rhinitis has been associated with more severe asthma, greater difficulty in controlling asthma, and substantial impairment in quality of life [30]. We also performed a case-only study to assess correlations between sequence variants of SNPs and allergic rhinitis, and observed a positive association between rs2381289 TC+TT and allergic rhinitis and a negative association between rs11466651 GA+AA and allergic rhinitis. Effects on asthma have been identified for SNPs in *TLR6* and *TLR10* [16,17,20]. Furthermore, the functional study by Kormann et al [20] showed that primary cells derived from carriers of protective *TLR6* and *TLR10* variants enhanced inflammatory responses, increased T_H1 cytokine expression, and reduced T_H2-associated IL-4 production after specific

stimulation. Association studies on *TLR6* and *TLR10* variants and the risk of allergy will provide useful information in this area.

In our study, the asthma phenotype was classified following restrictive criteria. All asthmatic patients and controls had a similar ethnic background, thus limiting possible confounding effects. To guarantee the quality of laboratory procedures, SNPs were closely scrutinized and retyped to ensure that they were genotyped correctly. Patients and controls were not genotyped in separate batches, and samples for controls and patients were not coded separately; therefore, analysis was double blind. These measures may be help to ensure positive results.

Our study had the limitations that are typical of all case-control studies. First, 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. We calculated the power to detect the association between *TLR2*/rs7656411 and the risk of asthma with 317 cases and 351 controls (1 genotyping failure) at a significance level of $P<.05$. We observed that our study had an 87.6% power to detect relative risks of 0.6 for minor allele frequencies of 0.4. Second, environmental factors other than smoking (eg, occupational exposure, pets, allergen exposure, and domestic endotoxin levels) might interact with the *TLR2* genotype or act as potential confounders in the analysis. Third, rigid adherence to an empirical significance level of $P<.05$ may be too conservative and obscure true-positive associations.

In summary, this study supports the association between *TLR2* subfamily polymorphisms and the risk of asthma in the study population. In order to confirm the significance of these polymorphisms, we are endeavoring to increase the sample size. Larger, better-designed studies are to be carried out using appropriate molecular and statistical methods to further analyze this association.

Acknowledgments

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