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Lack of Association between Toll Like Receptor-2 and Toll Like Receptor-4 Gene Polymorphisms and Other Features in Iranian Asthmatics Patients

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ABSTRACT

Asthma as a chronic inflammatory airway disease is considered to be the most common chronic disease that is involving genetic and environmental factors. Toll like receptors (TLRs) and other inflammatory mediators are important in modulation of inflammation. In this study, we evaluated the role of TLR2 Arg753Gln and TLR4 Asp299Gly polymorphisms in the asthma susceptibility, progress, control levels and lung functions in Iranian patients.

On 99 asthmatic patients and 120 normal subjects, TLR2 Arg753Gln and TLR4 Asp299Gly polymorphisms were evaluated by PCR-RFLP method recruiting Msp1 and Nco1 restriction enzymes, respectively. IgE serum levels by ELISA technique were determined and asthma diagnosis, treatment and control levels were considered using standard schemes and criteria.

Our results indicated that the genotype and allele frequencies of the TLR2 Arg753Gln and TLR4 Asp299Gly polymorphisms were not significantly different between control subjects and asthmatics and were not related to in asthma features such as IgE levels, asthma history and pulmonary factors.

Whereas some previous studies indicated TLRs and their polymorphisms might have some role in asthma incidence and features, our data demonstrated that TLR2 Arg753Gln and TLR4 Asp299Gly gene variants were not risk factors for asthma or its features in Iranian patients. Genetic complexity, ethnicity, influence of other genes or polymorphisms may overcome these polymorphisms in our asthmatics.

Keywords: Asthma; Polymorphism; RFLP-PCR; TLR2; TLR4

INTRODUCTION

Asthma is a complex and chronic disease in which allergen-induced inflammatory processes in the airways contribute to the development of symptoms, such as wheezing, cough, dyspnea and breathlessness.[1] Asthma is considered to be the most common chronic disease and the leading cause of hospitalization in schoolchildren.[2,3] Control of asthma and response to medication is different in patients, and different

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asthmatics show various levels of asthma severity and progress which depends on multiple factors especially genetic composition of patients. The need for management of medication and control of asthma make us improve our knowledge about pathogenesis of asthma and the role of different elements that contribute to airway inflammation or influence signs and symptoms.[4,5] Asthma is believed to be a complex disorder involving genetic and environmental factors.[6] Many allergens and environmental microorganisms have pattern molecules on their surfaces, and these molecules interact with pattern recognition receptors (PRRs), which are part of the innate immune system. Among the known PRRs for microbial products, toll-like receptors (TLRs) are an evolutionarily conserved group of molecules expressed in antigen-presenting cells and epithelial cells.[7] TLRs recognize microbial patterns and play a crucial role in linking innate and adaptive immunity inducing a proinflammatory immune response that may counterbalance allergic diathesis. 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.[8] Among TLRs, TLR4 recognizes lipopolysaccharide, a cell wall component of gram-negative bacteria. In contrast, TLR2 recognizes a wide spectrum of Pathogen Associated Molecular Patterns (PAMPs) such as membrane components of Gram-positive and Gram-negative bacterial cell wall, mycoplasma, mycobacteria, yeast and parasites.[9] A study showed the alterations in intestinal microflora balance promoted the maturity of Dendritic Cells (DCs) and raised the expressions of TLR2 and TLR4 on DCs in allergic mouse lung.[10] TLR2 signaling has been shown to induce Treg cell expansion accompanied by a loss of suppressive activity in vitro and in vivo.[11] A study mentioned protective effects of TLR2 polymorphisms on lung function among workers in swine operations and the possibility of its protective role in airway disease in individuals exposed to gram-positive organisms in the inhaled airborne dust.[12] In TLR4 deficient mice compared with wild-type mice, allergen-induced eczema were exacerbated and also increased allergen-induced skin levels of innate (IL-1β, TNF-α, and CXCL2) and Th17 genes (IL-17A and IL-17F) were observed.[13] All of these findings support role of TLRs in asthma susceptibility or asthma features as they determine type, severity and outcomes of asthma pathogenesis. On the other hand, the amounts of TLR synthesis in the cells or their functions are determined by some polymorphisms in their genes. As a result, polymorphisms in TLR genes might determine susceptibility or progress of disease and degree of asthma control. With regards to the proposed role TLRs in asthma, in this study we analyzed the genetic variants of TLR2 Arg753Gln, and TLR4 Asp299Gly in Iranian asthmatic patients and healthy controls to evaluate role of these polymorphisms in asthma susceptibility, progress, control and lung functions.

MATERIALS AND METHODS

Study Populations

In this study, 99 unrelated adult asthmatic patients (28 males and 71 females, mean±SD age of 44.15 ± 13.06 years) (Table 1), whose asthma was defined according to the criteria of the Global Initiative for Asthma (GINA)[14] were enrolled. Clinical history, physical examination, and pulmonary function test (PFT) in a standard fashion were assessed for all subjects. Asthmatics have had treatment in a standard scheme as inhaled corticosteroids and/or bronchodilator when necessary, and the asthmatic patients were subdivided into two control groups based on American Thoracic Society criteria[15] as Asthma Control Tests (ACT).

Smoking history of more than 10 pack-years, presence of parasitic infection, and pregnancy or breastfeeding were exclusion criteria. A total of 120 healthy volunteers were recruited from the general

Table 1. The Study populations

Variables	Asthmatic group	Control group
Number	99	120
Female/male ratio	71/28	83/37
Mean±SD of age	44.15 ± 13.06 years	46.6±14
Have clinical asthmatic symptoms	Yes	No

Table 2. PCR materials and cycles

Locus	PCR conditions
TLR2	35 cycle: 94 °C 30s, 58 °C 50s, 72 °C 40s
	50ng DNA, 200µmol dNTPs, 0.7mM $MgCl_2$
TLR4	35 cycle: 94 °C 20s, 61 °C 50s, 72 °C 50s
	50ng DNA, 200µmol dNTPs, 2mM $MgCl_2$

Table 3. Cytokine and internal control for PCR-RFLP primers

Locus	Primers	Method
TLR2	Forward: 5'- TAAACTTGGGAGAACATGGT -3'	Msp1 based RFLP
	Reverse: 5' - TGGGGAAAGATAGAGTAATA -3'	
TLR4	Forward: 5'- ACACAACTGTGTTCACTAGC -3'	Nco1based RFLP
	Reverse: 5'- CAACTTCATCCACGTTCACC -3'	

population. Controls had to meet the following criteria: good health status and matched with the cases for age, gender, and area of residence, no respiratory symptoms or history of asthma and allergy. The study protocol was approved by the ethics committee at Tarbiat Modares University and written informed consent was obtained from all participants.

Total Serum IgE Measurements

Serum was separated from 5 ml of patients' and normal subjects' blood, and total serum IgE levels were measured using the ELISA kit (Genesis Diagnostics, UK) according to the manufacturer's instructions.

DNA Preparation

Genomic DNA was extracted from peripheral blood leucocytes using a DNG^{plus} extractor WB kit (Cinagen, Iran) according to the manufacturer's instructions.

Determination of TLR2 Arg753Gln and TLR4 Asp299Gly Gene Polymorphisms

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for evaluation of TLR2 Arg753Gln, and TLR4 Asp299Gly polymorphism. PCR steps were performed using a thermal cycler (Techne, Genius, UK). PCR conditions, PCR cycles and primers (FERMENTAS Inc, 830 Harrington Court, Burlington Ontario, Canada) are summarized in Table 2 and 3. In brief, PCR materials were mixed according to table 2 and 3 and tubes passed thermal cycles as summarized in table 2. After conformation of single bands of PCR product in agarose electrophoresis, restriction enzymes were affected. In a final volume of 25 µl, PCR products were digested by Msp1 and Nco1 (Fermentas thermo scientific, USA) restriction enzymes on TLR2 and TLR4 products, respectively and then digestions were monitored by agarose gel electrophoresis and ethidium bromide staining.

Statistical Analysis

Allele and genotype frequencies were calculated in patient and control subjects by direct gene counting. Statistical evaluation was carried out using the Statistical Package for the Social Sciences (SPSS) version 15. The statistical significance of the difference was tested by a χ^2 analysis with one difference or by the two-tailed Fisher's exact test when the criteria for the χ^2 analysis were not fulfilled. The associations between genotypes and risk of asthma were estimated by computing the odds ratio (OR) and its 95% confidence interval (CI). For analysis of IgE and respiratory factors, differences in various gene variants, the one way analysis variance test (ANOVA) and t-test were used. *P*-Values<0.05 were considered statistically significant. An exact test was used to evaluate deviations from expected Hardy-Weinberg genotypic proportions.

RESULTS

The results of TLR2 Arg753Gln, and TLR4 Asp299Gly gene polymorphisms in asthmatic and normal subjects (Table 4), two levels of asthma controls (Table 5), sex, allergy history and familial history of asthma (Table 6) and serum IgE and

respiratory factors in asthma patients (Table 7) are shown. Our results didn't show any statistically significant difference between TLR2 Arg753Gln and TLR4 Asp299Gly genotypes and alleles of asthmatics and normal subjects (p>0.05). Other features of asthma as asthma control levels, serum IgE, history and pulmonary factors also were not different in TLR2 Arg753Gln and TLR4 Asp299Gly different variants (p>0.05).

Table 4. Results of TLR2 Arg753Gln and TLR4 Asp299Gly SNPs determined in asthmatics and normal subjects

Gene	Genotype	Asthma %(N)	Normal %(N)	*P* value	OR	%95CI
TLR2	GG	%92.9(92)	%94.2(113)	0.709	0.814	0.276-2.405
	GA+AA	%7.1(7)	%5.8(7)			
	G allele	%96.5(191)	%97.1(233)	0.714	0.820	0.283-2.378
	A allele	%3.5(7)	%2.9(7)			
TLR4	AA	%85.9(85)	%86.7(104)	0.863	0.934	0.431-2.022
	AG+GG	%14.1(14)	%13.3(16)			
	A allele	%92.9(184)	%93.3(224)	0.868	0.939	0.446-1.974
	G allele	%7.1(14)	%6.7(16)			

N, absolute number; CI, Confidence Interval; OR, Odds ratio

Table 5. Results of TLR2 Arg753Gln and TLR4 Asp299Gly SNPs distribution in controlled and uncontrolled asthmatics

Gene	Genotype	Controlled Asthma % (N)	Uncontrolled Asthma % (N)	*P* value	OR	%95CI
TLR2	GG	%95.3(41)	%91.1(51)	0.411	2.010	0.371-10.898
	GA+AA	%4.7(2)	%8.9(5)			
	G allele	%97.7(84)	%95.5(107)	0.419	1.963	0.371-10.369
	A allele	%2.3(2)	%4.5(5)			
TLR4	AA	%86.0(37)	%85.7(48)	0.962	1.028	0.328-3.220
	AG+GG	%14.0(6)	%14.3(8)			
	A allele	%93.0(80)	%92.9(104)	0.964	1.026	0.342-3.075
	G allele	%7.0(6)	%7.1(8)			

N, absolute number; CI, Confidence Interval; OR, Odds ratio

Table 6. Distribution and *P* values for TLR2 Arg753Gln and TLR4 Asp299Gly variants in gender, allergy history and familial history of asthma groups

Gene	Genotype	Distribution (N)			*P* value		
		Sex M/F	Allergy P/N	History P/N	Sex	Allergy	History
TLR2	GG	26/66	63/29	44/48	0.986	0.077	0.800
	GA+AA	2/5	7/0	3/4			
	G allele	54/137	133/58	91/100	0.986	0.077	0.803
	A allele	2/5	7/0	3/4			
TLR4	AA	25/60	59/26	39/46	0.539	0.485	0.434
	AG+GG	3/1	11/3	8/6			
	A allele	53/131	129/55	86/95	0.555	0.502	0.452
	G allele	3/11	11/3	8/6			

M: Male; F: Female; P: Positive; N: Negative.

Table 7. IgE concentrations and *p* values for association between TLR2 Arg753Gln and TLR4 Asp299Gly polymorphisms and serum IgE and respiratory factors of asthma patients

Gene	Genotype	IgE Cons. (IU/ml)	*P* value						
			IgE	Age	FEV1 (%P)	FVC (%P)	FEV1/FVC (%P)	FEF 25-75% (%P)	PEF (%P)
TLR-2	GG	113.84±251.38	0.258	0.658	0.928	0.731	0.663	0.282	0.436
	GA+AA	241.61±564.32							
	G allele	119.16±268.71	0.265	0.662	0.929	0.733	0.256	0.665	0.440
	A allele	241.61±564.32							
TLR-4	AA	117.37±257.65	0.584	0.371	0.158	0.295	0.948	0.427	0.238
	AG+GG	116.20±433.55							
	A allele	120.94±271.91	0.596	0.388	0.174	0.315	0.949	0.440	0.257
	G allele	166.20±433.55							

FEV1: Forced Expiratory Volume in 1 Second, FVC: Forced Vital Capacity, PEF: Peak Expiratory Flow, FEF25-75: Forced Expiratory Flow 25-75%.

DISCUSSION

Asthma is a multifactor chronic inflammatory disorder of the airways and a variety of genetic and environmental factors contribute to its pathogenesis. Immune and inflammatory elements are important factors in induction, progress and clinical outcomes of asthma.[6] TLRs are PRRs, are highly polymorphic, and play an important role in both innate and adaptive immunity.[9,16] TLR2 mainly responds to cell wall structure components from gram-positive bacteria, such as peptidoglycan, and TLR4 mainly recognizes microbial membrane components from gram-negative bacteria, such as lipopolysaccharide (LPS) or endotoxin.[9,17] In a study to quantify messenger RNA (mRNA) and protein expression of TLR2, TLR3, and TLR4 in the nasal mucosa of patients with seasonal allergic rhinitis before and after challenge with relevant pollens identified, protein expression for all three TLRs was increased in patients with rhinitis after challenge. This study raises the possibility that TLR polymorphisms may have a role in the development of allergic airway inflammation.[17] The reduced immune responses of TLR2 polymorphisms have also been investigated in experimental studies using human cells or animal models.[18,19] All of these data support the role of TLR molecules and also their polymorphisms in asthma features. With regard to these findings, we evaluated two main polymorphisms as TLR2 Arg753Gln, and TLR4 Asp299Gly in Iranian asthmatic patients and then analyzed correlation of these variants with serum IgE levels, respiratory factors, patient's allergy and familial history and also standard control levels of asthma in these patients. Analysis of our results did not show any positive correlation of TLR2 Arg753Gln, and TLR4 Asp299Gly polymorphisms with asthma risk, IgE levels, lung functions, control level or even familial allergy/asthma history. For explanation of these results, we looked up at some previous studies. A study on Chinese asthmatics which analyzed four SNPs in TLR4 gene showed that polymorphisms in TLR4 gene were associated with asthma severity but not susceptibility.[20] These researchers in another paper on the same patients reported positive correlation of TLR2/rs7656411 TT variant and risk of asthma.[21] In a study, SNPs in the TLR2 (4 SNPs) and TLR4 genes (9 SNPs) were genotyped in asthmatic children. In these subjects, Two TLR2 SNPs and four TLR4 SNPs significantly modified the effect of air pollution on the prevalence of doctor-diagnosed asthma.[22] A study reported protective effects of TLR2-16933T/A polymorphisms on lung function among workers in swine operations while in these subjects there were no significant differences between Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene and lung function values.[23] In another study on Danish asthmatic farmers, three CD14 SNPs, three TLR2 SNPs (-16934 A/T, Pro631His C/A and Arg753Gln C/T), and two TLR4 SNPs (Asp299Gly A/G and Thr399Ile C/T) were evaluated and their results indicated no associations between CD14, TLR2, or TLR4 genotypes and new-onset asthma.[24] In a quick look to the previous studies and with attention to probable role of TLRs in asthma, there are several

studies that have reported polymorphisms or other SNPs in TLR2 and TLR4 genes which are involved in asthma and meanwhile there are also several studies that have showed no such association. These controversies may be due to several explanation such as: genetic complexity and/or nature of asthma pathogenesis and pathophysiology with involvement of various factors including genetic and environmental factors, ethnicity and different genetic background of studied subjects, overcoming of other molecules and their genetic variations on TLRs functions and even effectiveness of other polymorphisms in TLR2 and TLR4 genes more than two TLR2 Arg753Gln and TLR4 Asp299Gly studied polymorphisms in Iranian asthmatic patients. It is noteworthy to mention that in our previous study, in almost the same asthmatics, we showed effectiveness of cytokine polymorphisms in asthma susceptibility and its features in Iranian asthmatic patients.[25] In conclusion, our results in this study do not confirm TLR2 Arg753Gln and TLR4 Asp299Gly polymorphisms as a risk factor for asthma in Iranian patients. These studies on SNPs also did not affect asthma features such as IgE levels, lung functions, control level or even familial allergy/asthma history. With regard to positive correlations in some of other studies and supposed roles of TLR2 and TLR4 in modification of immune and inflammatory responses further studies on other SNPs or other involved factors may be beneficial for clarification of controversies in asthma as a multifactorial disease.

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REFERENCES

1. Basehore M J, Howard T D, Lange L A, Moore WC, Hawkins GA, Marshik PL, et al. A comprehensive evaluation of IL4 variants in ethnically diverse populations: Association of total serum IgE levels and asthma in white subjects. J Allergy Clin Immunol 2004; 114(1):80-7.
2. Price D, Thomas M. Thomas, Breaking new ground: challenging existing asthma guidelines. BMC Pulm. Med 2006; 6 (Suppl 1):S6.
3. Georgy V, Fahim HI, El-Gaafary M, Walters S. Prevalence and socioeconomic associations of asthma and allergic rhinitis in northern Africa. Eur Respir J 2006; 28(4):756–62.
4. Aoki T, Hirota T, Tamar M, Ichikawa K, Takeda K, Arinami T, et al. An association between asthma and TNF-308G/A polymorphism: meta-analysis. J Hum Genet 2006; 51(8):677-85.
5. Barnes PJ. The role of inflammation and anti-inflammatory medication in asthma. Respir med 2002; 96 (Suppl A):S9-Sl5.
6. Ngoc PL, Gold DR, Tzianabos AO, Weiss ST, Celedon JC. Cytokines, allergy, and asthma. Curr Opin Allergy Clinical immunol 2005; 5(2):161-6.
7. Imler JL, Hoffmann JA. Toll receptors in innate immunity. Trends Cell Biol 2001; 11(7):304-11.
8. O'Neill LA, Bowie AG. The family of fi ve: TIR-domain-containing adaptors in Toll-like receptor signaling. Nat Rev Immunol 2007; 7(5):353-64.
9. Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999: 33419–25.
10. Sun D, Yang X, Zhao X, Liu E. Alterations in intestinal microflora balance induce maturity and increase the expressions of TLR2 and TLR4 of dendritic cells in mouse lung. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2013; 29(9):923-6.
11. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006; 116(2):485–94.
12. Gao Z, Dosman JA, Rennie DC, Schwartz DA, Yang IV, Beach J, et al. Association of Toll-like receptor 2 gene polymorphisms with lung function in workers in swine operations. Ann Allergy Asthma Immunol. 2013; 110(1):44-50.
13. Brandt EB, Gibson AM, Bass S, Rydyznski C, Khurana Hershey GK. Exacerbation of allergen-induced eczema in TLR4- and TRIF-deficient mice. J Immunol 2013; 191(7):3519-25
14. Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2006. [Cited 2007 March 21]. Available from: http://www.ginasthma.org.
15. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Available from: http://www.asthmacontrol.com.
16. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010; 11(5):373-4.
17. Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, Cardell LO. Upregulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. Respir Res 2005; 6:100.
18. Yang HZ, Cui B, Liu HZ, Chen ZR, Yan HM, Hua F, et

al. Targeting TLR2 attenuates pulmonary inflammation and fibrosis by reversion of suppressive immune microenvironment. J Immunol 2009; 182(1):692-702.
19. Poole JA, Wyatt TA, Kielian T, Oldenburg P, Gleason AM, Bauer A, et al. Toll-like receptor 2 regulates organic dustinduced airway inflammation. Am J Respir Cell Mol Biol 2011; 45(4):711-9.
20. Zhang Q, Qian FH, Zhou LF, Wei GZ, Jin GF, Bai JL, et al. Polymorphisms in toll-like receptor 4 gene are associated with asthma severity but not susceptibility in a Chinese Han population. J Investig Allergol Clin Immunol 2011; 21(5):370-7.
21. Qian FH, Zhang Q, Zhou LF, Jin GF, Bai JL, Yin KS. Polymorphisms in the toll-like receptor 2 subfamily and risk of asthma: a case-control analysis in a Chinese population. J Investig Allergol Clin Immunol 2010; 20(4):340-6.
22. Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. Thorax 2010; 65(8):690-7.
23. Gao Z, Dosman JA, Rennie DC, Schwartz DA, Yang IV, Beach J, et al. Association of Toll-like receptor 2 gene polymorphisms with lung function in workers in swine operations. Ann Allergy Asthma Immunol 2013; 110(1):44-50.
24. Smit LA, Bongers SI, Ruven HJ, Rijkers GT, Wouters IM, Heederik D, et al. Atopy and new-onset asthma in young Danish farmers and CD14, TLR2, and TLR4 genetic polymorphisms: a nested case-control study. Clin Exp Allergy 2007; 37(11):1602-8.
25. Daneshmandi S, Pourfathollah AA, Pourpak Z, Heidarnazhad H, Kalvanagh PA. Cytokine gene polymorphism and asthma susceptibility, progress and control level. Mol Biol Rep 2012; 39(2):1845-53.