ORIGINAL ARTICLE

Association of TNF-α -308 G/A and IL-4 -589 C/T Gene Promoter Polymorphisms With Asthma Susceptibility in the South of Iran

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Abstract

Background: Both tumor necrosis factor α (TNF-α) and interleukin (IL) 4 have been implicated in the pathogenesis of asthma. Furthermore, a G/A substitution at position -308 of the TNF-α gene promoter and a C/T substitution at position –589 of the IL –4 gene promoter have been associated with increased production of TNF-α and IL-4, respectively.
Objective: The aim of the present study was to analyze the association between TNF-α -308 G/A and IL-4 -589 C/T polymorphisms and susceptibility to asthma in a group of patients from southern Iran.
Methods: We analyzed the frequency of TNF- α -308 G/A and IL-4 -589 C/T polymorphisms in a total of 203 asthmatic patients compared to 113 nonasthmatic control subjects.
Results: An association was observed between the TNF-α –308 G/A polymorphism and susceptibility to asthma in patients with a ratio between forced expiratory volume in 1 second and forced vital capacity of less than 75% compared with normal subjects; however, the association did not achieve statistical significance (P = .054). The IL-4 –589 C/T polymorphism was associated with asthma susceptibility (P = .02). In addition, the association between this polymorphism and asthma severity approached statistical significance (P = .07).
Conclusion: These results provide further evidence for a role of TNF-α -308 G/A and IL- 4 –589 C/T polymorphisms in susceptibility to and severity of asthma. Further studies involving a larger number of patients may help to confirm our observations.

Key words: Asthma. Interleukin-4. Tumor necrosis factor-α. Polymorphism.

Resumen

Antecedentes: Tanto el factor de necrosis tumoral α (TNF-α) como la interleucina (IL) 4 se han implicado en la patogenia del asma. Una sustitución G/A en la posición -308 del promotor del gen del TNF-α y una sustitución C/T en la posición –589 del promotor del gen de la IL–4 se ha asociado con un incremento en la producción de TNF-α e IL-4, respectivamente.
Objetivo: El objetivo de este estudio fue analizar la relación entre los polimorfismos -308 G/A del TNF-α y -589 C/T de la IL-4 y la susceptibilidad al asma en un grupo de pacientes del Sur de Irán.
Métodos: Analizamos la frecuencia de los polimorfismos -308 G/A del TNF-α y -589 C/T de la IL-4 en un total de 203 pacientes asmáticos comparado con 113 sujetos control no asmáticos.
Resultados: Se observó una relación entre el polimorfismo –308 G/A del TNF-α y la susceptibilidad al asma en pacientes con una proporción entre el volumen espiratorio máximo en el primer segundo y la capacidad vital forzada de menos del 75% en comparación con los sujetos normales. No obstante, la relación no alcanzó significación estadística (P = 0,054). El polimorfismo –589 C/T de la IL-4 se asoció con susceptibilidad al asma (P = 0,02). Además, la relación entre el polimorfismo y la gravedad del asma se acercó a la significación estadística (P = 0,07).
Conclusión: Estos resultados proporcionan más pruebas sobre el papel del polimorfismo -308 G/A del TNF-α y -589 C/T de la IL-4 en la susceptibilidad al asma y la gravedad de esta enfermedad. Para confirmar nuestras observaciones, sería necesario realizar más estudios con un mayor número de pacientes.

Palabras clave: Asma. Interleucina-4. Factor de necrosis tumoral α. Polimorfismo.

Introduction

Asthma is an obstructive inflammatory disease of the airways caused by a combination of genetic and environmental factors [1,2]. Airway inflammation is a hallmark of asthma and is caused by the release of cytokines and mediators from a variety of cells. Tumor necrosis factor TNF-α is a cytokine believed to play a central role in airway inflammation and increased bronchial hyperresponsiveness [3,4]. The expression of TNF-α is upregulated in asthmatic individuals, as shown by its increased secretion in the airways and higher levels in bronchoalveolar lavage (BAL) fluid of symptomatic subjects [5-7]. This cytokine facilitates the migration of inflammatory cells to the respiratory epithelium, mainly through the enhanced release of chemotactic mediators and upregulation of adhesion-molecule expression [3,4]. Interestingly, a G/A substitution at position –308 in the promoter of the TNF-α gene has been associated with increased TNF-α production in vitro [8]. Although several studies have reported an association of the TNF-α -308 G/A polymorphism with asthma [9-12], other studies reject this association [13,14].

Interleukin (IL) 4 is another cytokine that plays a crucial role in the development of asthma through induction of immunoglobulin (Ig) E synthesis by B cells and differentiation of T helper type 2 lymphocytes [15]. IL-4 can also induce airway inflammation by induction of vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells [16]. Furthermore, asthmatic patients have been shown to have increased expression of IL-4 in both peripheral blood and BAL fluid [17,18]. IL-4 production is partially controlled by a functional C/T substitution at position –589 of the IL-4 gene promoter region. This functional polymorphism was reported to be associated with increased IL-4 production and high IgE levels in vivo [19]. Moreover, association of the IL-4 -589 C/T variant with asthma has been reported in some previous studies [19-21], but could not be confirmed by others [22].

Considering the evidence supporting a pathogenic role of TNF-α and IL–4 in asthma and the influence of the promoter polymorphisms on TNF-α and IL-4 production, we analyzed whether the TNF-α -308 G/A and IL-4 -589 C/T polymorphisms are associated with asthma susceptibility and severity in a group of individuals from the south of Iran.

Material and Methods

Subjects

One hundred twelve male asthmatic patients with a mean (SD) age of 43.7 (15) years (range, 15-79 years) and 91 female asthmatic patients with a mean age of 44 (2) years (range, 19-74 years) were recruited from 2 specialist outpatient respiratory clinics at Shiraz University of Medical Science (Shahid Motahari and Shahid Faghihi hospitals). To be eligible for inclusion in the study, patients had to have asthma as defined by the Global Initiative for Asthma with minor modifications [23]. At least 1 symptom of asthma was enough for asthma diagnosis, including cough, wheezing, breathlessness, and chest tightness. On physical examination, extensive wheezing was found in both lungs of the patients. Moreover, at their first visit, patients had to have a forced expiratory volume in 1 second (FEV_1) of between 40% and 80% of predicted normal values after at least 8 hours without inhaled β-agonist treatment. The FEV_1 in the absence of bronchodilator medications was used as an index of asthmatic airway obstruction [24]. Pregnant women and patients with a history of cigarette use in their lifetime were excluded from the study. One hundred five patients had a ratio of FEV_1 to forced vital capacity (FVC) less than 75% and 98 patients had FEV_1/FVC greater than 75% based on the lung function tests [25]. In the present study, according to the FEV_1 value, patients with FEV_1/FVC less than 75% were categorized as having severe (FEV_1 < 50%) or mild to moderate (FEV_1> 50%) asthma. In addition, 113 healthy nonasthmatic control individuals (67 male subjects with a mean age of 42 [14.4] years and 49 female subjects with a mean age of 38 [13.5] years) with no personal or family history of asthma or other allergic diseases were recruited from personnel of Shiraz University of Medical Sciences. All control subjects had normal spirometric values and had no respiratory symptoms. Moreover, controls were matched for sex and ethnicity with the patients. Patients and healthy individuals were genetically unrelated.

The study was approved by local ethics committees.

Typing of the TNF-α -308 G/A Gene Polymorphism

Genomic DNA was extracted from peripheral blood leukocytes by a salting out procedure [26]. DNA analysis was carried out by polymerase chain reaction (PCR) using the conditions previously described by Verjan et al [27], with some modifications. Three primers were used for the allele-specific PCR: the 3' primer (position-144/-164:5'-TCTCGGTTTCTTCTCCATCG- 3') was used in combination with either a 5' primer complementary to the TNFA1 allele (position -328/-308 G: 5'-ATAGGTTTTGAGGGGCATGG-3') or one that is complementary to the TNFA2 allele (position -320/-308A: 5'-ATAGGTTTTGAGGGGCATGA-3'). Allele-specific primers only differed in terms of their 3' terminal nucleotide. When the 3' nucleotide does not match the template DNA, amplification does not occur. As an internal control, primers against β-globin (5' primer, 5'-ACACAACTGTGTTCACTAGC; 3' primer, 5'-CAACTTCATCCACGTTCACC-3') were included in the reactions. Ten microliters of PCR reaction mixture was used, containing genomic DNA samples (250 ng), 200 μmol/L dNTPs, 2 mMol/L $MgCl_2$,1 μL 10X Taq DNA polymerase buffer, 1 unit of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), 10 pmol of each test primer and 10 pmol of internal control primers. Reaction conditions used were as follows: 95°C for 5 minutes; 31 cycles of 95°C for 90 seconds, 61°C for 150 seconds, and 72°C for 60 seconds, with a final extension step of 72°C for 10 minutes. PCR products were electrophoresed on 2.5% agarose gels containing 0.5 mg/mL ethidium bromide at 100 W for 45 minutes. The gels were visualized with a UV transilluminator imaging analyzer (Sigma-Aldrich, St. Louis, USA).

Typing of the IL-4 -589 C/T Gene Polymorphism

Allelic forms of the IL-4 gene were studied using a primer-induced restriction site assay [28]. The -589 region was amplified by PCR with the following primer pair: 5'-TAAACTTGGGAGAACATGT-3' and 5'-TGGGGAAAGATAGAGTAATA-3'. PCR was carried out under the conditions previously described [28], except that the $MgCl^2$ concentration was 2.2 mmol/L and the annealing temperature was set at 53°C for 50 seconds. PCR products were separated on a 2.5% agarose gel and visualized with a UV transilluminator (Sigma Aldrich Co). After amplification of the desired IL-4 promoter region, a restriction fragment length polymorphism assay was performed using *Ava* II restriction enzyme (FERMENTAS, Vilnius, Lithuania). The reaction was carried out in a 20 μL volume containing PCR product (10 μL), 10X buffer (2 μL), restriction enzyme (*Ava* II, 2.5 U per reaction), and distilled water (7.75 μL). Reactions were incubated at 37ºC overnight. The digested products were run on a 6% polyacrylamide gel containing ethidium bromide at 60 V for 3 hours. The T allele gave a single band of 195 base pairs and the C allele showed 2 fragments of 177 and 18 base pairs.

Statistical Analysis

Data were analyzed using χ^2 test and Fisher's exact test if the number of subjects was less than 5. All tests performed were 2-tailed. Statistical calculations were carried out using the Epi Info 2000 software. Statistical significance was established at a value of $P < .05$

Results

The genotype distributions of TNF-α -308 G/A and IL-4 -589 C/T polymorphisms were in Hardy-Weinberg equilibrium within each phenotype group. The frequency of genotypes and alleles of the TNF-α -308 G/A polymorphism in the asthmatic and nonasthmatic subjects are shown in Table 1. We found no significant difference in the prevalence of this polymorphism between patients and control subjects (Table 1). However, comparison of the genotype distribution of the TNF-α -308G/A polymorphism between patients with FEV1/FVC less than 75% and control subjects revealed a difference that did not quite achieve statistical significance ($P = .054$). We also investigated the association of the TNF-α –308 G/A polymorphism with disease severity. No significant association was found between TNF-α –308 G/A frequencies and asthma severity in patients with FEV_1/FVC less than 75% (Table 2).

Interestingly, analysis of the IL-4 -589 C/T polymorphism did reveal a statistically significant difference between both patient groups and healthy individuals (Table 1). Also, there was an apparent association between the IL-4 -589 C/T polymorphism and disease severity in patients with FEV_1/FVC less than 75%, although this association did not achieve statistical significance (Table 2). In fact, the high-producer allele of IL-4 (T allele) was observed more frequently in

Table 1. Genotypes and Allele Frequencies of the TNF-α -308 G/A and IL-4 -589 C/T Polymorphisms in Patients and Control Subjects*

Genotypes and Alleles	Patients Nº. (%)		Controls	*P*†	*P*‡
	FEV_1/FVC < 75%	FEV_1/FVC > 75%			
TNF-α					
AA	0 (0)	0 (0.0)	1 (0.9)	1	1
AG	19 (18.1)	9 (9.2)	9 (8)	.04	.94
GG	86 (81.9)	89 (90.8)	103 (91.2)	.07	.87
A	19 (9.05)	9 (4.6)	11 (4.9)	.12	.92
G	191 (90.95)	187 (95.4)	215 (95.1)	.12	.92
IL-4					
CC	70 (66.7)	69 (70.4)	93 (83)	.008	.04
CT	34 (32.4)	26 (26.5)	18 (16.1)	.008	.09
TT	1 (0.9)	3 (3.1)	1 (0.9)	1	.34
C	174 (82.9)	164 (83.7)	204 (91.1)	.016	.03
T	36 (17.1)	32 (16.3)	20 (8.9)	.016	.03

*TNF indicates tumor necrosis factor; IL, interleukin; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity.
†Difference between FEV_1/FVC<75% and controls by χ^2 test
‡Difference between FEV_1/FVC>75% and controls by χ^2 test.

Table 2. Genotypes and Allele Frequencies of the TNF- α -308 A/G and IL-4 -589 C/T Polymorphisms and Asthma Severity in Patients With FEV_1/FVC less than 75%*

Genotypes and Alleles	FEV_1, % Predicted, Mean (SD)	FEV_1 < 50%, No. (%)	FEV_1 > 50%, No. (%)	*P*†
TNF-α				
AA		0 (0.0)	0 (0.0)	
AG	45.33 (11.9)	14 (19.2)	5 (15.6)	.75
GG	44.14 (15.9)	59 (80.8)	27 (84.4)	.75
TNF-α				
A		14 (9.6)	5 (7.8)	.9
G		132 (90.4)	59 (92.2)	.9
IL-4				
CC	42.39 (14.3)	53 (72.6)	17 (53.1)	.08
CT	47.83 (17.6)	20 (27.4)	14 (43.8)	.15
TT	52‡	0 (0.0)	1 (3.1)	.3
IL-4				
C		126 (86.3)	48 (75.0)	.07
T		20 (13.7)	16 (25.0)	.07

*TNF indicates tumor necrosis factor; IL, interleukin; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity.
†Statistical difference between FEV_1 < 50% and FEV_1 > 50% by χ^2 test
‡The IL-4 -589 TT genotype was only detected in 1 patient.

patients with mild to moderate disease compared to those with severe disease (25% and 13.7%, respectively; *P* =.07).

Discussion

Previous studies have shown several linkages between asthma and different regions of the human genome [1]. Despite this, no specific polymorphisms in candidate genes have been definitively implicated in the pathogenesis of the disease. The reason could be that individuals who have asthma might carry genes with differing propensity that determine the susceptibility to asthma and modify the severity of the disease depending on their interaction with each other and with the environmental milieu. Among the many genes involved in the induction of airway inflammation, genes encoding TNF-α and IL-4 are potential candidates. Previous studies have shown an association between the TNF-α -308 G/A polymorphism and asthma [9-14]. In the present study we found an association between the TNF-α -308 G/A polymorphism and asthma in patients with FEV_1/FVC less than 75% compared with normal subjects, although this observation did not achieve statistical significance (*P* = .054). Our results suggest that the high-producer allele of TNF-α (A allele) could be considered as a genetic factor for susceptibility to obstructive asthma in our studied population. This association could be explained by proinflammatory effects of TNF-α that could upregulate the expression of adhesion molecules and facilitate the migration of inflammatory cells into the airways [3,4]. However, this study has been done as a case–control study with a modest sample size. The size of our sample may have accounted for our inability to demonstrate a statistically significant difference in the frequency of the TNF-α -308 G/A polymorphism between patients and control subjects, and future studies including a larger number of patients may provide confirmation of our results. Furthermore, the TNF-α -308 G/A polymorphism lies within the major histocompatibility complex region on chromosome 6. Therefore, the A allele of TNF-α may be in linkage disequilibrium with a functional variant that alters TNF-α expression or by itself determines the extent of inflammation and asthma development [29,30].

Previous studies have shown that the IL-4 –598 C/T polymorphism is associated with asthma [19-21]. In agreement with those studies, we found that the prevalence of the IL-4 –598 T allele was significantly increased in asthmatic patients with FEV_1/FVC greater than 75% and asthmatic patients with FEV_1/FVC less than 75% compared to control subjects (*P* = .03 and *P* = .016, respectively). Therefore, our results suggest that inheritance of the T allele of the IL-4 -589 polymorphism may be a risk factor for the development of asthma. Indeed, IL-4 is considered as an IgE class-switching factor and also induces the expression of VCAM-1 on endothelial cells [15,16].

Therefore, association of the high-producer IL-4 -589 T allele with asthma is not surprising. In the present study, among patients with FEV_1/FVC less than 75%, the presence of the IL-4 -589 T allele appeared to be associated with an FEV_1 less than 50% of predicted (P = .07). This borderline association may prove to be statistically significant if the number of cases or controls is increased. Interestingly, our finding is in contrast to the results of Estaban et al [31]. The reason for this discrepancy is not clear. However, it could be explained by probable differences in the genetic and environmental factors which influence the severity of airway obstruction in the study populations. Consistent with this possibility, FEV_1 below 50% of predicted was not significantly associated with the IL-4 -589 T allele among African Americans, even in the study of Esteban et al [31]. Whatever the explanation for this difference, it has been shown in some studies that inflammation in the airway of patients is decreased in the presence of high levels of IL-4 [32]. Therefore, patients who are genetically predisposed to produce high levels of IL-4 may experience less airway inflammation in the long term and thus develop mild to moderate asthma. Further support for this hypothesis was provided by Borger et al [33], who showed that higher IL-4 mRNA levels are associated with less severe adenosine-monophosphate responsiveness.

In summary, the results of this study suggest that TNF-α –308 G/A is a risk factor for asthma susceptibility but not asthma severity. We have also shown that the TNF-α -308 G/A polymorphism may influence lung function in subjects who have asthma. Furthermore our data suggest that IL-4 –589 C/T might play a role both in conferring susceptibility to asthma and modulating its severity. Further studies with larger samples are required to prove these associations.

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