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Association between +4259 T>G and -574 G>T Polymorphisms of TIM-3 with Asthma in an Iranian Population

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ABSTRACT

T-cell immunoglobulin and mucin domain (TIM)-3 have been shown to negatively regulate Th1 cell-mediated immunity. Activation of TIM-3 by galectin-9 induces Th1 cell apoptosis, which may contribute to skewing of immune response towards Th2-dominant immunity. The aim of this study was to determine whether certain genetic variations of TIM-3 influence predisposition to asthma in a sample of Iranian population.

This case-control study was conducted on 209 patients with asthma and 200 healthy controls. The +4259 T>G and -574 G>T polymorphisms were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system-PCR (ARMS-PCR). Total serum IgE was further measured with ELISA.

Notably, +4259T > G and-574G>T polymorphisms of TIM-3 were significantly associated with the susceptibility to asthma. In addition, the present study showed a significant difference between the distribution frequency of the GT + TT genotype and T allele on the +4259 T>G and -574 G>T locus between the groups. However, no correlation between the +4259 T > G and -574G > T polymorphisms and total serum IgE levels were observed.

Together these results suggest that the TIM-3 +4259 T>G and -574 G>T polymorphisms are greatly associated with the susceptibility of Iranian population to asthma, which could open up new horizons for better understanding of the pathophysiology, diagnostic, prognostic and therapeutic approaches of asthma.

Keywords: Asthma, Immunoglobulin E; Iranian population; Single nucleotide polymorphism; T cell immunoglobulin and mucin domain 3 (TIM-3)

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INTRODUCTION

Asthma is a serious chronic airway inflammatory disease caused by a combination of genetic and environmental factors affecting 300 million people worldwide.[1-3] Due to worldwide rise in its prevalence, extensive genome-wide association studies as well as more targeted searches for specific genes have been used to discover common genetic variations associated with asthma.[4]

The T cell immunoglobulin and mucin domain-containing molecules (TIMs) are type 1 transmembrane proteins expressed on various immune cells.

Three functional TIM genes have been identified so far in the human genome including TIM-1, TIM-3 and TIM-4.[5,6] TIM gene family located in chromosome 5q33.2 and have been shown to play an important role as a human susceptibility gene for several inflammatory diseases such as asthma, allergy and autoimmunity disease by regulating Th1 and Th2-cell-mediated immunity. All of the TIM molecules encode type 1 cell-surface glycoproteins with common structural motifs including signal peptides, immunoglobulin variable region-like domains, mucin-like domains, transmembrane regions, and intracellular tails.[7]

Among different members, TIM-3 has been reported to be expressed at the highest level on Th1 cells but not on Th2 cells. Interaction of TIM-3 with its main ligand, C-type lectin, galectin-9 (Gal9), has been shown to promote the apoptosis of Th1 cells. Thus, TIM-3 is a negative regulatory molecule important for Th1-mediated immune responses and TIM3/Gal9 interaction may ameliorate autoimmune diseases and asthma by indirectly modulating the balance between Th1 and Th2 type responses.[8-10]

The polymorphisms of TIM-3 has been associated with the susceptibility of human to Th2-mediated allergic diseases since it affects the Th1/Th2 balance and skew the immune response towards Th2-dominant immunity.[11,12] However, it has not yet been clearly determined whether the variations within the coding or non-coding regions of TIM-3 are involved in the protein expression, stability or function of this protein.[13]

Previous investigations demonstrated the effect of different polymorphisms on allergic diseases in different populations. As none of the TIM-3 polymorphisms have yet been evaluated in Iranian patients with asthma; in the current study, we aimed to analyze the association of -574G>T and + 4259T>G polymorphisms of TIM-3 in asthma patients and non-atopic controls. We also assessed the relationship between these polymorphisms and serum IgE levels in asthmatic patients. This study may open up new horizons for better understanding of the pathophysiology, diagnostic, prognostic and therapeutic approaches of asthma.

MATERIALS AND METHODS

Subjects

This hospital-based case-control study included a total of 209 patients with confirmed asthma and 200 healthy controls. The 209 asthmatic patients had symptoms such as coughing, wheezing, episodic breathlessness as well as chest tightness, and were diagnosed according to the global initiative for asthma guidelines (GINA). Participants of the current study were recruited between October 2014 to May 2015 at the Amin hospital, Isfahan, Iran. Healthy individuals (n= 200) were selected from general population based on comprehensive medical screening at Amin hospital. All controls were examined for asthma and a family history of asthma; and did not have any symptoms of allergic and respiratory diseases in their history or past physical check-up. Current pregnancy or breast-feeding as well as presence of parasitic infection were exclusion criteria for both groups.

All subjects participated voluntarily in this study and signed written informed consent. Experimental protocols of this study were approved by the Isfahan University of Medical Sciences Ethic Committee (No. 392448) and were performed in compliance with the provisions of the Declaration of Helsinki.

DNA extraction

Peripheral blood specimens were collected from all subjects in vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). Genomic DNA was extracted from white blood cells of each samples using a genomic DNA extraction kit (Genotbio, Korea) according to the manufacturer's instructions. DNA concentration and purity were determined using a nucleic acid spectrometer (Bio-Rad, CA, USA). DNA samples with A260/280 ratio with expected values from 1.8 to 2.0 were used for polymerase chain reaction (PCR) templates.

Serum concentrations of total IgE

Total serum IgE level was measured with human IgE ELISA kit (Euroimmun, Germany) according to manufacturer's instruction.

TIM3 -574 G>T genotyping

The -574 G>T polymorphism was genotyped by amplification refractory mutation system (ARMS-PCR) analysis. Two reactions were performed; each containing a common reverse primer (R1 5'-GGTGTCTGATTGCCAGTGATTC-3') and one allele-specific primer (F1:5'-GGCTTATGCTGGGAGTTGCT -3' or F2 5'-GGCTTATGCTGGGAGTTGCG -3').

F1 and R1 were used to amplify T allele fragments, and F2 and R1 were used to amplify G allele fragments; all the amplified fragments were499 base pair PCR with F1/R1 and F2/R1.

The reaction mixture included 2.5 µL 10× PCR buffer, 0.5 µL dNTP; 1 µL forward primer; 1 µL reverse primer, 0.6 µL $Mgcl_2$; 2 µL DNA templates, 0.5 µL Taq DNA polymerase. The final volume of the mixture was made up to 25 µL with double distilled water.

The reaction was performed with a Thermo Cycler (Eppendorf, Germany) in 95°C for 2 min, followed by 35 cycle of 94°C for 30 s, 64.6°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Next, 5 µL of the amplified products was used for 1.5% agarose gel electrophoresis. The PCR products were visualized by ethidium bromide (EB) staining.

TIM-3 +4259T>G genotyping

The TIM-3 4259T>G polymorphism was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primer sequences and restriction enzyme are shown in Table 1.

Mixes containing 2 ng of genomic DNA, 1 µL of each primer, 0.6 µL of Taq DNA polymerase, 2.5 µL 10x buffer, 0.9 µL $Mgcl_2$, 0.6 µL dNTP and double distilled water was further added to reach the final volume. The reaction mixture was heated in a Thermo Cycler (Eppendorf, Germany) to amplify the target amplicons. The PCR cycle parameters were as follows: general denaturation at 94°C for 5 min, 1 cycle, followed by 35 cycles of 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. DNA digestion condition was as follows: 1 units of PST 1, 2X buffer and 10 µL PCR product. The final volume was made up to 20 µL with double distilled water. The samples were incubated at 37° for 90 min with restriction enzyme. This enzyme digests the 649 bp PCR products into 474 and 175 fragments when the G allele is presented. Digested fragments were separated on 8% polyacrylamide gel electrophoresis (PAGE) at 100 V for 3 h and were visualized by silver nitrate staining.

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 software package (IBM Corp Armonk, NY, USA). Genotype and allele frequencies were analyzed using chi square test. Odds ratios (OR), *p* value and 95% confidence interval (CI) were also calculated. Results were considered statistically significant when $p< 0.05$.

RESULTS

Characteristics of the Study Population

The characteristics of the study population recruited to groups of patients with asthma (n=209) and the controls (n=200) are summarized in Table 2. As it is shown in Table 2, there were no significant variations in mean and distribution of age and sex between the groups (p=0.399 and p=0.142, respectively). Patients with asthma showed higher level of total serum IgE compared to the control group(p=0.001). In addition, smoking status was checked and no significant difference was observed between case and control groups (p=0.726).

We further evaluated TIM-3 -574 G>T and +4259 T>G genotypes distributions of both groups of patients with asthma (n=209) and the controls (n=200) (Table 3) and found significant differences in genotype and allele frequencies between 2 groups (p=0.000 and p=0.001, respectively).

Genotype and Allele analyses of the -574 G>T Polymorphism

At this position, G and T allele were defined as wild-type and mutant alleles, respectively. After agarose gel electrophoresis, the GG genotypes were characterized as wild GG homozygotes, TT genotypes as mutant TT homozygotes and GT genotypes as GT heterozygotes. In the present study, 180 asthmatic patients had the GG genotype, and other asthmatic patients had the GT genotype. However, GG genotype was found in 196

Table 1. Primer sequence, restriction enzyme, alleles and fragment sizes of the region containing 4259 T>G polymorphism

Polymorphism	Primer sequence 5′→ 3′	Restriction enzyme	Alleles	Fragment sizes
4259 T>G	F: GGGAAGGTGATGGGCTTT	PstI	G	474;174
	R: GGCAGGTTTGGAAGCTGA		T	474;159.16

Table 2. Clinical and demographic characteristics of patients and controls evaluated for association between +4259 T>G and -574 G>T polymorphisms of TIM-3 with asthma in an Iranian population

	Patients(n=209)	Control (n =201)	*p* value
Age (mean ± SD)	43.167±14.89	41.96±14.11	0.399
Gender			
Male/Female	69/140	81/120	0.142
Total serum IgE log10 (mean ± SD)	1.74±0.65	0.75±0.38	0.001
Smoking			
No /Yes	202/7	193/8	0.726

Table 3. Genotype and allele analyses of the -574 G>Tand +4259 G>T polymorphisms among controls and patients with asthma

	Genotype/Allele	Asthma group (n=209),n(%)	Control group (n=200), n (%)	Odds ratio	95% CI	*p* value
-574G>T	GT+TT	29+0 (13.87)	4+0 (1.99)	7.935	(2.73, 3.011)	0.000
	GG	180 (86.12)	197 (98)			
	T	29	4	7.418	(2.58, 21.29)	0.000
	G	389	398			
+4259G>T	GT+GG	69+0 (33.01)	37+0 (18.40)	2.185	(1.38 ,3.45)	0.001
	TT	140 (66.98)	164 (81.59)			
	G	69	37		(1.27, 2.98)	0.002
	T	349	365	1.95		

control subjects, while only 4 subjects had the GT genotype. TT genotype was identified neither in patients with asthma nor in the control subjects (Figure 1). In the asthma group, at the -574 G>T position on TIM-3, the genotype frequencies for GG and GT were 86.12 and 13.87%, respectively. In the control group, 98.01% of the subjects had the GG genotype, and the remaining 1.99% had the GT genotype. Differences of the genotype frequencies between 2 groups were statistically significant (Table 3).

Genotype and Allele Analyses of the +4259 T>G Polymorphism

At the +4259 T>G position, the genetic polymorphism was demonstrated as 2 genotypes (GT and TT). Because PCR products of the GG genotype make a recognition site of PstI endonuclease; after digestion, they could be cleaved into 2 DNA fragments known as 474 bp and 174bp. Alternation of this position (from T to G) disrupts the recognition site of PstI endonuclease and the PCR products of the TT genotype have 474, 159 and 16 bp fragments, while the PCR products of GT genotype could be split into 4 DNA fragments including 474, 174, 159 and 16 bp (Figure 2).

Unfortunately, due to their low molecular weight, the 16 bp DNA fragments could not be visualized by agarose gel electrophoresis. Here, 140 patients had the TT genotype, and the other patients (n=69) had the GT genotype; meanwhile, in the control group, 164 had the

TT genotype and 37 had the GT genotype.

In the asthma group, at +4259 G>T position on TIM-3, the TT and GT genotypes had frequencies of 66.9% and 33.0%, respectively. In contrast, 81.5% of the control subjects had the TT genotype, and the other 18.4% had the GT genotype. There was significant statistical difference between these 2 groups (Table 3).

We further investigated the relationship between the genotypes of each polymorphic site of *TIM-3* with total serum IgE levels in asthmatic patients. These two polymorphisms of TIM-3 in asthma patients were showed to have no significant association with the levels of IgE (Table 4).

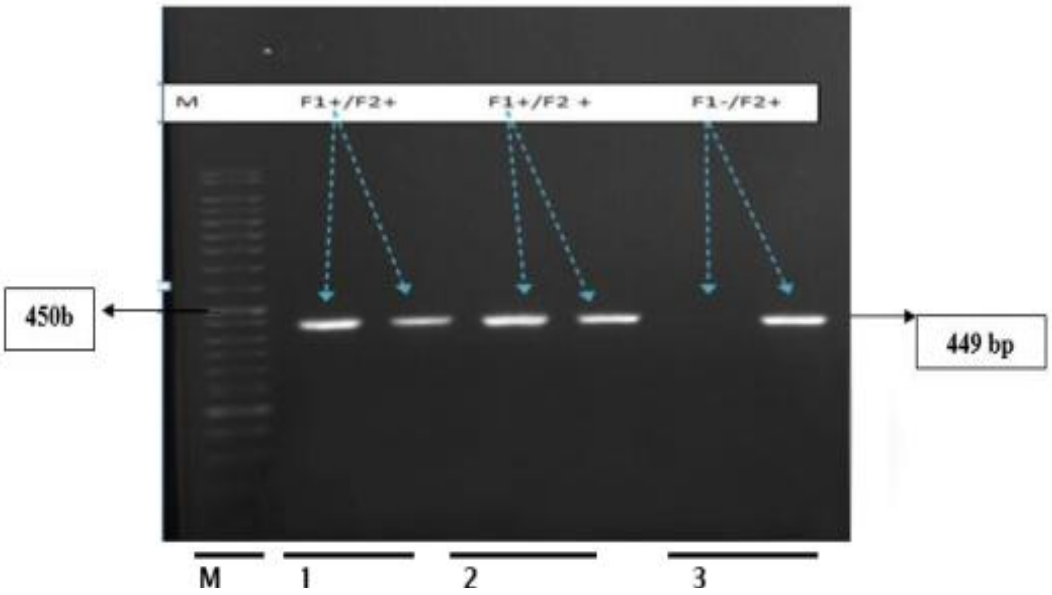

Figure 1. Genotype analyzed by ARMS-PCR method for the -574G>T. M: represents for 50 bp DNA marker; 1 and 2 represents genotype of GT; 3 represents genotype of GG. [F1 amplify mutant allele and F2 amplify normal allele]. The TT genotype was not identified in the patients with asthma or in the control subjects.

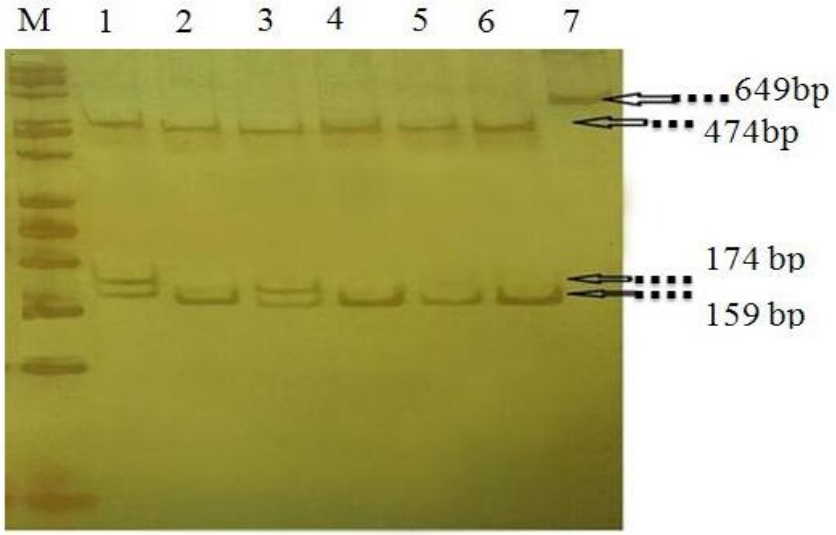

Figure 2. Genotype analyzed by PCR-RFLP method for the+4259G>T polymorphism of the TIM-3 gene. The PCR amplified products were digested with PstI: Electrophoretogram of +4259 G>T TIM-3 gene. M represents 50 bp DNA size marker; 1 and 3 represent genotype of GT; 2, 4-6 represents genotype of TT. Number 7 represents PCR product.

Table 4. Association between total serum immunoglobulin (Ig) E levels and TIM-3 -574 G>T and TIM-3 +4256 G>T polymorphisms in patients with asthma

	Genotype	Total serum IgE (log10 value)	*p*.value
-574G>T	GG	1.619 ± 0.606	> 0.05
	GT	1.730 ± 0.647	> 0.05
+4259G>T	TT	1.579 ± 0.608	> 0.05
	GT	2.05 ± 0.652	> 0.05

TIM-3: T cell immunoglobulin and mucin domain 3

DISCUSSION

Asthma is a heterogeneous chronic inflammatory disorder characterized by episodes of reversible airway narrowing, bronchial hyperresponsiveness and airway remodeling.[14] The pathophysiology of asthma is complex with both genetic as well as environmental risk factors and involves both innate and adaptive immune responses, various inflammatory cells, cytokines and pattern recognition receptors. Upon inhalation of an allergen, antigen presenting cells in the airway migrate to the draining lymph nodes where they present the antigen to precursor T-helper cells. Precursor T-helper cells then maturate and differentiate into Th2 cells leading to the production and activation of eosinophils, the release of cytokines such as IL-4 and IL-13, activation of B cells and subsequent of IgE-producing plasma cells.[15-19]

It has been speculated that genes that are involved in the regulation of Th1/Th2 balance could be potentially considered as asthma predisposing factors. TIM-3, as an example, is known to negatively regulate the Th1 cell-mediated immunity and it is located on human chromosome 5q33; a region that has been reported to be associated with asthma susceptibility. TIM-3 is highly expressed on terminally differentiated Th1 cells but not on Th2 cells it is therefore identified as a Th1-specific marker. TIM-3 binds to its ligand, Gal9, and TIM3-Gal9 interaction induces Th1 apoptosis and downregulates Th1 responses. Subsequently, it shifts the immune response towards Th2-dominant immunity.[20,21]

In the present study, the frequency of two polymorphisms at TIM-3 gene promoter and coding region (-574 G>T and +4259 T>G, respectively) in an Iranian population with asthma was investigated.

The current study demonstrated that the frequencies of GG, GT and TT genotypes in -574 G>T polymorphism in the TIM-3 promoter region were 86.12, 13.87 and 0%, respectively in the asthma population, while these frequencies were 0.98.01, 1.99 and 0%, respectively in the healthy population. Hence, our findings indicated that the T allele in -574 G>T polymorphism is a risk factor for asthma susceptibility.

The role of TIM-3 promotor polymorphisms in allergic phenotypes has been investigated in a number of studies. Chae et al. investigated the frequency of -574 G>T TIM-3 polymorphism in asthma and allergic rhinitis in patients from a Korean population. They showed that −574 T allele was found only in asthma and rhinitis patients at the frequency of 1% and 1.5%, respectively, while all healthy controls carried the G allele. Although, this polymorphism was associated with symptoms of allergic asthma or rhinitis, a significant correlation was observed neither with serum IgE level nor with blood eosinophil counts, which was in agreement with our study.[12]

In Zhang et al. study, polymorphisms of TIM-3 in promotor region (574 G/T , -882 C/T , -1516 G/T , -1571delC, -1766G/T and -1922 G/A) was not associated with asthma or related phenotype. [(22)] Also In Caucasian and African-American populations no association was seen between TIM3 polymorphisms with any phenotype.[23,24] Study conducted by Graves et al. reported a positive correlation between atopic diseases in children from white or Hispanic parents and 882 C>T polymorphism in promoter region of TIM-3. In addition, −882 C>T polymorphism has been shown to be associated with atopic dermatitis.[25]

Therefore, such polymorphisms in promoter region of TIM-3 might positively affect its expression which in turn elevates Th2 responses.

In the present study, the frequencies of TT, GT and GG genotypes of +4259 T>G polymorphism were 66.98, 33.01, 0% and 81.5, 18.4 and 0% in the asthmatic and healthy population, respectively. The GT

genotype and G allele frequencies of +4259 T>G polymorphism between asthma patients and controls were significantly different; therefore, it could be speculated that the polymorphism of +4259 T>G may contribute to the asthma pathogenesis.

Contrary findings have been reported in different studies, which looked into possible correlation between +4259 T>G polymorphism and asthma. In the Study conducted by Chae et al on Korean population, the minor T allele of +4259 T>G was found more frequently in rhinitis patients, but not in asthma patients. However, in another study performed on children from white or Hispanic parents, neither atopic dermatitis nor asthma was associated with +4259 T>G polymorphism.[12] This discrepancy may be due to the different ethnic and regional group studied in these reports.

Linkage between IgE phenotypes and chromosome 5q have been previously evidenced.[26] In the present study, we investigated the relationship between these single nucleotide polymorphism (SNPs) of TIM-3 with total serum IgE levels in asthma patients. In the current study, no significant association between serum IgE levels and polymorphisms of TIM-3 were observed in asthma patients. This finding was in a line with the study of Chaee et al., who did not find an association between TIM-3 polymorphisms and total serum IgE levels in a Korean population.[12]

In conclusion, we demonstrated that TIM-3 -574 G>T and 4259 T>G polymorphisms may play an important role as genetic risk factors in the immunopathophysiology of asthma. However, it has not been yet investigated whether variations within the coding or non-coding regions of TIM-3 lead to increased TIM-3 protein expression, stability or its function**.** It is also important to find out whether or not these polymorphisms are replicable in other populations and to examine the roles of associated variants in TIM-3 expression and function. It would also be interesting to analyze the allele frequency of these polymorphisms with larger sample size in patients with autoimmune and allergic diseases.

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