

## Association analysis of –416 G&gt;C polymorphism of T-cell immunoglobulin and mucin domain-1 gene with asthma in Iran

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### Summary

TIM (T-cell immunoglobulin (Ig) and mucin domain)-1, one of the members of TIM family, expresses on Th2 cells and promotes the production of Th2 signature cytokines. This can increase a series of responses in these cells which could be one of the causes of asthma or asthma-related phenotypes. The aim of this study was to investigate whether a TIM-1 promoter single nucleotide polymorphism (SNP), –416 G>C, is associated with asthma in Iranian population. In this case-control study, existence of the –416 G>C polymorphism was assessed using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) in 300 patients with asthma (97 atopic, 203 nonatopic) and 309 healthy volunteers. Additionally, the relationship between these polymorphism genotypes and total serum IgE levels in this Iranian population was evaluated. We discovered a significant association between the –416 G>C polymorphism and atopic asthma susceptibility in the population, but this SNP showed no connection with nonatopic asthma ( $P < 0.05$ ). However, our results showed significant relation between this polymorphism and serum IgE level ( $P < 0.05$ ). Our results suggest that –416 G>C polymorphism in TIM-1 gene could

be a predisposing factor for atopic asthma in Iranian population, and CC genotype of this SNP can be associated with increased level of IgE in patients with asthma in the same population.

### Introduction

Asthma is a serious problem worldwide with an estimated 300 million affected persons (Masoli *et al.*, 2004; Bateman *et al.*, 2008). Asthma is a chronic inflammatory disorder of airways resulted from abnormal immune responses and induced by environmental factors in genetically susceptible individuals (Palmer & Cookson, 2000). When uncontrolled, could place severe limitations on life and occasionally could be fatal (Beasley, 2004; Bateman *et al.*, 2008). It is proved that CD4+ T helper (Th) 2 cells play crucial roles in the development of allergen-induced airway hyper-reactivity (AHR) and in the immunopathogenesis of asthma (Mosmann *et al.*, 2005). Activated Th2 cells produce a series of cytokines, including IL-4, IL-5 and IL-10 (Mosmann *et al.*, 1986; Cohn *et al.*, 1997). Thus, they make B cells to produce antibodies, especially IgE (Finkelman *et al.*, 1986; Stevens *et al.*, 1988; Kuhn *et al.*, 1991), which have the ability of causing asthma and other allergic diseases (Kuchroo *et al.*, 1995; Hofstra *et al.*, 1998).

Several human linkage analyses have implicated at least 15 genetic loci in human atopy and asthma, so far (Steinke *et al.*, 2003; De Souza *et al.*, 2005). One human genetic area which has received a great deal of attention is located on 5q23–35. Further analysis identified a family of similar genes, named TIM, consisting of three members (TIMs 1, 3 and 4) on human chromosome 5q33.2 (McIntire *et al.*, 2001; Kuchroo *et al.*, 2003). TIM genes encode surface glycoproteins with common structural features containing an extracellular immunoglobulin (Ig)-like domain, a mucin domain close to the membrane, a single transmembrane domain, and cytoplasmic regions of various lengths (Kuchroo *et al.*, 2003; McIntire *et al.*, 2004). On naive CD4+ T cells, TIM-1 is not expressed (McIntire *et al.*, 2001; Umetsu *et al.*, 2005), but becomes upregulated within hours of TCR engagement

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Received 16 October 2014; revised 10 March 2015; accepted 8 May 2015

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Financial resources: Shahrekord University of Medical Sciences

(McIntire *et al.*, 2001), and preferentially expressed on Th2 cells (Meyers *et al.*, 2005; Umetsu *et al.*, 2005). Hence, TIM-1 has become a good candidate susceptibility gene for Th2-related diseases, such as asthma (Li *et al.*, 2006). A number of different population studies have indicated that the genetic variations in TIM-1 might be associated with susceptibility to allergic diseases (Chae *et al.*, 2003; McIntire *et al.*, 2004; Gao *et al.*, 2005; Wu *et al.*, 2009).

However, no such correlation has been found in other populations (Noguchi *et al.*, 2003; Li *et al.*, 2006). One of these polymorphisms is  $-416\text{ G}>\text{C}$  which is found to be associated with development of allergic rhinitis in a Chinese Han population (Mou *et al.*, 2010), and with asthma susceptibility in the same population (Liu *et al.*, 2007), while is not correlated  $-416\text{ G}>\text{C}$  with asthma in Korean population (Chae *et al.*, 2003), but this SNP was associated with the risk of allergic rhinitis in Mazandaran, Iran (Hassannia *et al.*, 2011). To determine whether the  $-416\text{ G}>\text{C}$  SNP in TIM-1 promoter is linked with atopic and nonatopic asthma susceptibility in Iranian population, we planned a population-based analysis. We also evaluated the relationship between genotypes of this polymorphism and total serum IgE levels in atopic and nonatopic patients with asthma.

## Materials and methods

The study was conducted on 300 patients (203 nonatopic, 97 atopic) offered to Hajar and Amin Hospitals from Chaharmahal-o Bakhtiari and Isfahan Provinces, respectively. As controls, we enrolled 309 age-matched healthy individuals. Controls did not have any of the symptoms and personal or family history of allergic and respiratory diseases. All subjects with asthma were diagnosed according to the GINA (Daraei *et al.*, 2012).

This study is approved by the ethical committee of the Shahrekord University of Medical Sciences, and informed consent was obtained from all controls and patients, and this work was conformed to the provisions of the Declaration of Helsinki (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2566407/pdf/11357217.pdf>).

### Sample preparation and DNA extraction

Whole-blood samples supplied with EDTA were collected from all subjects. Genomic DNA was extracted from peripheral blood leucocytes using a Genomic DNA Isolation Kit (GENET BIO, Korea) according to the manufacturer's directions.

### PCR amplification and sequencing

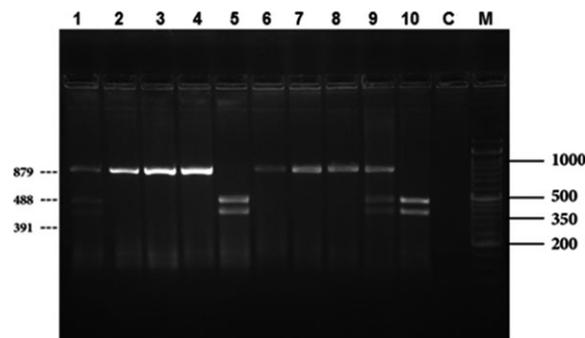
The genotype of  $-416\text{ G}>\text{C}$  polymorphism was identified with restriction fragment length polymorphism (RFLP) method. A 879-bp fragment of TIM1 promoter was amplified by PCR. The primers used to amplify

the fragment containing the SNP were forward 5'-AG TTGGTTGATTCATATGAGCC-3' and reverse 5'-GG AGGTGTAGTCTGAAGCATG-3'. PCR was carried out in a total volume of 25  $\mu\text{L}$  containing the PCR mixture of 150 ng genomic DNA, 2.5  $\mu\text{L}$  10 $\times$  PCR buffer, 1 U Taq DNA-polymerase, 200  $\mu\text{mol L}^{-1}$  dNTPs and 400  $\text{nmol L}^{-1}$  each primer. Amplification was performed with an initial denaturation step at 94  $^{\circ}\text{C}$  for 4 min, followed by 35 cycles of denaturation at 94  $^{\circ}\text{C}$  for 30 s, annealing at 63 for 45 s and extension at 72  $^{\circ}\text{C}$  for 45 s. A final extension time was performed at 72  $^{\circ}\text{C}$  for 5 min.

For genotyping the SNP, TaqI restriction enzyme (Fermentas, Germany) was applied. PCR products containing the SNP were incubated overnight at 65  $^{\circ}\text{C}$  with TaqI restriction enzyme according to the manufacturer's instructions. Then, restricted products were electrophoresed on 1.5% agarose gel and stained with DNA green viewer and visualized under illumination. TaqI cut the 879-bp PCR product into two fragments of 488- and 391-bp length. Fragments 488 bp and 391 bp indicated the presence of homozygous  $-416\text{GG}$  genotype, a single 879-bp band represented the presence of homozygous  $-416\text{CC}$  genotype, and three fragments of 879 bp, 488 bp and 391 bp displayed the presence of heterozygous  $-416\text{GC}$  (Fig. 1). The accuracy of the genotyping results was approved by direct sequencing of several randomly selected samples to Macrogen Company in Korea.

### Statistical analysis

The spss 16 software package (SPSS Company, Chicago, IL, USA) was used to carry out statistical analyses. The chi-square test was first applied to compare the frequency distribution of gender, and smoking status between cases and controls and two cases together groups. In addition, this test was used to compare the genotype distributions and the allele frequencies between tree groups. Association between this polymorphism and asthma was declared as odds ratios (OR) estimates with 95% confidence intervals (95%



**Figure 1.** Genotyping of TIM-1  $-416\text{ G}>\text{C}$  polymorphism by PCR-RFLP. Lane M: 50-bp DNA marker; Lane C: negative control, lanes 5, 10: homozygous GG, lanes 1, 9: heterozygous GC genotype, lanes 3–4, 6–8 homozygous CC genotype, Lane 2: PCR; product (undigested).

CI). Logistic regression analysis was used to predict the relation of the -416 G>C SNP with susceptibility to asthma. Furthermore, age, eosinophil percentage and total serum IgE levels between groups were compared by means of analysis of variance (ANOVA) and for comparison mean serum IgE levels and eosinophil percentage. Between genotype groups, ANOVA and analysis of covariance (ANCOVA) were used. *P*-value <0.05 was considered as significant in all of the tests.

## Results

In this study, we analysed -416 G>C SNP on TIM-1 gene from 300 patients with asthma (203 nonatopic and 97 atopic) and 309 healthy controls of an Iranian population. Selected characteristics of groups are shown in Table 1. As shown in Table 1, the tree groups are suitably matched in age and gender. A successful genotyping for the SNP was performed (Fig. 1) and verified by direct sequence analysis. The genotype distribution in all of the subjects was agreed with what expected by Hardy-Weinberg equilibrium.

In the atopic patients with asthma, the frequency of -416G/G was higher than C/G and CC genotypes and C/G genotype was the lowest frequency, and this pattern repeated in nonatopic patients with asthma. In addition, in atopic patients with asthma, frequencies of C and G alleles were 36% and 64% and these frequencies in the nonatopic asthma group were 14.8%, 21.2%, 64% and the frequencies of C and G alleles were 25% and 75%. The multivariate logistic

regression analysis was applied to investigate the association between the -416 G>C polymorphism genotypes and asthma. However, as indicated in Table 2, the CC genotype was associated with the risk of atopic asthma (crude OR = 2.465, 95%, CI = 1.379–4.405, *P* = 0.002) but none of the genotypic forms had a predisposing or protective impact on nonatopic asthma (*P* > 0.05). Furthermore as shown in Table 3, CC genotypic forms also had a predisposing effect on atopic asthma after odds ratio (OR) adjustment (adjusted OR = 0.477, 95%, CI = 0.214–1.063, *P* = 0.070) and GG, GC genotypes had no effect on relation to asthma in any way (Table 2). G allele showed protective effect against atopic asthma (crude OR = 0.401, 95%, CI = 0.225–0.715, *P* = 0.002) but not for nonatopic asthma (*P* = 0.302).

Furthermore, the difference of log<sub>10</sub> of total serum IgE level among genotype groups was investigated by one-way ANOVA test, and a significant difference in serum IgE level observed among these groups (Table 4). Bonferroni correction showed that this difference is related to CC genotype. In other words, CC genotype has a significant association with total serum IgE level. After adjusting for variables age, gender and smoke status, using ANCOVA test, this association survived (Table 5).

## Discussion

In developed countries, atopic diseases such as asthma are the main causes of morbidity, while their frequency

**Table 1.** Distributions of the frequency of the selected variables and characteristics of the studied population

	Atopic (n = 97)	Nonatopic (n = 203)	Control (n = 309)	<i>P</i> -value
Age (mean ± SD)	41.37 ± 15.07	43.43 ± 14.44	42.94 ± 14.68	0.518
Gender				<i>P</i> <sup>a</sup> = 0.433
Male	42 (43.3%)	79 (38.9%)	120 (38.8%)	<i>P</i> <sup>b</sup> = 0.985
Female	55 (56.7%)	124 (61.1%)	189 (61.2%)	<i>P</i> <sup>c</sup> = 0.469
Eosinophils (mean ± SE) 10 <sup>3</sup> per μL	0.31 ± 0.02	0.23 ± 0.02	0.09 ± 0.02	0.000*
Total serum IgE log <sub>10</sub> (mean ± SD)	2.49 ± 0.30	1.36 ± 0.42	0.77 ± 0.40	0.000*
Smoking				<i>P</i> <sup>a</sup> = 0.918
No	87 (89.7)	183 (90.1%)	276 (89.3%)	<i>P</i> <sup>b</sup> = 0.746
Yes	10 (10.3)	20 (9.9%)	33 (10.7%)	<i>P</i> <sup>c</sup> = 0.902

\**P* < 0.0001.

<sup>a</sup>Patients with atopic asthma versus controls; <sup>b</sup>Patients with nonatopic asthma versus controls; <sup>c</sup>Patients with atopic asthma versus nonatopic asthma.

**Table 2.** Genotype and allele frequencies of TIM-1 -416 G>C polymorphism

	Atopic (n/%)	Nonatopic (n/%)	Control (n/%)	Crude odds ratio (95% CI) <sup>a</sup>	<i>P</i> <sup>a</sup>	<i>P</i> <sup>b</sup>
Genotype						
CC	24 (24.7)	30 (14.8)	36 (11.7)	2.493 (1.399–4.442)	0.002	0.302
GG	52 (53.6)	130 (64)	188 (60.8)	0.744 (0.470–1.178)	0.206	0.466
GC	21 (21.6)	43 (21.2)	85 (27.5)	0.728 (0.423–1.254)	0.252	0.106

<sup>a</sup>Patients with atopic asthma versus controls; <sup>b</sup>Patients with nonatopic asthma versus controls.

**Table 3.** Adjusted odds ratios with 95% confidence interval (CI) in TIM-1 -416 G>C genotypes with adjustment for age, gender and smoke status

	Adjusted odds ratios	P <sup>a</sup>
Groups		
CC	2.465 (1.379–4.405)	0.002
GG	0.751 (0.473–1.192)	0.224
GC	0.723 (0.417–1.251)	0.246

<sup>a</sup>Patients with atopic asthma versus controls.

**Table 4.** Association between total serum immunoglobulin (Ig) E levels and Tim-1 -416 G>C polymorphism in patients with atopic asthma

	Total serum IgE, log10 values Mean ± SD	F	P-value
Genotype group			
CC	2.089 ± 0.685	11.59	0.000*
GG	1.619 ± 0.606		
GC	1.730 ± 0.647		

\*P < 0.0001.

has been increasing (Gergen & Weiss, 1992). Asthma is affected by environmental and genetic factors (Sears *et al.*, 1991). Several studies have investigated the association between different TIM-1 polymorphisms and the risk of various allergic diseases such as asthma, so far (Chae *et al.*, 2003; Gao *et al.*, 2005; Li *et al.*, 2006; Wu *et al.*, 2009; Mou *et al.*, 2010).

We observed a contradiction in studies about the association between a SNP in promoter of TIM-1 gene, -416 G>C, and asthma in Korean and Chinese population (Chae *et al.*, 2003; Liu *et al.*, 2007). On the other hand, this polymorphism was associated with allergic rhinitis; therefore, we suspected this SNP can be linked with the development of asthma in Iranian population. In current study, we investigated the frequency of -416 G>C polymorphism among Iranian population with asthma compared with normal controls, and for the first time, we examined the correlation of this SNP with atopic as well as nonatopic asthma. A significant association was found between the frequency of -416 G>C genotype with atopic asthma in the target population and CC genotype has predisposing impact on atopic asthma but none of the CC, GG, GC genotypes had effect on the risk of non atopic asthma. These effects also were observed before adjusting for confounding effect of variables age, sex and smoking; hence, we can see lack of the effect of environmental factors on connection between -416 G>C polymorphism and asthma. Moreover, because of the association between this polymorphism and atopic asthma (but not with nonatopic form of the disease), is likely that this SNP can contribute to asthma susceptibility through the pathways leading to allergy.

A few studies have investigated for possible connection between -416 G>C and asthma in different

**Table 5.** Analysis of covariance of the effect of -416 G>C on total serum immunoglobulin (Ig) E levels in patients with atopic asthma after adjustment for age and gender and smoke status

	Total serum IgE, log10 values Mean ± SD	F	P-value
Genotype group			
CC	2.089 ± 0.685	11.49	0.000*
GG	1.619 ± 0.606		
GC	1.730 ± 0.647		

\*P < 0.0001.

populations, but their findings were contradictory. Chae *et al.* have reported that there is no association between -416 G>C and asthma in Korean population (Chae *et al.*, 2003), which is not consistent with our results. However, our findings are consistent with the (Liu *et al.*, 2007) report which have shown an association between -416 G>C and asthma in Chinese Han population. Moreover, our results about asthma are compatible with the (Hassannia *et al.*, 2011) findings about allergic rhinitis in an Iranian population. This harmony with the last study can be due to the similar genetic background in Iranian population. Overall, these divergent findings in these four studies may be due to the difference in sample size, ethnic diversity with particular genetic background and interaction of distinct environmental factors which may affect the impact of this polymorphism as a predisposing factor. We were able to find any report about the relation of the SNP with atopic and not with nonatopic in order to compare with our results, and this report can be due to the relationship between TIM-1 and atopy.

We also for first time investigated the effect of this polymorphism on total serum IgE level of patients with asthma. The results showed that -416 G>C CC genotype is significantly correlated with increased total serum IgE level in atopic patient. Based on our survey in literatures, there was no similar report for comparison with this finding. All together, there is a significant association between -416 G>C and atopic asthma susceptibility in Iranian population, and this polymorphism could be an important factor for increasing the level of total IgE as one of the important players in asthma pathogenesis.

### Acknowledgements

This work was financially supported by Shahrekord University of Medical Sciences, Shahrekord, Iran. We wish to thank all the patients and normal individuals who kindly and voluntarily took part in this work.

### Conflict of Interest

We hereby declare that there is no conflict of interest among the authors of this work.

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