

A Functional Polymorphism in the TIM-1 Gene Is Associated with Asthma in a Chinese Han Population

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Key Words

TIM-1 • Promoter • Polymorphism • Single nucleotide polymorphism • Asthma

Abstract

Background: TIM-1, a member of the T cell immunoglobulin and mucin domain (TIM) gene family was implicated as an asthma susceptibility gene in previous studies. TIM-1 is selectively expressed on activated CD4⁺ T cells and its expression is sustained preferentially on T helper type 2 (Th2) cells, which suggests that TIM-1 is associated with T cell differentiation and the development of Th2-biased immune response. **Methods:** In order to evaluate the effects of the promoter polymorphism in the TIM-1 gene on asthma susceptibility in a Chinese Han population, 2 promoter polymorphisms, –416G>C and –1454G>A, from 2 alternative promoter regions, were genotyped in 409 unrelated asthma patients and 305 healthy controls by using polymerase chain reaction and restriction fragment length polymorphism. Also, we analyzed the functional significance of –416G>C using the luciferase reporter gene assay. **Results:** We found that –416G>C was associated with asthma susceptibility in our study population ($\chi^2 = 9.88$, $p = 0.002$, odds ratio 1.41, 95% confidence interval 1.14–1.75). No statistically significant difference in the distribution of genotype and

allele frequency of the –1454G>A site was observed. The –416G>C substitution increased the transcriptional activity of the TIM-1 gene. **Conclusion:** Our findings suggest that the –416G>C variation site in the human TIM-1 promoter region is associated with asthma susceptibility in a Chinese Han population.

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Introduction

Asthma is characterized by periodic obstruction of the airways and respiratory symptoms of wheeze, cough and breathlessness caused by acute and chronic bronchial inflammation. Asthma is thought to arise from an imbalance in T helper type 1 (Th1)-Th2 immune regulation, resulting in the driving of the development of Th2-biased immune responses and the overproduction of cytokines such as interleukin-4 (IL-4), IL-5, IL-9 and IL-13, which mediate allergic inflammation [1, 2]. Although environmental factors play important roles in the development of asthma, there is a strong genetic predisposition in asthma patients. Several asthma susceptibility genes have been identified through whole genome scan and candidate gene approaches [3, 4]. TIM-1, a member of the T cell immunoglobulin and mucin domain (TIM) gene family, is

Table 1. Clinical and demographic characteristics of patients and controls

	Patients	Controls
Subjects	409	305
Mean age, years	42.84 (14–73)	40.36 (18–68)
Sex, male/female	197/212	167/138
FVC, % predicted	67.7 ± 17.2	80.6 ± 15.4
FEV ₁ , % predicted	56.4 ± 16.8	85.9 ± 12.2
Changes in FEV ₁ by bronchodilator, %	28.2 ± 14.6	4.3 ± 3.9

Figures in parentheses are ranges. FVC = Forced vital capacity; FEV₁ = forced expiratory volume in 1 s.

one such gene that figures prominently in such investigation [5–8].

The TIM gene family is located on human chromosome 5q23-35, a region repeatedly linked to asthma [9–13]. It contains 3 members in humans (TIM-1, TIM-3 and TIM-4), all of which have recently been implicated in the control of T cell-mediated immune responses [5, 14]. TIM-1 is selectively expressed on activated CD4⁺ T cells and its expression was sustained preferentially on Th2 cells. The TIM-1 gene contains many polymorphisms, of which exon 4 variations have been intensively studied for evaluating the role in pathogenesis of allergic diseases, though the results were not all consistent [6, 8, 15–17]. Relative to most obvious effects caused by mutations in coding sequences, common diseases are more likely to be caused by subtle mechanisms to affect gene function. Such mechanisms may affect upstream sequences for the promotion and initiation of gene transcription and intronic sequences, which regulate both the timing and cellular or tissue expression of the gene and the factors that regulate splicing. Therefore, the identification and evaluation of the promoter polymorphism regulating the susceptibility to asthma is of great value. Chae et al. [18] identified several promoter polymorphisms in the TIM-1 gene using a Korean population and found that a promoter polymorphism, –1637A>G, was associated with susceptibility to rheumatoid arthritis. In order to determine the association of TIM promoter polymorphisms and asthma susceptibility in a Chinese Han population, 2 single nucleotide polymorphisms (SNPs; –416G>C and –1454G>A), from 2 alternative promoter regions, were genotyped in 409 unrelated asthma patients and 305 healthy controls. We found that –416G>C was associated with asthma susceptibility in our study population. Fur-

thermore, we analyzed the functional significance of –416G>C using the luciferase reporter gene assay, and found that the –416G>C substitution increased the transcriptional activity of the TIM-1 gene.

Materials and Methods

Study Subjects

We recruited 409 unrelated asthma patients (197 males, 212 females) from the Weifang Asthma Hospital and the Shandong Provincial Hospital. Asthma was diagnosed according to the criteria of the American Thoracic Society (1987). An extended medical history was recorded including occurrence and duration of wheezing symptoms. We excluded patients who had a history of other atopy-related diseases such as allergic rhinitis or atopic dermatitis. A total of 305 individuals (167 males, 138 females) were enrolled as controls when meeting the following criteria: (1) no symptoms or history of asthma or other pulmonary diseases; (2) no symptoms or history of atopy; (3) absence of first-degree relatives with a history of asthma or atopy. All subjects were Chinese Han who lived in the same area. Clinical and demographic characteristics of the patients and controls are given in table 1. Blood samples were obtained from all participants in this study after informed consent. Genomic DNA was extracted from leukocytes in peripheral blood by a standard method.

SNP Genotyping

Two SNPs in the TIM-1 promoter region, –1454G>A and –416G>C, were genotyped by polymerase chain reaction (PCR) and restriction fragment length polymorphism. The primer pair 5'-AATCATAGCCTCCAACTGC-3' and 5'-CCCACATGCGTTAAATCG-3' was used to amplify the –1454G>A polymorphism. The –416G>C polymorphism was amplified using the primers described by Chae et al. [18]. PCR was performed in a total volume of 25 µl solution containing 50 ng of genomic DNA, 200 µM of each dNTP (mixture of dATP, dTTP, dCTP, dGTP), 0.2 µM of each primer, 1.5 mM of MgCl₂, 10 mM of Tris-HCl (pH 8.3) and 1 U of Taq DNA polymerase. Cycling conditions included an initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 40 s, 55°C for 40 seconds, 72°C for 60 s, and a final extension at 72°C for 10 min. PCR products were digested with locus-specific restriction enzymes according to the manufacturer's instructions (Msp I for –1454G>A and Taq I for –416G>C). Digestion products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Luciferase Assay

Two luciferase reporter constructs, pGL3/–416G and pGL3/–416C, were generated by PCR combined with restriction digestion. Briefly, 325-bp fragments of the TIM-1 promoter corresponding to –621 to –288 were amplified from 2 individuals, whose genotypes were homozygous for –416G and –416C, using the primers 5'-CGAGCTCGAGACGGAGTCTTGCTCTG-3' and 5'-GGAAGATCTGACTCCTGGTCCTGATAC-3', which incorporate specific cleaving sequences for the restriction endonucleases Sac I and Bgl II, respectively. The amplified PCR products were digested with Sac I and Bgl II, and the cleaved product was cloned into promoterless pGL3/basic plasmid (Promega). Plasmid

Table 2. Genotype and allele frequencies of 2 promoter polymorphisms of the TIM-1 gene in asthma patients and controls

SNP position	Genotype/allele	Control ¹	Patient ¹	Odds ratio ²	p
-416	CC	117 (38.36)	131 (32.03)	1.00	0.001
	CG	155 (50.82)	192 (46.94)	1.106 (0.789–1.534)	
	GG	33 (10.82)	86 (21.03)	2.328 (1.451–3.733)	
	C	389 (63.77)	454 (55.5)	1.00	
	G	221 (36.23)	364 (44.5)	1.411 (1.138–1.750)	
-1454	GG	200 (65.57)	238 (58.19)	1.00	0.100
	GA	90 (29.51)	152 (37.16)	1.419 (1.029–1.957)	
	AA	15 (4.92)	19 (4.65)	1.064 (0.527–2.149)	
	G	490 (80.33)	628 (76.77)	1.00	
	A	120 (19.67)	190 (23.23)	1.235 (0.955–1.598)	

¹ Figures in parentheses are percentages. ² Figures in parentheses are 95% confidence intervals.

constructs were verified by direct sequencing analysis. Plasmid DNA was prepared using a plasmid mega kit (Omega) for further transfection.

Luciferase activity was measured using the Dual Luciferase reporter assay system (Promega). Briefly, COS-7 and HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37°C in an atmosphere of 5% CO₂; 5 × 10⁴ cells/well were inoculated and cultured in a 24-well plate for 24 h before transfection. The subconfluent cells were cotransfected with 1 µg of each construct (pGL3/basic, pGL3/-416G and pGL3/-416C) and 0.04 µg of pRL-TK vector, an internal control for transfection efficiency, using Lipofectamine 2000 transfection reagent (Invitrogen). After 36 h, cells were collected and lysed with passive lysis buffer (Promega) according to the manufacturer's instructions. Luciferase activity was measured using a Microplate luminometer and normalized using the activity of the *Renilla* luciferase. The relative luciferase activity of the TIM-1 reporter construct was represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was repeated 3 times and each sample was studied in triplicate.

Promoter Database Analysis

Allele-specific transcription factor binding sites were identified using the MatInspector software (<http://www.genomatix.de/products/index.html>).

Statistical Analysis

Allele carrier frequency was defined as the percentage of the individuals carrying the allele among the total number of individuals. Each SNP was evaluated for Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test. Asthma patients and healthy controls were compared with the use of case-control association analysis. Fisher's exact test was applied to analyze the comparison of the frequency of discrete variables between asthma patients and healthy controls. The allele and genotype frequencies, odds ratios with 95% confidence intervals, and significance values were analyzed using SPSS for Windows (version 13; SPSS Inc.,

Chicago, Ill., USA). Pair-wise linkage disequilibrium (LD) was measured using the online SHEsis software (<http://202.120.7.14/analysis/myAnalysis.php>) [19]. The luciferase reporter assay data were evaluated using a nonparametric Mann-Whitney test (two-tailed). Statistical analysis was performed with the SPSS software. $p < 0.05$ was considered to indicate statistical significance.

Results

The fact that 2 splice variants of human TIM-1 exist suggests that the TIM-1 gene contains 2 alternative promoters, located upstream of exon 1 and intron 1 relative to the full-length isoform. SNPs genotyped in this study were chosen from 2 alternative promoters: -416G>C from intron 1 of the full-length isoform; 3 other SNPs, -3949A>G, -1637A>G and -1454G>A, from upstream of exon 1. Genotyping results indicated that the 3 SNPs from the upstream region were in complete LD (data not shown). Therefore, only the SNP -1454G>A from this block was included in association analysis.

Genotype and allele frequencies of each SNP are shown in table 2. The distributions of 2 SNPs were in agreement with the Hardy-Weinberg equilibrium ($p > 0.05$) in both patients and normal controls. The genotype and allele frequencies of each SNP were compared between the patients and normal controls. The G allele of the SNP -416G>C was significantly more common among patients with asthma than among the controls ($p = 0.002$). This result suggested that the SNP -416G>C in the TIM-1 promoter region was significantly associated with asthma in our Chinese cohort ($p = 0.001$). There was an in-

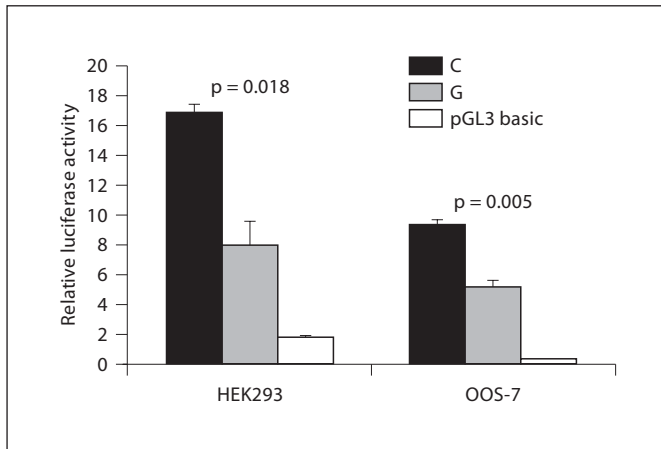


Fig. 1. Effect of -416G>C on the transcription activity of the human TIM-1 promoter. HEK293 or COS-7 cells were transiently cotransfected with pGL3/-416G or pGL3/-416C and pRL-TK vector. The relative luciferase activity of the TIM-1 reporter constructs is represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was conducted in triplicate for each sample and the results are expressed as means $\pm$ SD for 3 independent experiments. The p value was determined by the Student t test.

creased risk for asthma associated with a G allele (odds ratio = 1.41; 95% confidence interval 1.14–1.75). On the other hand, no significant difference of the genotype and allele frequencies for the SNP -1454G>A was detected between asthma patients and normal controls (table 2). We analyzed the LD among the 2 promoter SNPs and 2 previously analyzed exon 4 variants [17]. No significant LD was detected between 2 promoter SNPs and exon 4 variants, though 2 exon 4 variants were in strong LD (table 3).

The potential functional role of the -416G>C polymorphisms in transcription was explored using the MatInspector software. The G-to-C substitution at position -416 is predicted to generate a Yin and Yang 1 (YY1) transcription factor binding site (5'-CCAT-3'). To determine the effect of the G/C polymorphism at -416 on promoter activity, allele-specific constructs, pGL3/-416G and pGL3/-416C, were generated and transiently expressed in COS-7 and HEK293 cells. Luciferase activity in cell extracts was analyzed 36 h after transfection and standardized against internal control *Renilla* activity. Results indicated that the -416G construct showed a significant decrease in luciferase reporter activity compared with the -416C construct ($p = 0.005$ and $p = 0.018$ for COS-7 and HEK293 cells, respectively; fig. 1).

Table 3. LD coefficient ($|D'|$) among each polymorphism locus in the TIM-1 gene

Locus	-1454G>A	-416G>C	V1	V2
-1454G>A	–	0.403	0.341	0.150
-416G>C	–	–	0.129	0.131
V1	–	–	–	0.993

Data indicate the TIM-1 exon 4 5383–5397 ins/del and 5509–5511 ins/del CAA variant sites, respectively. V1 = 5383–5397 ins/del; V2 = 5509–5511 ins/del CAA.

Discussion

Asthma is thought to arise from an imbalance in Th1-Th2 immune regulation [1, 2]. Therefore, the genes involved in the regulation of CD4+ T cell differentiation might be the asthma susceptibility genes. TIM-1 is one of the recently identified genes implicated in the regulation of Th1 and Th2 immune responses. McIntire et al. [5] found that TIM-1 gene polymorphisms were strongly associated with Th1/Th2 differentiation and the development of airway reactivity and IL-4 production by T cells. They also found that the 6-amino-acid insertion in TIM-1 exon 4 appears to be related to hepatitis A virus (HAV) infection and atopy in a US population [6]. In contrast, Gao et al. [15] observed the association of the presumed 'protective' allele with asthma independent of HAV status in an African American population. No association was observed between the insertion allele and asthma in 3 Asian populations (Korean, Japanese and Chinese), in which the insertion allele is less common compared with the Caucasians and African Americans, though the endemicity for HAV infection varied among the 3 Asian populations [8, 16, 17].

The promoter polymorphisms might influence physiologic functions that are known to be relevant disease mechanisms in asthma through regulating the gene expression. To determine the role of the TIM-1 promoter polymorphisms in the pathogenesis of asthma, we selected 2 SNPs from 2 alternative promoter regions for an association study on a Chinese asthma population. The SNP -416G>C is located within intron 1 of the full-length isoform, while -1454G>A is upstream of exon 1. Moreover, the SNP -1454G>A is located in a 3-kb block containing several SNPs, including -1637A>G, which was associated with susceptibility to rheumatoid arthritis in a Korean population. Our results showed that the TIM-1

promoter -416G>C was significantly associated with risk of asthma. The frequency of allele G was significantly higher in asthmatic patients than in controls. To our knowledge, this is the first report of an association of the -416G>C polymorphism with asthma. In a Korean population, association studies of promoter polymorphisms, including -416G>C and -1454G>A, were conducted with a small group of asthma and 269 rheumatoid arthritis patients [7, 18]. Both studies failed to find any association of -416G>C and -1454G>A polymorphisms with asthma susceptibility and rheumatoid arthritis. Sample size might explain the conflicting results with asthma. Another explanation for the discordance with our study is unrecognized differences in environmental exposures.

We further examined the potential functional role of this promoter polymorphism, and found that the G-to-C substitution at -416 generated a YY1 transcriptional factor binding site. YY1 is a ubiquitously expressed zinc finger DNA binding protein. It can act as a transcriptional repressor or activator. YY1 binding sites have been reported in a wide variety of promoters and exhibit considerable diversity in their sequence, but contain a conserved 5'-CCAT-3' core flanked by variable regions [20]. Luciferase reporter assays confirmed that the -416G>C SNP altered the transcriptional activity of the TIM-1 gene. Unexpectedly, the -416C reporter construct was transcriptionally more active than the -416G construct in HEK293 and COS-7 cells. Thus, it appeared that -416G was associated with decreased transcriptional activity of the TIM-1 gene. The mechanism by which the -416G>C SNP affects TIM-1 expression may be explained by the potential differential transcription factor binding of YY1, since the -416G>C SNP is located at the core sequence of the YY1 binding site. Although additional studies are necessary to prove that YY1 plays a role in the transcription regulation of the TIM-1 gene, our data suggest that

loss of YY1 regulation with the -416GG genotype may decrease TIM-1 expression on the cell surface and lead to changes in T cell differentiation and function.

The pathophysiology of asthma is thought to arise from inappropriate or excess Th2-biased immune response to environmental antigens [1, 2]. It is also suggested that TIM-1 is selectively expressed in Th2 cells and directs Th2 cytokine production [14]. Surprisingly, our results have shown a significant increase in the -416G allele, the putative lower expression of TIM-1, among asthmatic patients compared with controls. Another TIM family member, TIM-3, was supposed to express on Th1 cells and direct Th1 differentiation. However, as variants were found in both TIM-1 and TIM-3 in congenic mice with airway hyperreactivity, and the promoter polymorphism (-574T>G) of the TIM-3 gene was associated with asthma and rheumatoid arthritis, the exact mechanisms by which the various TIM family members regulate T cell cytokine production remain to be determined.

To summarize, we analyzed the genetic association between the 2 promoter polymorphisms of TIM-1, -416G>C and -1454G>A, and asthma in a Chinese Han population. Our results showed a significant association between the -416G>C polymorphism and asthma. We further examined the functional role of this promoter polymorphism, and found that the single base substitution at -416 impaired a functional promoter element and affected the transcriptional activity of the TIM-1 gene.

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