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Original Article

Association between gene polymorphisms in TIM1, TSLP, IL18R1 and childhood asthma in Turkish population

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Abstract: Many immunologic and inflammatory mechanisms play a role in asthma etiology. The aim of this study was to investigate the susceptibility of asthma patients in the Turkish population with demonstrating genes for polymorphisms in TIM1, TSLP and IL18R1. All of the genomic DNA samples were isolated from blood samples according to a standard salting-out protocol. DNA samples were stored at -20°C until the genotype analysis was performed. rs3806933 (TSLP -847 C > T) and TIM1 -416G > C were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The rs3806933 (TSLP -847 C > T) was genotyped by PCR using our new primers and HphI restriction enzyme digestion. rs2287033 (IL18R1 c. 1270+150 A > G), rs3213733 (IL18R1 c. 626-196 G > T), and rs3771166 (IL18R1- c. 302+1694 C > T) were genotyped using SYBR green dye based real time PCR assay. Results: The allele frequencies of 5 SNPs in *TSLP*, *TIM-1,* and *IL18R1* genes were determined in 139 asthmatic patients and 126 healthy controls of in Turkish population. The investigated SNPs are as follows; rs3806933 (TSLP -847 C > T), TIM1 -416G > C, rs2287033 (IL18R1 c. 1270+150 A > G), rs3213733 (IL18R1 c. 626-196 G > T), and rs3771166 (IL18R1- c. 302+1694 C > T). Results suggest that IL18R1 c. 626-196 G > T (rs3213733) and TIM1 -416G > C are significantly associated with asthma in patients in Turkish population. Patients with AA genotypes of rs2287033 (IL18R1 c. 1270+150 A > G), have significantly less total serum IgE levels when compared with patients having GG or GA genotypes ($p < 0.012$; 381.77±239.46 vs 557.52±549.96, respectively). Conclusion: This study showed that IL18R1 c. 626 -196 G > T (rs3213733) and TIM1 -416G > C are significantly associated with asthma patients in Turkish population.

Keywords: Asthma, genetics, SNP polymorphism

Introduction

Asthma is a heterogeneous disease commonly presented in childhood. As a chronic inflammatory disease of the lungs, many immunologic and inflammatory mechanisms play a role in asthma etiology. Enviromental factors with genetic abnormalities are important in both development and severity of asthma. Genetic studies about asthma are aimed the determination of individuals who will be at risk for disease and to established the severity of disease in every case. Several studies have showed the contribution of specific genes and single nucleotide polymorphisms (SNPs) in the development of the asthmatic phenotype. About Twenty five genes associated with asthma have been duplicated in 6 or more samples [1].

The TIM (T-cell immunoglobulin domain and mucin domain) proteins are expressed on T lymphocytes and are related with the regulation of T helper (Th) cells, immune responses and some allergic diseases including asthma. The TIM gene family is encoded on chromosome 5q33.2 in humans. Three members of the family have been identified in humans: TIM-1, TIM-3, and TIM-4 [2]. Development of polymorphisms in TIM-1 and TIM-3 were associated with Th1-Th2 differentiation and airway hyperreactivity. TH1 phenotype cells stimulate cellular immune responses; On the other hand, Th2

phenotype cells favor antibody production specially IgE, related with allergic asthma.

IL-18, synthesized form Kupffer cells, macrophages, dendritic cells, T cells and B cells, is a cytokine that activate and differentiate Th2 cells which may turn increase the levels of IL-4, IL-13 and IgE [3, 4]. IL18R1 is responsible for IL-18 binding which triggers activator protein 1 and NF-kB activation that cause Th2 cell activation for cytokine production. Significant polymorphisms (rs2287033, rs3213733, rs3771 166) that may responsible for excess activation of this process were detected in asthma patients [5].

The thymic stromal lymphopoietin (TSLP), produced in skin cells and airway epithelium, is an IL-7–like cytokine TSLP that induces myeloid dendritic cells to stimulate the differentiation of naive CD4+ T cells to Th2 cells [6]. Together with this differentiation, chemokine production (IL-4, IL-5, IL-13) from Th2 cells increase and initiate response to allergens [7]. In the airway epithelium, the entry site for allergens, TSLP produced for trigger DC-mediated inflammation and expression of Th2-related chemokines. TSLP -847 C > T polymorphism increase the expression of TSLP mRNA and a risk factor for allergic rhinitis.

The aim of this study was to investigate the susceptibility of asthma patients in the Turkish population with demonstrating genes for polymorphisms in TIM1, TSLP and IL18R1.

Material and methods

Samples

All of the genomic DNA samples were isolated from blood samples according to a standard salting-out protocol. DNA samples were stored at -20°C until the genotype analysis was performed.

All of the samples had been genotyped for the SNPs rs3806933 (TSLP -847 C > T), TIM1 -416G > C, rs2287033 (IL18R1 c. 1270+150 A > G), rs3213733 (IL18R1 c. 626-196 G > T), and rs3771166 (IL18R1- c. 302+1694 C > T) using real time PCR or restriction enzyme digestion.

Genotyping

rs3806933 (TSLP -847 C > T) and TIM1 -416G > C were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Genotyping of TIM1 -416G > C was performed as descibed by Mou et al [8]. rs3806933 (TSLP -847 C > T) was genotyped by PCR using our new primers and HphI restriction enzyme digestion. rs2287033 (IL18R1 c. 1270+150 A > G), rs3213733 (IL18R1 c. 626-196 G > T), and rs3771166 (IL18R1- c. 302+1694 C > T) were genotyped using SYBR green dye based real time PCR assay.

Primer design

The real-time PCR assay was used to genotype the 3 SNPs in IL18R1 gene. Primers were designed according to the published sequences) using web-based software. We used the 'BLAST' program at http://www.ncbi.nlm.nih.gov/blast to determine the specificity of the primers. The primers used in this study were listed in **Table 1**.

Real-time PCR method

Real-time PCR was performed in a total volume of 20 µl containing 10 µl 2X LC480 SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany), 1 µl of each primer per reaction, 4 µl of the genomic DNA dilution (10 ng/µl), and distilled water. The PCR protocol on Light Cycler (LC480) (Roche Diagnostics, Mannheim, Germany) was as follows: an initial denaturation step (95°C for 7 min) followed by amplification and quantification steps repeated for 30-40 cycles (95°C for 10 sec, 60°C for 10 sec, 72°C for 20 sec, with a single fluorescence measurement at the end of the elongation step at 72°C), a melting curve program analyzed the data, and the reaction was terminated by cooling to 40°C.

Melting curves were constructed by lowering the temperatures to 65°C and later increasing the temperature by 0.2°C/s to 98°C while continuously measuring the change in fluorescence. Tm values were manually assigned from a plot generated by the Roche Light Cycler 480 instruments of the negative derivation of fluorescence versus temperature (-dF/dT) of the melting curve for amplification products measured at 530 nm.

Table 1. The primers used in this study

Primer
TSLP-847CT-F: 5'-AGCTCAGGACAGCATCGTCT-3'
TSLP-847CT-R: 5'-TAGGGGCAGGAACGATAAGA-3'
TIM1-416GC-F: 5'-AAT GAC CAA GAT TGA C-3'
TIM1-416GC-R: 5'-CTC ACT CTA GAC TGT CCT TCT-3'
IL18R1-rs2287033-A: 5'-GGAAGCTCATTAGTTTGTTTTCTCAATA-3'
IL18R1-rs2287033-G: 5'-AAGAAATAACCACGTAAGTCAACAATCTAC-3'
IL18R1-rs2287033-CF: 5'-AGTGAGGATTAACTAGTCCACACATCA-3'
IL18R1-rs2287033-CR: 5'-AAGTTATGCATATTTGAAAGGGATGTAG-3'
IL18R1-rs3771166-T: 5'-TCCTAAAAATTAAAGAATTACTGTTCTCAT-3'
IL18R1-rs3771166-C: 5'-ACAGACTTTACACCTGAAAATTTCACG-3'
IL18R1-rs3771166-CF: 5'-TAGGATTGGTTTATTAGATTTCTGTGTG-3'
IL18R1-rs3771166-CR: 5'-GGAGCATAGAAGATACAGACATATTCAA-3'
IL18R1-rs3213733-G: 5'-GCACCTTGTATCTGGTTTTCTCTCACTTAG-3'
IL18R1-rs3213733-T: 5'-AGTTGAATTGAGCAGAAGCTGCAGGTA-3'
IL18R1-rs3213733-CF: 5'-CATGGTTAAGCTTTGGTCCAAGAAGAAC-3'
IL18R1-rs3213733-CR: 5'-AGCTGAGGAGGACAGATGGAGAGAAT-3'

Statistical analysis

Allele carrier frequency was defined as the percentage of individuals carrying the allele of the total number of individuals. The χ^2 and Fisher exact tests were used to test for deviations from the Hardy-Weinberg equilibrium, and to compare the frequency of discrete variables between patients with asthma and healthy controls. SPSS 13.0 for Windows (SPSSInc., Chicago, Illinois, USA) was used to compare allele and genotype frequencies, calculate odds ratios with their 95% confidence intervals, perform multivariate logistic regression analysis, and analyze significance. Comparisons of the levels of total serum IgE for the different genotypes were performed using the analysis of variance test and Kruskal-Wallis Test. The haplotypes and haplotype frequencies were also calculated by logit loglineer analysis. To investigate the effect of each individual SNP on the asthma, binary logistic regression analysis was performed using Backward Stepwise (Likelihood Ratio) method. A P value of < 0.05 was considered statistically significant. For stringently significant analysis, Bonferroni correction was applied.

Results

In present study, the allele frequencies of 5 SNPs in *TSLP*, *TIM-1,* and *IL18R1* genes were determined in 139 asthmatic patients and 126 healthy controls of in Turkish population. The investigated SNPs are as follows; rs3806933 (TSLP -847 C > T), TIM1 -416G > C, rs2287033 (IL18R1 c. 1270+150 A > G), rs3213733 (IL18R1 c. 626-196 G > T), and rs3771166 (IL18R1- c. 302+1694 C > T).

When demographic data of the asthmatic patients and the healthy controls were compared, the mean ages was 13.12±2.53 in control and 12.47±2.19 in asthma group ($p > 0.05$). The ratio of gender in each group show a significant difference ($p < 0.01$) that the female gender was 66.7% in control and 46% in asthma group.

Genotype and allele frequencies of the SNPs are shown in (**Tables 2**-**4**). Genotyping analysis revealed that two SNPs (rs3213733 and TIM1 -416G > C) were statistically different between asthmatic patients and healthy controls. GG and GT genotype and G allele of the IL18R1 c. 626-196 G > T (rs3213733) were statistically different between asthmatic patients and healthy controls. There was an increased risk for asthma associated with G allele ($p < 0.028$, OR 1.676; 95% CI 1.055-2.662). IL18R1 c. 626-196 GG genotype was significantly more common in asthmatic children than in controls ($p < 0.011$).

The G allele of the SNP TIM1 -416G > C was significantly more common in asthmatic children ($p < 0.001$). TIM1 -416 CC genotype was significantly less common in patients than in controls ($p < 0.001$).

However, no significant difference of the genotype and allele frequencies for the SNP rs3806933 (TSLP -847 C > T), rs2287033 (IL18R1 c. 1270+150 A > G), and rs3771166 (IL18R1- c. 302+1694 C > T) were detected between asthmatic and control groups. These results suggest that IL18R1 c. 626-196 G > T (rs3213733) and TIM1 -416G > C are significantly associated with asthma in patients in Turkish population.

We next analyzed the haplotypes of all SNPs between patients and control group. Among the all the possible haplotypes, four of them having small p values are shown in **Table 5**. Statistically

Table 2. Genotype distrubition in controls and asthma patients

Position	Genotype	Control N (%)	Asthma N (%)	X^2	P
rs3213733 g > t	GG	75 (41.7)	105 (58.3)	7.193	< 0.027
	GT	45 (60.0)	30 (40.0)		< 0.027
	TT	3 (42.9)	4 (57.1)		> 0.05
rs2287033 a > g	AA	27 (21.4)	39 (28.1)	1.555	> 0.05
	AG	72 (57.1)	73 (52.5)		> 0.05
	GG	27 (21.4)	27 (19.4)		> 0.05
TIM1 -416 g > c	GG	24 (20.5)	39 (30.7)	13.547	> 0.05
	GC	60 (51.3)	75 (59.1)		> 0.05
	CC	33 (28.2)	13 (10.2)		< 0.001
TSLP -847 c > t	CC	45 (39.5)	39 (28.9)	3.120	> 0.05
	CT	63 (55.3)	87 (64.4)		> 0.05
	TT	6 (5.3)	9 (6.7)		> 0.05
rs3771166 c > t	CC	45 (40.5)	51 (46.8)	1.260	> 0.05
	CT	15 (13.5)	16 (14.7)		> 0.05
	TT	51 (45.9)	42 (38.5)		> 0.05

Table 3. Allele frequencies in controls and asthma patients

Position	Allele	Control N (%)	Asthma N (%)	X^2	P	OR (95% CI)
rs3213733 g > t	G	190 (79.2)	242 (86.4)	4.847	< 0.028	1.676 (1.055-2.662)
	T	50 (20.8)	38 (13.6)			
rs2287033 a > g	A	123 (50.0)	153 (54.6)	1.132	> 0.05	1.205 (0.855-1.698)
	G	123 (50.0)	127 (45.4)			
TIM1 -416 g > c	G	105 (45.7)	155 (60.3)	10.481	< 0.001	1.809 (1.262-2.549)
	C	125 (54.3)	102 (39.7)			
TSLP -847 c > t	C	149 (66.8)	166 (61.3)	1.638	> 0.05	0.785 (0.542-1.138)
	T	74 (33.2)	105 (38.7)			
rs3771166 c > t	C	104 (47.7)	118 (53.6)	1.540	> 0.05	1.268 (0.871-1.846)
	T	114 (52.3)	102 (46.4)			

significant differences were found in the haplotype frequency distribution of GGGCC between the asthmatic patients ($p < 0.036$, OR 1.80; 95% CI 1.285-2.522). Although not significant, the haplotypes GAGTT, GACCC, and GGCTT are more common in asthmatic patients (p=0.082, 0.096, and 0.111, respectively).

To further determine the association between investigated polymorphisms and asthma, we analyzed the association between the SNPs and total serum IgE levels. As illustrated in **Table 6**, patients with AA genotypes of rs2287033 (IL18R1 c. 1270+150 A > G), have significantly less total serum IgE levels when compared with patients having GG or GA genotypes ($p < 0.012$; 381.77±239.46 vs 557.52 ±549.96, respectively). There was no significant difference in total serum IgE levels between other asthmatic patients. Although it was not primary aim of this study, we also investigated the relation between clinical parameters and SNPs. Statistically significant difference was found with atopy and rs2287033 between the asthmatic patients ($p < 0.039$). There was positive correlation between atopy and AA genotype of rs2287033 (Linear-by-Linear Association: $p < 0.012$). The occurance of recurrent upper respitory infection was found to be significantly different between patients with CC genotype and other genotypes of rs3806933 (TSLP -847 C > T) ($p < 0.043$). We have found positive correlation between recurrent upper respitory infection and TT genotype of TSLP -847 C > T (Linear-by-Linear Association: $p < 0.013$).

Table 4. Genotypes in controls and asthma patients

Position	Genotype	Control N (%)	Asthma N (%)	χ^2	P	OR (95% CI)
rs3213733 g > t	GG	75 (61.0)	105 (75.5)	6.437	< 0.011	1.976 (1.163-3.358)
	GT+TT	48 (39.0)	34 (24.5)			
rs2287033 a > g	AA	27 (21.4)	39 (28.1)	1.553	> 0.05	1.430 (0.814-2.513)
	AG+GG	99 (78.6)	100 (71.9)			
TIM1 -416 g > c	GG+GC	84 (71.8)	114 (89.8)	12.853	< 0.001	1.809 (1.262-2.549)
	CC	33 (28.2)	13 (10.2)			
TSLP -847 c > t	CC	45 (39.5)	39 (28.9)	3.098	=0.078	0.623 (0.367-1.057)
	CT+TT	69 (60.5)	96 (71.1)			
rs3771166 c > t	CC	45 (40.5)	51 (46.8)	0.873		1.290 (0.756-2.200)
	CT+TT	66 (59.5)	58 (53.2)			

Table 5. Frequencies of haplotypes in controls and asthma patients

Haplotypes					Frequency		χ^2	P	OR (95% CI)
rs3213733	rs2287033	TIM1 -416	TSLP -847	rs3771166	Asthma	Control			
G	A	G	T	C-T	5-4	9-0	2.893	=0.082	2.801 (1.426-5.650)
G	A	C	C	C-T	12-1	12-9	2.763	=0.096	9.000 (0.982-82.496)
G	G	C	T	C-T	7-17	12-9	2.538	=0.111	3.236 (0.943-11.111)
G	G	G	C	C-T	7-15	0-12	4.536	< 0.036	1.800 (1.285-2.522)

Discussion

Asthma is one of the most common chronic respiratory disease diagnosed in children. Several susceptibility genes and environmental factors are responsible for asthma development. 60-80% of asthma is caused by genetic factors, and 20-40% of disease is caused by environmental factors. Most recent data indicate that 300 million asthma patients have been reported and prevalence of disease is rising especially in developing countries.

Asthma pathophysiology is critically based on the excess amount of IL-4, IL-5 and IL-13 production from Th2 cells. Based on this data, research for gene polymorphisms that regulate the amount of Th2 cells and chemokines which synthesized by Th2 cells were decided. Association of TIM4 -1419G > A polymorphism with asthma risk was defined for the first time. IL18 as a cytokine has immunoregulatory functions by binding to IL18 receptor (IL18R) which is a member of the *IL1* receptor superfamily. IL18R is an important regulator of Th-1 cells located on chromosome 2q12. It consists of IL18R1 and IL18R2. Th-1 cytokine production is triggered by the binding of IL-18 to IL-18R.

There are sudies that showed strong associations with SNPs located in *IL18R1* and asthma and atopic diseases [4, 9, 10]. Higa S et al showed that the frequency of the 105A/C polymorphism of the IL18 gene was significantly higher in asthmatic patients than in controls [11]. Zhu G et al [4], studied the association between polymorphisms in IL18R1 and asthma. They examined seven SNPs in 294, 342 and 100 families from Denmark, United Kingdom and Norway. The relation of SNP's with asthma, atopic dermatitis and bronchial hyper-reactivity (BHR) were evaluated. Imada Y et al [12] conducted a study on 288 asthmatics and 1032 control patients. SNP's on Interleukin (IL18R1) (rs3213733) were associated with asthma in the 1st and 2nd stage analyses. Wu H [5] et al studied 11 SNPs in IL1RL1 and 9 SNPs in IL18R1. Eleven of the 20 SNPs were associated with asthma in the Mexican population. IL18R1 (rs3213733) was also one of the polymorphism associated with asthma in this study.

TIM1, member of T-cell immunoglobulin and mucin domain protein family, expressed on all activated Th1 and Th2 cells and located as a membrane receptor. TIM4, receptor on dentritic cells and macrophages, ligates TIM1 to drive the proliferation and expansion of T cells, and stimulates TIM1 production. Thus TIM1 and TIM4 has significant role on asthma pathogen-

Table 6. The relationship between IG E levels and SNP's

Position	Genotype/Allele	N	IG-E	P
rs3213733 g > t	G	230	522.34±507.88	> 0.05
	T	34	400.88±257.80	
	GG	100	540.43±533.42	> 0.05
	GT	28	392.71±276.20	> 0.05
	TT	3	439.00±175.31	> 0.05
	GG	100	540.43±533.42	> 0.05
	GT+TT	31	397.19±266.27	> 0.05
rs2287033 a > g	A	146	480.33±465.54	> 0.05
	G	118	539.32±506.76	> 0.05
	AA	38	381.77±239.46	> 0.05
	AG	68	589.09±616.926	> 0.05
	GG	25	471.64±293.98	> 0.05
	AA	38	381.77±239.46	< 0.012
	AG+GG	93	557.52±549.96	< 0.012
TIM1 -416 g > c	G	145	544.97±575.72	=0.067
	C	99	437.06±328.94	=0.067
	GG	35	633.11±736.10	=0.065
	GC	72	433.82±277.18	=0.065
	CC	13	363.31±179.28	=0.065
	GG+GC	107	499.01±483.82	> 0.05
	CC	13	363.31±179.28	> 0.05
TSLP -847 c > t	C	156	504.58±448.17	> 0.05
	T	100	497.27±551.86	> 0.05
	CC	36	570.53±452.60	> 0.05
	CT	82	446.11±445.34	> 0.05
	TT	9	730.33±897.70	> 0.05
	CC	36	570.53±452.60	> 0.05
	CT+TT	91	474.22±507.35	> 0.05
rs3771166 c > t	C	116	483.75±494.26	> 0.05
	T	94	530.24±512.42	> 0.05
	CC	50	436.61±392.70	> 0.05
	CT	16	778.38±870.63	> 0.05
	TT	38	478.08±398.02	> 0.05
	CC	50	436.61±392.70	> 0.05
	CT+TT	54	567.06±586.75	> 0.05

esis and triggers Th2 cell development and expression of proinflamatory cytokines from Th2 cells. Studies showed that TIM1 -416G > C polymorphism increase the production of TIM1 and associated with asthma, allergic rhinitis and IgE levels [8, 13, 14]. TIM4 -1419 G > A promoter polymorphism is studied for the first time in Chinese population and this polymorphism observed significantly more common in asthmatic children. Moreover, -1419A allele correlation with asthma is confirmed by genotype frequencies. This polymorphism may change transcription regulation site and upregulate the transcription of TIM4.

In this study, we designed to select 139 asthma cases and 126 controls from Vakıf Gureba Training And Research Hospital, in order to check their genes for polymorphisms in TIM1, TIM4 and TSLP and 3 polymorphisms in IL18R1. We aimed the comparing and contrasting genetic data of Turkish population and other ethnic origins, and adds new statistical data to help explain the effect of these polymorphisms.

Genotyping analysis revealed that two SNPs (rs3213733 and TIM1 -416G > C) were statistically different between asthmatic patients and healthy controls However, no significant difference of the genotype and allele frequencies for the SNP rs3806933 (TSLP -847 C > T), rs2287033 (IL18R1 c. 1270 + 150 A > G), and rs3771166 (IL18R1- c. 302 + 1694 C > T) were detected between asthmatic and control groups.

In conclusion, this study showed that IL18R1 c. 626-196 G > T (rs3213733) and TIM1 -416G > C are significantly associated with asthma in patients in Turkish population.

Disclosure of conflict of interest

None.

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