Association of the STAT-6 rs324011 (C2892T) variant but not rs324015 (G2964A), with atopic asthma in a Saudi Arabian population

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ARTICLE INFO

ABSTRACT

Background: The signal transducer and activator of transcription 6 (STAT6) transduces signals in response to IL-4 and IL-13 cytokine stimulations, resulting in many cell-specific responses. Some common STAT6 SNPs were associated with asthma predisposition and/or IgE levels, although discrepancies have also been reported.

Objective: To determine whether STAT6 rs324011 and rs324015 polymorphisms are associated with atopic asthma in Saudi Arabian patients.

Methods: A total of 536 Saudi individuals aged 11–70 years old (230 atopic asthmatics, 306 healthy subjects) were recruited. DNA was purified from peripheral blood and genotyping for rs324011 and rs324015 polymorphisms was performed by PCR amplification, followed by cycle sequencing of the purified PCR fragments using BigDye chain terminator and capillary electrophoresis.

Results: By the contrast of alleles tests, no significant differences between asthma and healthy groups were detected for both variants (rs324011: $X^2 = 0.25$, Pearson's P-value = 0.617; rs324015: $X^2 = 0.068$, Pearson's $P = 0.814$).When testing for genotypes, rs324011 homozygous T/T genotype was significantly associated with asthma, when the Recessive model is considered (T/T vs. C/C + C/T) (adjusted, OR = 2.49, 95% CI = 1.18–5.25, Pearson's $P = 0.014^*$, Yates' $P = 0.022^*$). In contrast, rs324015 variant was not significantly associated with asthma.

Conclusions: Rs324011 homozygous T/T genotype was significantly associated with asthma risk whereas rs324015 genotypes were not in the Saudi population.

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1. Introduction

The signal transducer and activator of transcription factor 6 (STAT6) is member of a family of 7 related proteins, which transmit extracellular signals perceived by a cell-surface receptor into the nucleus, where it drives the expression of target genes [1]. STAT6 is ubiquitously expressed, and is a critical mediator of IL-4 and IL-13 cytokine signaling, leading to many cell-specific responses, including the differentiation of Th2 lymphocytes and immunoglobulin E (IgE) production [2]. Through IL-4 and IL-13 activation, STAT6 also promotes allergic disorders such as food allergy, atopic asthma and atopic dermatitis [3]. Asthma is a chronic airway inflammatory disorder characterized by bronchial hyper-responsiveness and intermittent narrowing of small airways, with subsequent reversible airflow obstruction; STAT6 plays a prominent role in its pathogenesis, which is characterized by infiltration of predominantly Th2 cells, eosinophils, monocytes/macrophages, mast cells, and elevated IL-4, IL-13 and IgE levels in the airways [4]. STAT6's prominent role in asthma was demonstrated in knock-out STAT6 mice, which displayed reduction of Th2-related features and suppression of IgE [5,6]. Hence, STAT6 is

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currently a therapeutic target to treat patients with allergic disorders, such as atopic asthma [7]. Also, considerable effort has been invested to determine whether single nucleotide polymorphisms (SNP) in the STAT6 gene enhance predisposition to asthma and to other atopic disorders. This gene is highly polymorphic, and thousands of SNPs within both coding and non-coding regions have been collected in the dbSNP database [8]. However, most, if not all missense variants in the STAT6 coding region are low or rare frequent [9]. Given the predominance of the 'common disease-common variant' model, genome-wide association studies are biased toward common variants [10]; likewise, most association studies of asthma with STAT6 polymorphism have focused on the possible effects of common variants, which are non-coding. For instance, rs324011 (C2892T) SNP is found in the second intron; interestingly, the C → T substitution seemingly creates an additional putative binding site for Nuclear Factor κB (NF-κB), therefore, enhancing NF-κB-mediated STAT6 gene transcription with concomitant elevated STAT6-mediated IgE production [11]. Another relatively frequent mutation in Caucasians, the rs324015 (G2964A), is located in the 3′ untranslated region (UTR) of exon 23, but its functional significance has not been determined unambiguously [3,12,13]. Although the biological significance of most STAT6's non-coding SNPs remains unknown, it is speculated that they could alter critical processing mechanisms, such as pre-mRNA splicing, mRNA stability, or transcription/translation activation [3,13,14]. Genome-wide association studies have linked asthma and atopy to 12q chromosome region, which is where STAT6 gene is located [15]. Furthermore, several case-control studies confirmed a significant association of STAT6 variants with asthma phenotypes and/or elevated IgE: in German Caucasians, both rs324015 and rs324011 SNPs were in weak association with total IgE levels [12,16]; in a Japanese population, rs324015 was strongly associated with mild atopic asthma [3]; in British Caucasians, rs324015 SNP was linked with increased risk of nut allergy [17]; in Czech children, rs324011 was associated with elevated IgE levels [18]. However, in other studies the association of STAT6 rs324015 SNP (G2964A) and rs324011 (C2892T) SNPs with asthma symptoms or serum total IgE levels could not be confirmed: in Caucasians from UK and Italy, the rs324011 was not significantly associated with asthma [19]; in a Finnish population, no significant association of both SNPs with asthma or high IgE serum levels was observed [13]; in a Chinese population, rs324015 was not significantly associated with asthma [20]; in a British population, atopic asthma or IgE levels were not correlated with the rs324015 variant [3]; in Czech asthmatic children, the rs324011 was not associated with asthma [18]. Epidemiological studies suggested that many factors, including age, gender, multiple genes, allergens and other environmental agents, influence asthma pathogenesis. Possibly, the large variation observed in responses to medical treatment, and discrepant results in genetic association studies, could reflect differences in the genetic background of ethnic groups; therefore, further studies are needed to replicate previous findings in different ethnic groups. In Saudi Arabia, about 11% of the population suffers from asthma, yet few studies have been performed to evaluate and identify susceptibility loci in possible association with this disorder [21]. The objective of this case-control study was to determine whether STAT6 rs324015 (G2964A) and rs324011 (C2892T) polymorphisms are linked with atopic asthma in a Saudi Arabian population.

2. Materials and methods

2.1. Patients and controls

A total of 536 individuals including atopic asthma patients and healthy subjects (aged 11–70 years old) were recruited for blood donation at the King Khalid University Hospital (KKUH), Riyadh, Saudi Arabia. Of these, 230 were atopic asthmatics diagnosed by a physician and classified according to criteria described by the American Thoracic Society (ATC) [22]. According to ATC criteria, the proportions of intermitent, mild, moderate and severe asthmatics were 28%, 25%, 20% and 27%, respectively. About 81% of patients presented asthma symptoms during daytime, with an average of 3.4 days per week; 62% of patients reported night-time awakenings due to asthma exacerbations (average of 2.8 nights/week); exercise-induced symptoms were reported by 64% of patients; allergic rhinitis was most frequently reported (58% of patients), and other allergies were eczema (25%), food allergy (21%), and medicines (aspirin, penicillin, etc.) (<1%). All patients were taking 1–2 daily doses of inhaled corticosteroids; 94% and 14% were taking a short-acting or a long-acting beta2 agonist, respectively, of which 38% used it when symptoms appeared; 6% were taking systemic steroids and 29% of patients were taking a leukotriene receptor antagonist. Healthy subjects ($n = 306$) of equivalent age range were also recruited, non-smokers nor having cronic lung diseases. To the best of our knowledge, all recruited asthmatic and healthy subjects were unrelated. This study was approved by the Ethical Committee of the KKUH.

2.2. Genotyping

DNA was purified from whole blood Peripheral blood, using a QIAamp DNA Blood Midi Kit (QIAGEN), following the manufacturer's instructions. A total of 536 subjects (45% were females and 55% males) categorized into 230 asthmatics and 306 healthy controls, were successfully genotyped for rs324011 (C2892T) SNP. Regarding rs324015 (G2964A), 462 individuals were successfully genotyped (204 asthmatics and 258 healthy subjects; 51% females and 49% males). The difference in numbers was due to either DNA unavailable for some samples, or a technical difficulty in obtaining sequencing data. The two gene regions encompassing the variants were amplified by PCR using Taq Polymerase (Quick-Load *Taq* 2X Master Mix; New England BioLabs) and specific primers; primers for rs324011: 5′-TCT ACT GCC CTG TCT CCC TTT (right) and 5′-GAC ATG ATC TGG GAC TTG GAG (left); primers for rs324015: 5′-CCA ATC CAC TCC TTC CTT TCT (right) and 5′-CCC TAA CCT GTG CTC TTA CCC (left). The purified PCR segments were then cycle sequenced using the BigDye Terminator kit, and separated by capillary electrophoresis (Sequencer model 3730XL; Applied Biosystems).

2.3. Statistical analysis

Statistical analysis of both allele and genotype frequencies were performed as described previously [21]. For allele frequencies, Chi square tests with 1 degree of freedom (df = 1) were performed, and Chi square values reported are corrected with Yates's continuity correction test; significance reported is based on Pearson's test, and Yates' Continuity correction P values also shown. For genotype frequencies non-adjusted, Pearson's Chi square tests with df = 2 were performed, with significance based on Pearson's, and Yates' correction P values also shown, for comparative purpose to the readers. All P values reported are two-tailed, with P-values < 0.05 were considered statistically significant. The dominant and the recessive models were tested for each SNP individually, with frequencies adjusted by gender; a Bonferroni correction was applied, with the new threshold of significance set at $P < 0.025$. Hardy–Weinberg equilibrium tests, linkage disequilibrium, analysis of interaction with covariate gender and best fit genetic model test (dominant, recessive) were performed using SNPStats tool [23]. Odds ratios (OR) and confidence intervals (95% CI) were calculated using an interactive calculation tool [24].

3. Results

3.1. Allele and genotype frequencies

Frequencies for both rs324011 (C2892T) and rs324015 (G2964A) polymorphism were distributed in Hardy–Weinberg equilibrium. The frequencies of the minor alleles, T and A (rs324011 and rs324015, respectively), were very similar in both asthmatic and healthy groups, or 22% in average (Table 1). In the contrast of allele frequencies, Pearson's Chi square test revealed no significant differences between asthmatic and healthy groups, for either SNPs. Regarding genotype frequencies, a borderline significant difference was observed between asthmatic and healthy groups for rs324011 variant only (X^2 = 6.0; Pearson's P-value = 0.049*), suggesting a possible link with asthma predisposition; however, the more stringent Yates' test suggests no significance (Table 1). A strong linkage disequilibrium was observed (D' = 0.685), with the haplotype C/C + G/G (rs324011 and rs324015, respectively) being the most common, or 68% in average for both asthmatic and healthy groups; however, the frequency of co-inherited homozygous T/T and A/A genotypes (of rs324011 and rs324015, respectively) was rare, both in asthmatics (<1%) and in healthy subjects (3%) (P-value = 0.06).

3.2. Association of SNP with asthma

We analyzed the genotype frequencies by testing the dominant and the recessive models, with frequencies adjusted for gender (Table 2). In the case of rs324011 (C2892T) SNP, when the recessive model is considered (T/T vs. C/C + C/T), the odds for asthmatics were over 2-fold of being carriers of the T/T homozygous genotype, suggesting that two copies of the minor allele, T, are necessary to increase the risk of asthma (OR = 2.49, 95% CI = 1.18–5.25, Pearson's P = 0.014*, Yates P = 0.022*). This association remained significant after Bonferroni correction of alpha significance threshold (α = 0.025) (Table 2). For comparative purpose, in the dominant model, odds for C/T + T/T genotypes combined were not significantly associated with asthma (OR = 0.96; 95% CI = 0.66–1.39; Pearson's P = 0.84). Concerning rs324015 (G2964A) polymorphism, odd ratios showed no significant association of genotype frequencies with asthma (Table 2).

3.3. Interaction gender with SNP genotypes

We analyzed the possible interaction of either SNP with covariate gender, by testing the same genetic models of inheritance. In the case of rs324011 (C2892T) SNP, with genders nested within genotypes, no significant differences between females and males were observed in genotype frequencies, all models considered (Table 3). In the case of rs324015 (G2964A) SNP, with G/A and A/A genotypes combined in the dominant model, our analysis revealed that females had significantly lower odds of being asthmatics than males (OR = 0.32, 95% CI = 0.17–0.61; Pearson's P < 0.001*); in addition, when G/G and G/A genotypes are combined in the recessive model, odds were smaller for females of being associated with asthma, relative to males (OR = 0.47, 95% CI = 0.32–0.70; Pearson's P < 0.001*) (Table 3).

4. Discussion

Asthma is an important inflammatory disorder, afflicting about 10% of the world population, and its incidence continues to increase. In Saudi Arabia, the number of asthmatics is significantly

Table 1
Frequencies and odd ratios for allele and genotypes.

SNP	Allele or genotype	Asthmatics N (%)	Healthy N (%)	% Deviations[†]		Chi square value[§]	OR (95% CI)	P value[*]
				Asthma	Healthy			
rs324011	C	361 (0.78)	488 (0.80)	−0.8	0.6	0.25 (0.182)	1.08 (0.80–1.45)	0.617 (0.669)
	T	99 (0.22)	124 (0.20)	2.9	−2.2			
	C/C	150 (0.65)	194 (0.63)	1.6	−1.2	6.0 (5.06)		0.049* (0.079)
	C/T	61 (0.27)	100 (0.33)	−11.7	8.8			
	T/T	19 (0.08)	12 (0.04)	42.8	−32.2			
rs324015	G	334 (0.78)	399 (0.77)	0.4	−0.3	0.068 (0.033)	0.96 (0.70–1.30)	0.814 (0.855)
	A	94 (0.22)	117 (0.23)	−1.2	1.0			
	G/G	137 (0.64)	156 (0.60)	3.1	−2.6	2.234 (1.722)		0.327 (0.422)
	G/A	60 (0.28)	87 (0.34)	−10.0	8.3			
	A/A	17 (0.08)	15 (0.06)	17.2	−14.2			

[§] Pearson's Chi square values are shown, with Yate's Chi square values within parenthesis for comparative purpose.
[*] Significance is set at *P*-value < 0.05. Two-tailed *P*-values are based on Pearson's test, and *P* values based on Yates's continuity correction are shown within parenthesis.
[†] Percentage deviation is calculated as [((observed − expected)/expected) × 100]. For odds ratios calculations, the minor alleles T and A for rs324011 and rs324015, respectively, were considered as 'exposure'.

Table 2
Association of STAT6 rs324011 and rs324015 SNPs with asthma.

SNP	Model	Genotypes	Asthmatics N (%)	Healthy N (%)	OR (95% CI)[†]	P-values[*]	AIC/BIC[§]
rs324011	Dominant	C/C	131 (64.2)	192 (63.4)	1.00	0.845 (0.920)	687/700
		C/T + T/T	73 (35.8)	111 (36.6)	0.96 (0.66–1.39)		
	Recessive	C/C + C/T	185 (90.7)	291 (96)	1.00	0.014* (0.022*)	681/694
		T/T	19 (9.3)	12 (4)	2.49 (1.18–5.25)		
rs324015	Dominant	G/G	121 (63.7)	155 (60.3)	1.00	0.468 (0.531)	601/614
		G/A + A/A	69 (36.3)	102 (39.7)	0.87 (0.58–1.27)		
	Recessive	G/G + G/A	177 (93.2)	242 (94.2)	1.00	0.664 (0.812)	602/614
		A/A	13 (6.8)	15 (5.8)	1.18 (0.55–2.55)		

[*] Statistical significance threshold was set at Bonferroni corrected *P*-value < 0.025. Two-tailed *P*-values are based on Pearson's Chi square test; *P*-values based on Yates' Continuity correction are shown within parenthesis.
[†] OR and 95% CI were calculated for the Dominant or Recessive genetic models adjusted for gender.
[§] AIC and BIC, Akaike and Bayesian information criterion.

Table 3
Interaction analysis of STAT6 rs324011 and rs324015 SNPs with covariate gender, with genders nested within genotypes.

SNP	Genetic model	Genotype	Gender	Frequencies N		OR (95% CI)[†]	P values[*]	Interaction P-value
				Asthmatics	Healthy			
rs324011	Dominant	C/C	F	56	92	1.00	0.360 (0.422)	0.90
			M	75	100	0.81 (0.52–1.27)		
		C/T + T/T	F	29	51	1.00	0.405 (0.496)	
			M	44	60	0.78 (0.41–1.47)		
	Recessive	C/C + C/T	F	75	136	1.00	0.187 (0.218)	0.67
			M	110	155	0.78 (0.54–1.13)		
		T/T	F	10	7	1.00	0.755 (0.949) 0.08	
			M	9	5	0.79 (0.18–3.41)		
rs324015	Dominant	G/G	F	54	88	1.00	0.045 (0.060)	0.11
			M	67	67	0.61 (0.38–0.99)		
		G/A + A/A	F	23	62	1.00	0.0004[*] (0.0007[*])	
			M	46	40	0.32 (0.17–0.61)		
	Recessive	G/G + G/A	F	72	143	1.00	0.0002[*] (0.0003[*])	0.61
			M	105	99	0.47 (0.32–0.70)		
		A/A	F	5	7	1.00	0.662 (0.956)	
			M	8	8	0.71 (0.16–3.23)		

[*] Two-tailed P-values are based on Pearson's Chi square test; P values based on Yates' test of Continuity are also shown within parenthesis. Statistical significance is set at P-value < 0.05; with Bonferroni correction, the threshold of significance is P < 0.025.
[†] Odd ratios (OR) and confidence intervals (95% CI) were calculated with females considered as 'exposure'.

high [21]; yet, few genetic studies in the Saudi Arabian population in relation to asthma susceptibility have been done so far. With a relatively elevated frequency of consanguineous marriages [25] and distinctively hot, dusty environment, it brings up an interesting opportunity to advance our understanding of genetic-environment interactions, and how this related with asthma incidence; in fact, mutant alleles of IL-17A, IL-17F and ADAM33 genes were found significantly associated with asthma in Saudi patients [21,26]. Our knowledge on asthma pathogenesis has improved considerably in the last decade. Recognition of the critically important role of Th2 cytokines IL-4 and IL-13 and their signaling pathway mediated by STAT6 on asthma pathogenesis and allergic disorders, has raised interest for the development of therapeutic antagonists targeting these factors [2,7]. However, success in this line of research will likely depend on our better understanding of the role or impact of genetic polymorphisms, both at the individual and the ethnic levels. Considerable discrepancies among gene association studies have been reported, which probably reflect the influence of different ethnic backgrounds; for instance, variant rs324015 (G2964A) was not significantly linked with atopy or with asthma phenotype in a British population; yet, in a Japanese population, this variant was strongly associated with mild atopic asthma, but not with severe atopic asthma [3].

Our study revealed a significant association of the rs324011 variant with asthma when the recessive model of inheritance (T/T versus C/C + C/T) was considered: odd ratios for asthmatics were over 2-fold of being homozygous T/T carriers, relative to healthy controls, suggesting that two copies of the minor allele, T, are necessary to increase the risk of asthma. In support of this, an additive model (T/T versus C/C) confirmed higher risk of asthma for homozygous T/T carriers than for C/C carriers (OR = 2.32; 95% CI = 1.09–4.94; Pearson's P = 0.026[*], Yates' P = 0.041[*]). In contrast, no significance was observed when the dominant model (C/T + T/T vs. C/C) was tested. Thus, these results are in good agreement with other reports indicating an association of rs324011 variant with asthma risk [9,13,15]. In particular, a meta-analysis pointed out that homozygous T/T carriers had 26% higher risk of asthma than other genotype carriers (C/C + C/T) [27].

In contrast, no significant association of rs324015 variant with asthma was found, as shown by testing these genetic models. This result is in agreement with some reports which found no association with asthma susceptibility, in both Caucasian and Asian populations, including British, Finish and Chinese [3,13,19,20,27]. However, a few studies did find a significant link of this variant

with mild atopic asthma risk in a Japanese population [3], with nut allergy in British Caucasians [17], and with IgE levels in Germans [12,16]. Thus, these contrasting results underline the need of further studies to confirm or to rule out a possible association of rs324015 variant with asthma.

In the contrast of alleles, no significant association of rs324015 and rs324011 variants with asthma risk in Saudi Arabian asthmatics was found. A similar conclusion was reported in a recent meta-analysis of six studies involving different ethnicities [27]. These two minor alleles, T and A, are common in the Saudi population (both 22% in average), although their frequencies are lower relative to other ethnic groups, including Finnish (rs324011, 41% in average; rs324015, 25% average) [13], British Caucasians (rs324015, A allele is 26% in average) [17], German Caucasians (rs324011, T allele is 37–44%) [12,16], and Japanese (rs324015, A allele is 67% in average) [3]. However, low allele frequencies may not be the only reason for the lack of significant difference between asthmatics and healthy groups in our study; in fact, several studies in which no significant association with atopic asthma was found, the reported frequencies of minor alleles were higher than in the Saudi population [12,13,18,20,21,27], suggesting that other factors, including environment, could be involved.

Asthma is currently considered a multigenic disease, with genes in interaction with environmental factors. For instance, it is well known that gender is an important risk factor influencing asthma susceptibility, with women being more prone to asthma than males [28]. However, no significant gender-to-rs324011 interaction was observed, suggesting that males and females had a similar risk of asthma relative to that variant. On the other hand, even if a significant gender-to-rs324015 interaction was observed, this result is meaningless given that none of its genotypes was associated with asthma. This could also be deduced indirectly from the linkage disequilibrium test; in particular, the co-inheritance of the two minor alleles as double homozygous, T/T and A/A (for rs324011 and rs324015, respectively), was very rare in asthmatics (<1%). Besides, none of the haplotypes considered were significantly associated with asthma (P > 0.05), thus supporting the observation that the rs324015 SNP is not associated with asthma in this sample population.

A possible mechanism by which the rs324011 variant could influence the risk of asthma has been proposed: it is thought that the rs324011 intronic C2892T substitution creates a new putative NF-κB site, which is used by NF-kB and contributes to increased STAT6 expression. Although the experimental evidence is

compelling [11], it has not been replicated nor confirmed by others so far. In addition, *in vitro* experiments aiming to characterize single genetic variants not always confirmed a role or function, which, *in vivo*, could be tissue- or cell-specific [11,29,30], thus highlighting the need of further functional studies to demonstrate whether these STAT6 variants play a role or impact on signal transduction and how it could increase the risk of asthma and allergy predisposition. The challenge remains to replicate previous findings in different ethnic populations, and to understand the gene-environment interactions that contribute to the heterogeneity in the responses of patients to asthma medications.

Acknowledgments

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