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Association of *STAT6 rs324011* Gene Polymorphism with Susceptibility of Atopic Bronchial Asthma in Egyptian Children

Rehab M El-Gohary[1*], Ayman A Wagih[1], Hala E Hamouda[1], Salwa M El-Melegy[1] and Mohamed A Rowisha[2]

[1]Faculty of Medicine, Department of Medical Biochemistry, Tanta University, Egypt

[2]Faculty of Medicine, Department of Pediatrics, Tanta University, Egypt

[*]**Corresponding author:** Rehab M Elgohary, Faculty of Medicine, Department of Medical Biochemistry, Tanta University, Egypt, Tel: +201062980773; E-mail: rehab.elgohary@med.tanta.edu.eg

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Abstract

Background: Bronchial asthma is a common disease with multiple determinants that include genetic variation. Signal transducer and activator of transcription 6 (STAT6) is a central molecule in the signal transduction pathway used by interleukin-4 (IL-4) in immunoglobulin E (IgE) isotype switching. Study Objective was to investigate whether single nucleotide polymorphism (SNP) in *STAT6* is associated with atopic asthma susceptibility in Egyptian children and to correlate the effect of *STAT6* gene polymorphism on IL-4 and total IgE levels.

Methods and Findings: the study was conducted on 60 children with atopic asthma in addition, 30 healthy children as controls. The enzyme-linked immunosorbent assay technique (ELISA) was used to measure serum IL-4 and total IgE levels. Genotyping pattern for the *STAT6 rs324011* polymorphism was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

Discussion: Our data revealed that *rs324011* TT genotype was significantly associated with increased susceptibility of developing bronchial asthma (OR=5.55, 95% CI=1.43-21.44) in comparison to *rs324011* CC and CT genotypes. Meanwhile, in bronchial asthma group, serum IL-4 and total IgE levels were higher in patients with CT and TT genotypes compared to those with CC genotype.

Conclusion: The T allele variant of *STAT6 rs324011* polymorphism may be associated with increased susceptibility of the development of atopic bronchial asthma in Egyptian children; however, further studies are needed to verify these preliminary results.

Keywords: Bronchial asthma; Interleukin-4; IgE; STAT6; Gene polymorphism

Introduction

Bronchial asthma is the most common chronic disease among children in the world. It affects up to 300 million people worldwide [1]. The prevalence among Egyptian children aged 3–15 years was 8.2% [2]. Bronchial asthma is influenced by genetic and environmental factors. The disease is defined by the presence of airway hyperreactivity, mucus overproduction, and chronic eosinophilic inflammation [3].

Asthma is often characterized by enhanced total serum IgE level upon the exposure to allergens, which is known as an atopy. Many family studies, through genome-wide linkage studies, confirmed the involvement of genetic predisposition in the development of atopy in asthmatic patients [4]. The elevated IgE production in asthmatic patients results in promotion of acute hypersensitivity responses, chronic eosinophil-predominant allergic inflammation with T helper-2 (Th2) cells cytokine production [5].

IL-4 serves as an essential proinflammatory cytokine in immune regulation mediated by activated T helper cells (Th) and facilitates immunoglobulin E isotype switching in B cells, growth, and differentiation of B cells and monocytes. As an important signal molecule, IL-4 can exacerbate airway inflammation through modulating eosinophils, lymphocytes, and air epithelial cells that play an important role in the pathogenesis of asthma. Moreover, IL-4 plays a pivotal role in phenotypic changes of bronchial asthma, such as airway hyperresponsiveness, eosinophil infiltration, and mucus overproduction [6]. Also STAT6, the signaling molecule from JAK/STAT pathway, activated by IL-4 and IL-13 cytokines, plays an important role in IgE production and allergic airway inflammation [5].

Signal transducer and activator of transcription (STAT) is a family of latent cytoplasmic transcription factors activated by specific cytokine receptor-mediated signal transducers. Selective activation of *STAT6* by IL-4 or IL-13 involves phosphorylation, dimerization and then translocation into the nucleus, where it binds to specific DNA elements TTC ($N_{3/4}$) GAA within the promoter region, activating gene transcription [7].

STAT6 gene, 19 kb with 23 exons and located on chromosome 12q13.3–q14.1, is highly polymorphic and thousands of SNPs within both coding and non-coding regions have been collected in the dbSNP database. *STAT6 rs324011* (2892C/T) SNP is found in the second intron; interestingly, the C→T substitution 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 [8].

Therefore, the present study was undertaken to investigate the relationship between *STAT6* gene polymorphism and the susceptibility to atopic asthma in Egyptian children and to correlate the effect of *STAT6* gene polymorphism on IL-4 and total IgE levels.

Methods

Study groups

The study included 90 Egyptian children ; Sixty children with atopic asthma diagnosed according to the criteria of the Global Initiative for Asthma (GINA), who were presented to the Outpatient Clinic of the Pediatric Department, Tanta University Hospital in the period from June 2015 to March 2016 and 30 healthy children with matched as a control group. All asthma patients were clinically stable. None of the participants received antihistamine, systemic or topical corticosteroids in 3 weeks prior to clinical evaluation. Subjects excluded from this study were those with heart failure, renal failure, liver failure, diabetes mellitus, malignancy and tuberculosis. Ethical approval of this study was obtained from the Ethical Committee of Faculty of Medicine Tanta University (Approval code 2820/10/14) and written informed consents were obtained from all children parents.

All participants underwent a full clinical examination, skin prick testing, routine and specific laboratory investigations.

Total immunoglobulin E (IgE) and Prick test assays

Atopy was diagnosed by a positive skin prick test (wheal diameter ≥3 mm) to at least one of 12 common aeroallergens and by measurement of the total IgE level. Positive values were taken to be ≥200 IU/ml. The control subjects had no history of asthma or other allergic diseases, negative skin prick tests and normal total IgE values.

Blood sample collection

Five milliliter venous blood were taken from every investigated subject under complete aseptic conditions and divided into 2 portions: one mL of whole blood was collected in sterile ethylenediaminetetraacetic acid (EDTA)-containing tubes for DNA extraction and eosinophil counts and stored at –80°C for further PCR amplification, and the remainder was left for 30 to 60 minutes for spontaneous clotting at room temperature before being centrifuged at 3000 rpm for 10 minutes. Serum samples were kept frozen at –20°C for determination of total IgE and IL-4 levels. Eosinophil counts were determined according to Burrows et al. [9].

Assay of serum total IgE and IL-4 levels

Serum total IgE was measured using Immunospec® human IgE sandwich enzyme linked immunosorbent assay (ELISA) kit (Cat# 10602, Vivantis International, Egypt). Serum IL-4 was estimated using Bioseps® human interleukin-4 ELISA kit (Cat# BEK1103, Vivantis International, Egypt). Both assays were carried out according to the manufacturers' instructions. Using the mean absorbance value for each sample, the corresponding concentration of IgE and IL-4 were determined from the standard curve.

Genomic DNA extraction

Genomic DNA was extracted from EDTA whole blood using a spin column method according to the manufacturer's instructions. (Cat# GF-BT-100, Vivantis Inc. USA). The quality of DNA was assessed by measuring absorbance at both 260 nm and at 280 nm. DNA was stored at − 20 °C till the time of use.

Genotyping for the *STAT6 rs324011* polymorphism

Polymerase Chain Reaction-restriction fragment length polymorphism (PCR-RFLP) was performed using the thermal cycler (Whatman Biometra, Germany) to determine the different genotypes of STAT6-2892C/T. The specific primers were: Fwd: 5'-CTCTTCCCACCCCTGTGTCTATC3'; and Rev: 5'-TCCCATAGATAGCCCTCCTAGGTAC-3' [10]. Briefly, the PCR mixture (total, 25 µl) containing 5 µL DNA, 3 µl of each primer, 12.5 µL 2X Taq master mix (containing Taq DNA Polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR) and 1.5 µL nuclease free water. The PCR mixture was incubated at 95˚C for 5 minutes, then 35 cycles of 95˚C for 30 seconds minute to denature, 72˚C for 40 seconds to anneal the primers and 72˚C for 8 minute to elongate the strand PCR, amplification kit was obtained from ((SIGMA Chemical Co, St. Louis, Missouri, USA).

Subsequently, the amplified products were digested by the BshNI (Cat# RE1106, Vivantis Inc. USA) restriction enzyme according to the manufacturer's instructions. Digested products were separated on 3% agarose gel stained with ethidium bromide. The gel was visualized under a UV transilluminator with a 10 bp marker ladder (Ultra Low Range DNA Ladder) (34-501 bp) served as reference for DNA fragment size (Cat# NM2415,Vivantis Inc. CA, USA) and photographed. The STAT6-2892C/T alleles were differentiated by BshNI restriction digestion (C allele, a single fragment of 132 bp; T allele, fragments of 107 and 25bp (obscure band)).

Statistical Analysis

The results for continuous variables were expressed as means ± SD and t-test and one-way analysis of variance (ANOVA) were used for their analysis. Genotype frequencies of *STAT6* genotypes in atopic asthmatic children and controls

were tested for Hardy–Weinberg equilibrium, and any deviation between the observed and expected frequencies was tested for significance using the Chi-squared test. The statistical significances of differences in frequencies of variants between the groups were tested using the χ2 test. In addition, the odds ratios (OR) and 95% confidence intervals (CIs) were calculated as a measure of the association of the polymorphic genotypes (CT and TT) with atopic asthma susceptibility. A difference was considered significant at P-values less than 0.05. All statistical calculations were performed using the computer program SPSS (Statistical Package for the Social Science) version 17 for Microsoft Windows.

Results

Demographic and biochemical data of the two studied groups is illustrated **Table 1.** There were no statistically significant differences among the two groups as regard age, sex distribution or exposure to passive smoking (p>0.05).

Table 1 Demographic and biochemical data of healthy control subjects and atopic asthmatic children.

Variables	Healthy control subjects (n=30)	Atopic asthmatic children (n=60)	Statistical test	
			t/ X2	P-value
Age	9.30 ± 1.82	8.90 ± 1.68	t= 1.03	>0.05
Sex (Males/Females)	11/19	26/34	X2=0.37	>0.05
Duration of Asthma (years)	NA	5.9 ± 1.5	-	-
Passive smoking%				
Negative	0.6	0.54	X2=0.73	>0.05
Positive	0.4	0.46		
Total IgE (IU/ml)	25.25 ± 19.21	670.08 ± 136.56	t= 25.67	<0.0001*
Eosinophils %	1.43 ± 0.74	8.53 ± 1.63	t= 22.67	<0.0001*
Data are presented as mean ± SD or percentages. P was calculated by Chi-square or unpaired t -test. P was considered significant at <0.05.				

The disease duration of the asthmatic group was 5.9 ± 1.5 years. Total serum IgE and peripheral blood esinophils % were significantly higher in atopic asthmatic children as compared to control subjects (p<0.0001). Also Interlukin-4 serum levels were significantly increased in atopic asthmatic children as compared to the healthy control subjects (p<0.0001) as depicted in **Figure 1**.

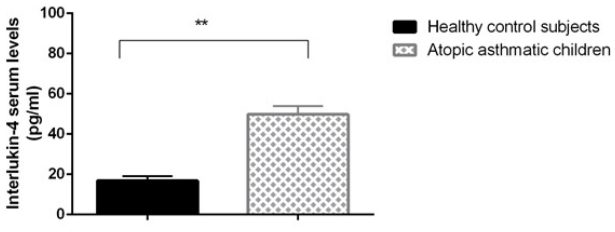

Figure 1 Interlukin-4 serum levels in healthy control subjects and atopic asthmatic children. ** P was considered significant at <0.05.

As regard the Genotypes of the STAT6-2892C/T polymorphism in the present study, three genotypes for STAT6-2892C/T polymorphism were recognized by genotyping; (CC) homozygous CC genotype with one band at 132 bp due to absence of the BshNI restriction site; homozygous TT genotype with 2 bands at 25 bp and 107 bp due to presence of the BshNI restriction site; and heterozygous CT genotype with 3bands at 25 bp, 107 bp, and 132 bp (**Figure 2**).

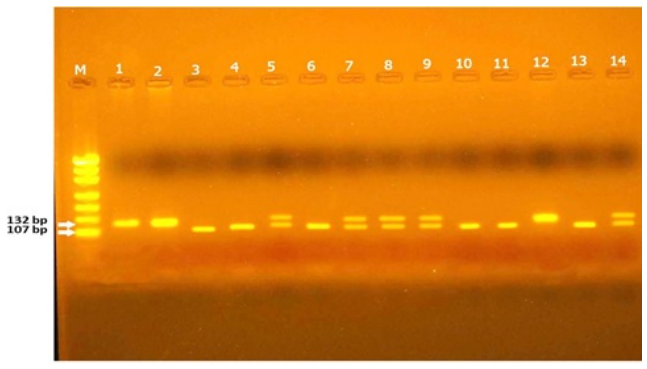

Figure 2 BshNI digestion for the STAT6-2892C/T polymorphism. M indicates 10 bp DNA molecular size marker. Lanes 1, 2& 12 are homozygous wild (CC) with single band at 132 pb; Lanes 3, 4, 6, 10, 11 & 13 are homozygous polymorphic (TT) with two bands at 107 & 25 pb (obscure band), lanes 5, 7, 8, 9 & 14 are hetrozygous (CT) with three bands at 132, 107 & 25 pb (obscure band).

The distribution of genotypes of STAT6-2892C/T polymorphism in controls were consistent with expectations under the Hardy–Weinberg equilibrium (X2 = 2.93, p= 0.089). In contrast, the observed genotype distributions deviated from

those predicted by the Hardy–Weinberg equilibrium in atopic asthmatic children (P<0.05) as shown in **Table 2**.

The genotypic and allelic frequencies of STAT6-2892C/T gene polymorphisms in healthy control subjects and atopic asthmatic children are demonstrated in **Table 3**. The CC genotype and the C allele were taken as references. The distribution of the STAT6-2892C/T genotypes and alleles differed significantly between atopic asthmatic children and healthy control subjects (X2 = 7.25, 10.58) respectively, p<0.05.

Table 2 Hardy Weinberg equilibrium for STAT6 (2892 C/T) gene polymorphisms in healthy control subjects and atopic asthmatic children.

Groups/Genotypes	Observed frequency			Expected frequency			X2	P-value
	CC	CT	TT	CC	CT	TT		
Healthy Control Subjects	20	7	3	18.4	10.2	1.4	2.93	>0.05
Atopic Asthmatic Children	24	16	20	17.05	29.87	13.08	10.86	<0.05

The frequencies of the CT genotype were insignificantly higher in atopic asthmatic children (26.7%) than healthy control subjects (23.3%), where the odds ratios were 1.90 (95% CI: 0.65 -5.54), p>0.05. However, TT genotype frequency was significantly higher in atopic asthmatic children (33.3%) than healthy control subjects (10%), where its odds ratios were 5.55 (95% CI: 1.43-21.44), p<0.05. Meanwhile, the frequency of T allele was significantly higher in atopic asthmatic children (46.7%) than healthy control subjects (21.67%), where odds ratios were 3.16 (95% CI: 1.55-6.44), p<0.05. For further assessment the association of different STAT6-2892C/T genotypes with serum IL-4 levels, atopic asthmatic children were subdivided according to C/T genotype where higher serum IL-4 levels were significantly associated with the minor T allele carriers (CT and TT genotypes) compared to the CC genotype, p<0.0001.

Table 3 Genotype and allele frequencies of STAT6 (2892 C/T) gene polymorphism in healthy control subjects and atopic asthmatic children.

Geno-typing	Healthy control subjects (n=30)		Atopic asthmatic children (n=60)		OR 95% CI		P value	Chi-Square	
	No.	Frequency	No.	Frequency				X2	P-value
CC	20	66.70%	24	40%	1		-		
					Reference				
CT	7	23.30%	16	26.70%	1.9	0.65-5.54	0.23	7.25	0.02*
TT	3	10%	20	33.30%	5.55	1.43-21.44	0.01*		
C allele	47	78.33%	64	53.30%	1		-	10.58	0.001*
					Reference				
T allele	13	21.67%	56	46.70%	3.16	1.55-6.44	0.001*		
*P was considered significant at <0.05. OR: odds ratio; CI: confidence interval									

Table 4 Association between the distribution of STAT6 (2892 C/T) genotypes and the studied parameters in Atopic asthmatic children.

Parameters	STAT6 (2892 C/T) genotypes			One-way ANOVA	
	CC (n=24)	CT (n=16)	TT (n=20)	F	P-value
IL-4 (pg/ml)	45.97 ± 2.19	49.55 ± 1.91#	52.67 ± 3.59#	34.28	<0.0001*
Total IgE (IU/ml)	576.70 ± 41.31	672.45 ± 74.11#	735.77 ± 164.15#	12.71	<0.0001*
Eosinophils %	8.00 ± 1.33	7.50 ± 2.16	9.35 ± 1.21# ¶	7.08	0.0018*
Data presented as means ± SD, *P was considered significant at <0.05, # significane Vs CC genotype, ¶ significane Vs CT genotype					

Moreover, the atopic asthmatic children with TT genotype had significantly higher IL-4 serum levels as compared to those with CT genotype, p<0.0001. Likewise, total serum IgE and peripheral blood esinophils% were significantly higher in atopic asthmatic children with CT and TT genotypes compared to the CC genotype (p<0.01) with being significantly higher in atopic asthmatic children with TT as compared to those with CT genotype, p<0.01. These data are summarized in **Table 4.**

Discussion

Asthma is one of the most common chronic diseases in childhood. For a long time, asthma has been known to cluster in families, and family studies were the first to suggest that the disease was genetically inherited [11]. One of the most replicated asthma candidate gene is signal transducer and activator of transcription 6 (STAT6), a regulator of IgE class switching. *STAT6* may promote the development of asthma by increasing serum IgE level and facilitating airway hyperresponsivness [12]. So the present study aimed to investigate whether single nucleotide polymorphism (SNP) in *STAT6* is associated with atopic asthma susceptibility in Egyptian children and to correlate the effect of *STAT6* gene polymorphism on IL-4 and total IgE levels.

Our data pointed out that the TT variant genotype of the *STAT6* polymorphism *rs324011* were significantly associated with an increased risk of atopic bronchial asthma with reference to the CC and CT genotypes, indicating a potentially crucial role for this gene in the pathogenesis of asthma. These data are consistent with the findings of Wu et al. [10] who reported that *STAT6 rs324011* may increase an individual's susceptibility to atopic asthma and contribute to the pathogenesis of asthma in middle China. Li et al. [13] performed a meta-analysis for evaluation of the association between *rs324011* polymorphism in the *STAT6* gene and the risk of bronchial asthma and their results draw the conclusion that the association between *STAT6 rs324011* polymorphism and bronchial asthma was definite and determined. They also documented that *rs324011* homozygous TT genotype contributed to the etiology or susceptibility to asthma. Moreover, Al-Muhsen et al. [14] found a significant association of *rs324011* homozygous TT genotype with the risk of atopic bronchial asthma whereas rs324015 genotypes were not in the Saudi population. Well in line, haplotype analysis of Godava et al. [15] revealed that *rs324011*, rs3024974 and rs4559 polymorphisms in *STAT6* were associated with total IgE elevation and increased risk of atopic bronchial asthma. *STAT6* intron 2 acts as a silencer regulatory element. The polymorphic T allele at *rs324011* increases *STAT6* promoter activity and gene expression of *STAT6* compared with the wild-type C allele. These effects correlate with the creation of a novel, T-allele-specific binding site for the transcription factor NF-κB in T cells [8].

However, some previous findings are inconsistent with our results, Pykalainen et al. [16] documented no significant association of *rs324011* to asthma or serum total IgE levels in the Finnish population. Moreover, the study Kavalar et al. [12] and Berenguer et al. [17] did not support such an association between the *STAT6 rs324011* and the risk of atopic bronchial asthma in Slovenian children and the Madeira island population, respectively.

The diversity of these studies has pointed out that environmental influences and epigenetic mechanisms as methylation and histone acetylation could pose important modifying factors [18]. Furthermore, gene-gene and gene-environment interactions are involved in the pathogenesis of asthma and more often present in patients with asthma or those with increased total IgE levels [19].

Moreover, the current study focused on the roles of IL-4 in the pathogenesis of bronchial asthma in children. Our results confirmed significantly higher serum IL-4 levels in atopic asthmatic children as compared to controls. Supporting our findings, Bao et al. [20], Shi et al. [21], Wu et al, [22] and Nesi et al. [23] revealed a significant increase in IL-4 in ovalbumin-induced asthmatic mice compared to the control. Our finding may also be reinforced in the light of observations of Antczak et al. [5] who found higher mRNA level of IL-4 and IL-13 genes in asthmatic patients when compared to controls. But they did not find statistically significant differences between controlled and uncontrolled bronchial asthma, even the highest IL-4 expression was recognized in uncontrolled atopic asthma patients. This observation may suggest that IL-4 expression may play a significant role in the course of asthma but it is not a sufficient inducer for asthma.

Interleukin-4 can exacerbate airway inflammation through modulating eosinophils, lymphocytes, and air epithelial cells that play an important role in the pathogenesis of bronchial asthma. Also, IL-4 is reported to play a pivotal role in phenotypic or functional changes of bronchial asthma, such as airway hyperresponsiveness, eosinophil infiltration, and mucus overproduction [24].

Emerging evidences have shown that IL-4 messenger ribonucleic acid and protein were highly expressed in airway mucosa of asthmatic patients; abnormal increase of IgE, promoted by IL-4, has been proved to be one of the pathogenesis of asthma, which indicates that IL-4 may induce asthma indirectly [25]. It has been revealed that, IL-4 is significantly overexpressed during the acute and plateau stage of asthma, indicating that immune dysfunction participates in the development and progression of asthma [26].

Immunoglobulin E is a key downstream biomarker of Th2 inflammation. IgE binds to the high-affinity IgE receptor FcεRI on mast cells and basophils, and antigen cross linking of IgE leads to degranulation and release of inflammatory mediators, including histamine, prostaglandins, and pro-inflammatory cytokines (IL-4, IL-5, IL-13). In the lower airways, this activity may result in eosinophilia, increased mucus production, and enhanced smooth muscle contractility [27].

In the present study, total plasma IgE levels were found to be significantly higher in atopic asthmatic children as compared to controls. Our findings are in accordance with the results of Ghadah [28], Noureldin et al. [29] and Alasandagutti et al. [30] who reported higher total IgE levels in atopic asthmatic group than normal controls. It has been revealed

that the production of IgE from B cells and the differentiation, maturation, migration and survival of eosinophils are induced by the increased pro-inflammatory cytokines, including interleukins IL-4, IL-5 and IL-13 [31].

Many types of cells are involved in the pathophysiology of asthma. The contribution of mast cells, lymphocytes, and eosinophils in the induction and effector phase has been well established [32]. Interestingly, eosinophils are the first cells recruited to the site of the allergic reaction and they play important roles in the pathogenesis of asthma in the way of releasing inflammatory mediators, including radical oxygen species, cysteinyl leukotrienes (CysLTs), major basic protein (MBP) and cytokines, that linked the presence of eosinophils to the persistence of inflammatory infiltrate, tissue damage and remodeling [33].

In the current study, as regards peripheral blood eosinophils, they were found to be significantly higher in atopic asthmatic children as compared to controls. In agreement with our findings Ma et al. [34], Wu et al. [22] and Malmström et al. [35] who found significantly higher peripheral eosinophil counts in asthmatic group. Also, Chen et al. [27] reported that there were statistically significant increase in the numbers of total leukocytes, lymphocytes, monocytes, eosinophils and basophils in the asthmatic group in comparison to control one.

Interestingly, IL-4 prolonges the survival of lung effector memory T cells, enhances Th2 cytokine production and also increases the expression of chemokine ligand 11 (CCL11), IL-5, IL-9, and IL-13 in ILC2 [36]. These Th2 cytokines and chemokines may attract eosinophils into the airway lumen and prolong survival of basophils and eosinophils. In addition, the upregulation of vascular cell adhesion molecule-1 (VCAM-1) via IL-4 promotes eosinophil recruitment to the target tissues [37].

Additionally, we revealed statistically significant positive correlations between the *rs324011* TT and CT genotypes, and total level of serum IgE. It could indicate the role of this polymorphism in atopic bronchial asthma pathogenesis. Although the single polymorphism *rs324011* is located in the second intron of the *STAT6* gene, it may have a functional importance. The second Intron was identified as a cis-acting silencing element leading to a reduction of *STAT6* promoter activity. *In vitro*, promoter activity is dependent on the allelic state of *rs324011* [8] confirmed that the T allele of *rs324011* increases *STAT6* promoter activity by creating a new site for NF-κB binding which is not detected in the wild C allele.

Furthermore, we also found significant positive correlation between *rs324011* TT and CT genotypes and serum IL-4 levels. In agreement with our findings, Gene expression analysis of Li et al. [38] showed that patients with 4 SNPs (rs2243250, rs1800925, rs1805010, and rs3224011) in *STAT6* gene had higher expression levels of IL-4, IL-13, and STAT6. It has been also detected that in bronchial asthma the over expression of IL-4 occurs via the activation of the *STAT6* pathway thus showing that *STAT6* plays a positive role, affecting the expression of IL-4 [39].

Our study has some limitations that worth mentioning. First, the sample size is relatively small; therefore, our findings should be interpreted with caution and verified using larger scaled studies. Also, because this study is restricted to the Egyptian population; these results may not be applied to the patients of other ethnic or racial groups.

Conclusion

Our study demonstrated that STAT6-2892C/T polymorphism contributes significantly to IgE production and the susceptibility of atopic asthma in Egyptian children, and highlights the etiological relationship between IL-4/*STAT6* signaling pathway and atopic bronchial asthma. Given that *STAT6* gene does not work alone as it cooperates with other genes in IL-4 /IL-13 signaling pathway, therefore the study of gene-gene and gene-environment interactions is greatly warranted in the future.

Conflict of Interest

The authors declare that there is no conflict of interest.

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