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# Transforming Growth Factor-ß1 C-509T Polymorphism and its Association with Prevalence and Severity of Asthma in Egyptian Children

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## ABSTRACT

***Background:*** Bronchial asthma is a complex genetic disorder regulated by the release of cytokines and inflammatory mediators. Transforming growth factor beta (TGF-ß1) cytokine plays a pivotal role in the inflammatory response of the airways. Differential production of this cytokine is associated with allelic variations in the transcriptional regulatory region of *TGF-ß1* gene.

***Aims:*** The objective of the present study was to investigate the C-to-T single-nucleotide polymorphism (C–509T) in the *TGF-ß1* gene promoter for its association with bronchial asthma in children.

***Material and Methods:*** DNA isolated from 84 asthmatic children and 55 control children was screened for this polymorphism using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

***Results:*** Genotype frequencies of the C-509T polymorphisms showed no statistically significant differences between cases and controls regarding both CC and TT genotypes, however, the heterozygous CT genotype showed a significant increase in controls more than cases with a *p*-value 0.04. The interaction between these polymorphisms revealed statistically significant association between the high producer T-allele of *TGF-ß1* (in TT & CT genotypes) and asthma severity with a *p*-value 0.03.

***Conclusion:*** Our results showed no significant association between the C-509T polymorphisms and the prevalence of asthma. However, our findings provide evidence that *TGF-ß1* plays an important role in determining disease severity in asthmatic children.

***Key Words:*** *Bronchial asthma – Transforming growth factor-ß1.*

## INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by recurrent episodes of airway obstruction and wheezing **[1]**. Airway inflammation and remodeling are critical pathophysiologic events in asthma. Genes involved in these processes are candidate genes for evaluation in association studies **[2]**. Transforming growth factor-ß1 (TGF-ß1) is a multifunctional cytokine that plays a critical role in cell growth and differentiation, immune modulation, airway development, and inflammation. TGF-ß1 is ubiquitously expressed in the lung and is involved in both normal cellular processes and numerous disease states **[3]**. Several lines of evidence suggest that TGF-ß1 contributes to the development of asthma, airway responsiveness, and airway remodeling. This cytokine has been also observed in the asthmatic airways and their bronchoalveolar lavage **[4]**. Differential production of this secreted cytokine is under genetic control and accumulating evidence indicates that functional polymorphisms in the *TGF-ß1* gene that affect its expression may modulate asthma occurrence. These allelic variations are attributed to the presence of single nucleotide polymorphisms (SNPs) in the *TGF-ß1* gene **[5]**. One such functional variant, a C-to-T base substitution at position –509 (i.e., C-509T) in the *TGF-ß1* gene promoter, increases *TGF-ß1* gene transcription **[6]** and plasma TGF-ß1 concentrations **[7]**. Although some epidemiologic studies found significant associations between C-509T and asthma occurrence in adults, data on childhood asthma are limited and inconsistent **[8]**.

The aim of this work was to investigate the C-to-T single-nucleotide polymorphism (C-509T) in the *TGF-ß1* gene promoter for its association with the incidence and severity of bronchial asthma in Egyptian children.

## PATIENTS AND METHODS

*Study population:*

Eighty four children with bronchial asthma were enrolled in this study. They included 51 males (60.7%) and 33 females (39.3%), their ages ranged from 0.5 to 12 years old (mean= 4.6±2.9, median=4). All patients were under follow-up in the Chest Clinic of New Cairo University Children's Hospital (group I). Bronchial asthma was diagnosed according to GINA guidelines 2009 **[9]**. They were assessed for history of chest wheezes, dyspnea, cough and respiratory distress. Other symptoms of atopy as eczema, urticaria, conjunctivitis or rhinitis were also determined. Fifty five age and sex matched healthy children were enrolled in the study as control group (group II). They included 33 males (60%) and 22 females (40%), their ages ranged from 0.5 to 5 years old (mean=2.5± 0.9, median=2.6). Informed consents were obtained from the parents of participating children before enrollment and the study was approved by the ethical committee of the Faculty of Medicine, Cairo University.

*Sample collection and laboratory investigations:*

Blood samples were drawn from each child in groups I and II by sterile venipunctures for performing; a complete blood count including manual differential, genotyping following DNA extraction and determination of serum IgE (IMX, Abott technology).

Stool and urine analysis were performed to exclude eosinophilia caused by parasitic infestations.

*Genotyping:*

Genomic DNA was purified from collected peripheral blood samples using DNA extraction kit (Qiagen). The *TGF-ß1* genotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique as described by Lario, et al. **[10]**.

*The primers' sequences for C509T gene locus are:*

5' GGGACACCATCTACAGTG 3' (forward) and 5' GGGGACACCATCTACAGTG 3' (reverse). The extracted DNA was amplified in a reaction mixture of 50µl containing 25µl Taq PCR Master Mix (2.5 units Taq DNA polymerase, 1X Qiagen PCR buffer, 200µM of each dNTP (Qiagen), 2µl of each primer (0.4µM final concentration) and distilled water. PCR reactions were initially denaturated at 94ºC for 5min. Amplification was then carried out for 35 cycles, each cycle consisting of denaturation at 94ºC for 30s, annealing at 60ºC for 20s, extention at 72ºC for 30s and finally a 5min extension at 72ºC. This produced a 455bp fragment.

The PCR products were digested by the restriction enzyme AoC I (Fermentas). Seventeen µl of the PCR product, 2µl of the restriction enzyme buffer and 1µl of the enzyme were mixed and incubated for 16 hours at 37ºC. Digested DNA fragments were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. A visible band at 429bp was significant for the wild CC genotype, a band at 455bp was significant for the homozygous TT genotype, while the presence of two bands at both 429bp and 455bp signified the heterozygous CT genotype.

*Statistical analysis:*

Data were collected and tabulated. Statistical Package for Social Science (SPSS) program version 17.0 was used for data analysis. Mean and standard deviation (SD) were estimates of quantitative data while frequency and percentage were estimates of qualitative data. Differences in clinical and biochemical characteristics were tested by Student's *t*-test or Mann Whitney U test, when appropriate, for continuous variables and by chi-square test for categorical data. A two-sided *p*-value <0.05 was considered statistically significant. Odds ratio were used to measure the strength of associations.

## RESULTS

In our study, children with asthma were more likely to be boys than girls (60.7% versus 39.3%). Respiratory symptoms and atopic manifestations were recorded in the asthmatic children with variable presentations which are described in Table 1(a). While, seasonal variation, pattern of asthma and precipitating factors of asthmatic episodes in the studied cases are shown in Table 1(b).

According to the severity of the symptoms on the basis of GINA guidelines 2008 **[11]**, we categorized our cases into 3 groups; those with mild persistent symptoms constituted 31% of cases, those with intermittent symptoms constituted 35.7% of cases, and those with moderate persistent symptoms constituted 33.3% of cases while none of them had severe persistent symptoms.

The peripheral blood eosinophilic count and serum IgE levels showed a highly significant increase in the cases as compared to controls ($p$=0.000 for both) as shown in Table (2).

Total serum IgE of patients showed a significant positive correlation with the peripheral blood eosinophils ($r$=0.3 and $p$=0.0009) as shown in Fig. (1).

Results of the PCR-RFLP are shown in Fig. (2) demonstrating the different patterns of polymorphisms of the *TGF-ß1* gene. Genotype frequencies of the C-509T polymorphisms showed no statistically significant differences between cases and controls except for the heterozygous CT genotype with a $p$-value 0.04 (Table 3).

The presence of the mutant T-allele (in TT & CT genotypes) showed a borderline significant increase in males than in females among the asthmatic children included in our study ($p$=0.05). We also recorded a significant association between the severity of asthma and the presence of T-allele in TT & CT genotypes ($p$=0.03). There were no other statistically significant associations of the different genotypes with either the eosinophilic count or the serum IgE levels (Table 4).

Table (1a): Distribution of symptoms among 84 bronchial asthma cases.

| (a) Symptoms | Number | Percentage |
|---|---|---|
| *Upper respiratory:* | | |
| Sneezing | 82 | 97.6 |
| Rhinorrhea | 77 | 91.6 |
| Snuffling | 22 | 26.2 |
| Itchy nose | 22 | 26.2 |
| *Chest symptoms:* | | |
| Wheezes | 84 | 100 |
| Dyspnea | 84 | 100 |
| Cough | 84 | 100 |
| Tightness | 35 | 41.6 |
| Respiratory distress | 79 | 94 |
| *Atopic manifestations:* | | |
| Eczema | 7 | 8.3 |
| Urticaria | 29 | 34.5 |
| Conjunctivitis | 7 | 8.3 |
| Rhinitis | 4 | 4.7 |

Table (1b): Characteristics of wheezing episodes in 84 asthmatic children.

| (b) Characteristics of wheezing episodes | Number | Percentage |
|---|---|---|
| *Season:* | | |
| Winter | 66 | 78.5 |
| Perennial | 15 | 17.8 |
| Spring | 2 | 2.4 |
| *Pattern:* | | |
| Paroxysmal | 84 | 100 |
| *Precipitating factors:* | | |
| Infection | 77 | 91.6 |
| Passive smoking | 71 | 84.5 |
| Exercises | 57 | 67.8 |
| Dust | 7 | 8.3 |
| Diet | 22 | 26.2 |
| *Severity:* | | |
| Intermittent | 30 | 35.7 |
| Mild persistent | 26 | 31 |
| Moderate persistent | 28 | 33.3 |

Table (2): Eosinophils and serum IgE levels in asthmatic children as compared to controls.

| Parameter | Cases (N=84) | Controls (N=55) | $p$-value |
|---|---|---|---|
| Eosinophilic count | 751.9±513.8 $^{+}$<br>11-2019 $^{++}$ | 311.8±120.7 $^{+}$<br>79-893 $^{++}$ | 0.000* |
| Ig E levels | 136.6±111.1 $^{+}$<br>5-500 $^{++}$ | 38.4±44.1 $^{+}$<br>6-320 $^{++}$ | 0.000* |
| IgE >100 N (%) | 38 (45.2%) | 3 (5.5%) | 0.000* |

+: Mean ± SD, ++: Range, *: $p$-value >0.01: Highly significant difference.

Table (3): *TGFß1* C-509T genotypic frequencies in 84 bronchial asthma cases as compared to controls.

| C-509T genotype | Cases N (%) | Controls N (%) | *p*-value | Odds ratio | 95% Confidence Interval |
|---|---|---|---|---|---|
| Wild type CC | 23 (27.4) | 8 (14.5) | 0.08 | 2.2 | 0.9-5.4 |
| Base exchange TT | 16 (19) | 8 (14.5) | 0.5 | 0.7 | 0.3-1. |
| Heterozygous CT | 45 (53.6) | 39 (70.9) | 0.04* | 2.1 | 1.03-4.4 |

Table (4): Association between the severity of asthma and the presence of TGF-ß1 genotype among 84 children with bronchial asthma.

| Genotype \ Severity of asthma | Intermittent N (%) | Mild persistent N (%) | Moderate persistent N (%) | *p*-value |
|---|---|---|---|---|
| Presence of T allele (TT or CT) | 23 (76.7) | 14 (53.8) | 24 (85.7) | 0.03* |
| Absence of T allele (CC) | 7 (23.3) | 12 (46.2) | 4 (14.3) | |

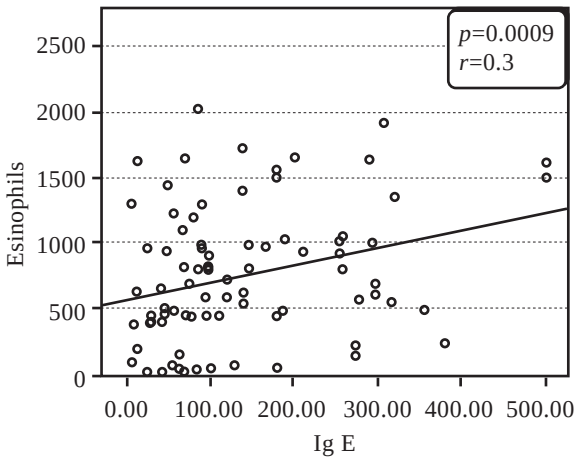


Fig. (1): Pearson correlation between IgE levels and eosinophilic counts among 84 children with bronchial asthma (*r*=0.3 & *p*=0.0009).

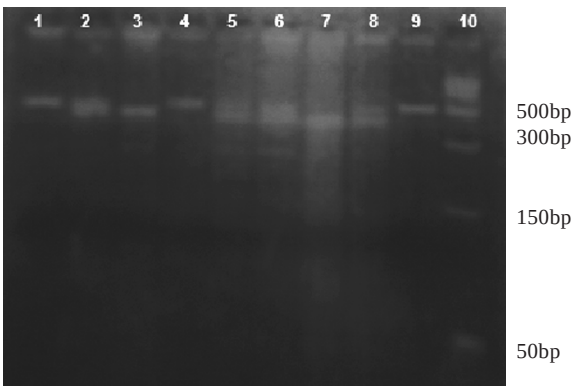


Fig. (2): PCR-RFLP of the *TGF-ß1* gene polymorphism.

- Lanes 1, 4, and 9 show homozygous TT genotype with single band at 455bp.
- Lanes 2, 5, 6 and 8 show heterozygous cases with two bands at 429 and 455bp respectively.
- Lanes 3 and 7 show homozygous wild CC genotype with single band at 429bp.
- Lane 10: Molecular weight marker (Promega), with the following base pairs: 50,150,300,500,750 and 1000 respectively.

## DISCUSSION

Asthma is known as a complex genetic disease with a broad range of phenotypes distinguished by hyper responsiveness and airway inflammation to various extrinsic and intrinsic stimuli **[12]**. Quite clearly, more emphasis is now laid on the role of immunological mechanisms involved in the process of inflammation. Cytokines are cell signaling proteins playing a central role in immunological and inflammatory mechanisms by relaying the necessary instruction to their target cell via specific receptor(s) in an autocrine, paracrine and endocrine fashion **[5]**.

TGF-ß1 is a multifunctional cytokine that is increased in the airways of individuals with asthma compared with those without asthma and is further increased in patients with status asthmaticus **[13,14]**. The increased TGF-ß1 is localized principally in the extracellular connective tissue of the subepithelial space of the airways in association with the binding proteoglycan decorin **[15]**; however, the precise cellular source of increased TGF-ß1 in the airways of individuals with asthma is unknown **[6]**. TGF-ß1 is secreted as a latent complex that must be cleaved via proteases, acid, or reactive oxygen species to become active **[16]**. Although multiple mechanisms are involved, transcriptional mechanisms have a very important role in the control of TGF-ß1 activity, and are regulated by inflammatory molecules found in the airways of individuals with asthma **[17]**.

The expression of TGF-ß1 is influenced by polymorphisms in the *TGF-ß1* gene, and some of these polymorphisms may be associated with

asthma and other diseases **[18,19,20]**. In particular, there is a C-to-T promoter polymorphism at base pair position –509 that alters a Yin Yang 1 (YY1) transcription factor consensus binding site (–CCATCTC/TG–) and is associated with higher circulating concentrations of TGF-ß1 in plasma. Grainger and colleagues **[7]** reported that the C-509T SNP accounted for 8.2% of the additive genetic variance of plasma TGF-ß1 concentrations. It has been hypothesized that the T allele enhances the YY1 binding site on the *TGF-ß1* promoter and is responsible for increased *TGF-ß1* transcription. Silverman and colleagues **[6]** demonstrated that the –509T SNP increases the TGF-ß1 promoter affinity for the transcription factor Yin Yang 1 by 30% resulting in a 30% increase in basal promoter activity. Taken together, findings from these studies indicate that the –509C SNP is likely to be a genetic determinant of levels of TGF-ß1 and support involvement of this SNP in asthma pathogenesis **[2]**.

In the present study, we utilized the PCR-RFLP technique to determine the C–509T gene polymorphism in the *TGF-ß1* gene promoter and to assess its association with the incidence and severity of bronchial asthma in Egyptian children compared to a control group of similar age and sex distribution and with no history for asthmatic episodes.

Regarding the age groups on which the study was performed, it was limited to childhood as this age harbors most of asthma reporting cases and high prevalence **[21]**; moreover, asthma exacerbations are among the leading causes of morbidity in children and have resulted in increased healthcare expenditures in the pediatric population over the last decade **[22]**.

Asthmatic children, included in our study, were more likely to be boys than girls (60.7% versus 39.3%). This increased risk of recurrent wheezing in males coincides with other studies that stated that asthma is more common in males compared to females **[2]**. Gissler et al., (1999) **[23]** reported that this could be related to the narrower airways, increased airway tone and possibly higher IgE levels in boys.

On studying the genotypic analysis of both groups, we found that genotype frequencies of the C-509T polymorphisms were as follows; the homozygous CC wild genotype was more prevalent in cases than controls (27.4% Vs 14.5%) while the TT mutant homozygous genotype was slightly increased in cases than controls (19% Vs 14.5%) but without statistically significant differences for both genotypes, however, there was a significant increase in the heterozygous CT genotype within controls when compared to cases (70.9% Vs 53.6%, *p*-value 0.04). When comparing the presence or absence of T allele between cases and controls, we found that the T allele being the high producer of TGF-ß1 was detected more among controls (85.5%) than cases (72.6%); however, this difference is not statistically significant (*p*-value 0.08, odds ratio 2.2). This is in accordance with some authors who failed to find significant associations between C-509T and asthma occurrence in children **[24,25]** or adults **[26,27]**; however, these findings are against many studies that found that the TT genotype is associated with an increased risk of asthma **[2,6,8,28,29]**. Also, Kumor, et al. **[5]** observed that individuals carrying T allele are at greater risk of developing asthma. However, in a study by Sharma et al., the TT and CT genotypes of a coding polymorphism in *TGF-ß1* were inversely associated with asthma exacerbations **[4]**.

The plausible reason for this controversy could be either the diverse ethnic background of the studied populations or the diverse etiologies that may contribute to the development of asthma, which might suggest that the physiological basis of this polymorphism might have a broader role in the susceptibility to asthma. Furthermore, determination of the levels of TGF-ß1 in serum or bronchoalveolar lavage fluid might prove helpful in better understanding of the role of this cytokine in asthma.

Another explanation would be attributable to the fact that there are at least two possible mechanisms by which TGF-ß1 may impact the development and severity of asthma. Some studies suggest that increased TGF-ß1 has a beneficial role in asthma by suppressing airway inflammation and hyper-responsiveness through the inhibition of T lymphocytes, dendritic cells, eosinophils, and mast cells. In this way, TGF-ß1 may be part of a negative-feedback loop, turning off inflammation that augments its production **[30]**. Under this paradigm, genetic variants that are associated with increased TGF-ß1 activity, such as the T allele of C–509T, would

be expected to be associated with decreased asthma prevalence or decreased asthma severity. Other studies suggest that TGF-ß1 has harmful effects in the airways of individuals with asthma. TGF-ß1 is profibrotic, and its sustained elevation may stimulate airway remodeling. Under this paradigm, genetic variants that are associated with increased TGF-ß1 activity, such as the T allele of C–509T, would be expected to be associated with higher asthma prevalence or increased asthma severity **[6]**. We have detected a statistically significant association between the presence of the high producing T allele and asthma severity in our asthmatic children (*p*-value 0.03). Pulleyn and coworkers **[31]**, also reported an increased frequency of homozygous 509T allele in severely asthmatic patient group compared to the mild and control groups included in their study. The same results were obtained in a study by Salam and coworkers **[2]** who mentioned that the TT genotype had a 5 fold increased risk of early persistent asthma. These findings are also supported by the study of Silverman and coworkers **[6]**.

The presence of the mutant T-allele (in TT & CT genotypes) showed a borderline significant increase in males than in females among the asthmatic children included in our study ($p$=0.05). But this may be due to unequel gender distribution among the studied population.

As regards the absolute eosinophilic count and the total IgE levels, there was a highly significant increase in these two atopic markers within the cases compared to the control group ($p$=0.000 for both). This is in accordance with several authors who stated that increased IgE levels and absolute eosinophilia are hallmarks for the diagnosis of bronchial asthma. A particular challenge is to distinguish children with transient wheezing from those whose wheezing persists and later develop asthma. Airway tissue inflammation leading to airway remodeling occurs at an early age and is fundamental for the development of asthma. Thus characteristic features of inflammation such as eosinophilia and increased IgE levels can be used to distinguish asthma-related wheezing from wheezing caused by viral infection **[32]**.

We found no association between 509T allele and IgE levels or eosinophil count. Our results are in agreement with other studies that found inconsistent associations between C-509T polymorphism and atopic markers like serum IgE, eosinophil count and positive skin test to allergens **[6,8,25]**. Therefore, it is unlikely that the relationship of *TGF-ß1* C-509T with asthma is primarily due to effects of this polymorphism on atopy.

In conclusion our study failed to find an association between the *TGF-ß1* C-509T polymorphisms and the prevalence of asthma taking into consideration the relatively limited number of the studied groups. However, our findings provide further evidence that TGF-ß1 plays an important role in determining disease severity in asthmatic children.

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