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Impact of Lymphotoxin alpha Gene Polymorphisms on Childhood Asthma

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ABSTRACT

Background: Bronchial asthma, a common allergic disorder among children, is a chronic airway inflammatory disease. Clinically, it tends to occur with recurrent symptoms mainly involve dyspnea, wheezing, cough, and chest distress.

Objective: The aim of the study was to evaluate the association between lymphotoxin alpha (LTα) gene polymorphisms and childhood bronchial asthma.

Patients and methods: A case control study was carried out on 116 children divided to 29 control group and 87 cases. Detection of lymphpotoxin alpha gene polymorphism was assessed.

Result: There was statistically significant difference between asthma group and control group regarding genotypes (P-value 0.044). There was a statistically significant negative correlation between FEV1 and IgE level, and a significant positive correlation between FEV1 and eosinophilic count.

Conclusion: The genetic predisposition is considered one of the important attributable risk factors of childhood asthma. In addition, the polymorphism of lymphotoxin alpha A/G (rs2844484) is correlated with the risk of childhood asthma.

Keywords: Lymphotoxin alpha Gene Polymorphisms, lymphotoxin alpha, Childhood Asthma, Risk factors.

INTRODUCTION

Bronchial asthma, a common allergic disorder among children, is a chronic airway inflammatory disease. Clinically, it tends to occur with recurrent symptoms mainly involve dyspnea, wheezing, cough, and chest distress **(1)**.

Asthma is the most common chronic disease in childhood and the main cause of childhood leading morbidity of chronic disease years, the incidence rate of childhood asthma has been increasing each year, which means a serious risk to the physical and mental health of children **(2)**.

According to global statistics, it is among the top 10 chronic conditions in children aged 5-14 years. Bronchial asthma mortality rate in children globally ranges from 0 to0.7 per 100 000 **(1)**.

Lymphoxin alpha (LTα) is an immune-related cytokine that belongs to the tumor necrosis factor family. It has a molecular structure and an active region similar to those of tumor necrosis factor-α (TNF-α). LTα is located on the MHC class III region of the short arm of chromosome 6. It is produced in autoimmune diseases and tumors after the stimulation of mitogen and lymphocyte antigen **(3)**.

It can affect cell apoptosis and regulate inflammatory immunity. Recently, some studies have showed that TNF-α may be one of the crucial candidate sites for the susceptibility to bronchitis but the role of LTα has not been proved yet **(3)**.

The objective of our study was to study the genetic polymorphism of lymphotoxin alpha (LTα) in children with bronchial asthma.

PATIENTS AND METHODS

This was prospective case control study conducted at Pulmonology & Allergy Unit of Pediatric Department in Pediatric Hospital, and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University during the period from November 2020 to July 2021.

Sample size:

A total of 116 children were included in our study. The recruited children were divided into two groups: The first group included 29 non-asthmatic children, and the second group included 87 asthmatic children.

Participant children were classified according to **GINA 2018 (4)** into:

- 29 well controlled asthmatic children.
- 29 partially controlled asthmatic children.
- 29 uncontrolled asthmatic children.

Inclusion criteria: Children with bronchial asthma, approval to participate in the study, both sexes, and age from 5-15 years.

Exclusion Criteria: Refusal to participate in the study, asthmatic children younger than 5 years old or older than 15 years old, children with accompanied inflammatory diseases as (inflammatory bowel, rheumatic fever, vasculitis), and children suffering from liver, renal or other chronic diseases rather than bronchial asthma.

Methods:

All patients were subjected for:

A) Detailed history taking from patients or parents.

B) Physical examination includes anthropometric measures, weight in kg and height in cm.

C) Laboratory investigations: Complete blood count, C-reactive protein, liver function tests, and kidney function tests.

D) Pulmonary function test: It was performed for asthmatic patients with 5 years and more using forced spirometry by D-97024 Hochberg, Germany, which is a program that allows a fast and reliable

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determination of the respiratory resistance on the basis of a tidal breathing analysis.

Forced spirometry is one of the basic measurements in pulmonology. At various breathing maneuvers, ventilated lung volumes and maximal respiratory flows are normally measured by means of a pneumotachograph [(5)].

- **Forced Vital Capacity (FVC):** Volume, which can be forced, exhaled after maximal inspiration with maximal effort and duration. In healthy, the value is equal to IVC.
- **Forced Expiratory Volume in one second (EFV1):** Volume of air, forced and suddenly exhaled in the first second, after a maximal inhalation.
- **PEF:** Peak-flow; maximal expiratory flow attained during an FVC-maneuver.
- **FVC%, FCV1% and PEF%:** Percentage refers to percentage from predicted.

E) Specific investigations:

Detection of lymphpotoxin alpha gene polymorphism: Identification of different genotypes of the lymphotoxin alpha gene was carried out using polymerase chain reaction - restriction fragment length polymorphism (PCR-RELP) technique.

Blood sampling: Two ml of venous blood were withdrawn from each study participant under aseptic conditions in sterile EDTA containing tubes, samples were stored in the same tubes at -20°C to be used for extraction and analysis of DNA.

Genomic DNA extraction:

Genomic DNA was extracted from whole blood with a commercial purification kit according to the manufacturer's instructions (**Gene JET Genomic DNA Purification Kit, Thermo Scientific™, USA)**.

Polymerase chain reaction (PCR):

Amplification of the LT-α gene was carried out using the LT-α-NcoI primers 5`-CCGTGCTTCGTGCTTTGGA CTA-3`and 5`-AGAGCTGGTGGGGA CATGTCTG-3`. Primers were purchased as lypophilized agents from **(operon, Invitrogen)**, reconstituted with sterile deionized water to make stock. Then, dilutions were made from this stock to reach 10 µM concentrations.

The PCR reaction was performed in a total reaction mixture of 25 µl, that contained 12.5 µl PCR Master Mix **(iTaqTM, iNtRON, Korea)**, 1.25 µl forward primer, 1.25 µl reverse primer, 9 µl DNA extract, and 1 µl nuclease free water. PCR was performed using the recommended thermal cycling conditions starting with 95º C for 5 min followed by 40 cycles of denaturation for 15 s at 95º C, annealing for 30 s at 55º C, and extension for 30 s at 72º C with a final extension at 72º C for 5 min. This reaction yielded amplification products of 740 bp size that were analyzed using 1.5% agarose gel electrophoresis.

Restriction fragment length polymorphism (RFLP):

Following amplification, 10 µL of PCR products were digested with 1 µL of NcoI endonuclease at 37°C for 16 hours according to the manufacture's protocol **(NcoI; Thermo ScientificTM, USA)**. The resultant products were analyzed on 2% agarose gel with ethidium bromide. Wild type LT-α- gene containing allele (A) was identified by a single 740 bp band, while variant LT-α- gene containing allele (G) was identified by 195 bp and 545 bp fragments. The three possible patterns of NcoI PCR-RFLP of LT-α- gene.

Ethical consent:

An approval of the study was obtained from Zagazig University Academic and Ethical Committee. The guardians of the participated children signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

The collected data were coded, processed and analyzed using the SPSS (Statistical Package for Social Sciences) version 20.0 for Windows® (IBM SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) and Fisher's exact test to calculate difference between two or more groups of qualitative variables. Quantitative data were expressed as mean and standard deviation (SD). Independent samples t-test was used to compare between two independent groups, and ANOVA for more than two groups. The correlation between two variables was examined using Pearson's correlation test. P value was set at ≤ 0.05 for significant results, and ≤ 0.001 for high significant results.

RESULTS

Table 1 shows that there were no statistically significant differences between the four studied groups as regard age (years) and sex. However, there was a statistically significant difference between the studied groups as regard the family history as the majority of asthmatic patients had positive family history while none of controls had positive family history.

Table (1): Basic characteristics of the four studied groups:

Variable	Uncontrolled group (n=29)	Partially controlled group (n=29)	Well controlled group (n=29)	Control group (n=29)	Tests	
					F-test	P value
Age (years)						0.6
Mean ± SD	7.96±3.6	7.9±2.8	8.4±2.8	8.83±2.82	0.6	(NS)
Range	(5-14)	(5-14)	(5-15)	(5-14)		
Variable	**No (%)**	**No (%)**	**No (%)**	**No (%)**	**X2**	**P value**
Sex						0.9
Female	13 (44.8)	12 (41.4)	15 (51.7)	13 (44.8)	0.66	(NS)
Male	16 (55.2)	17 (58.6)	14 (48.3)	16 (55.2)		
Family history						
Negative	1 (3.4)	4 (13.8)	8 (27.6)	29 (100)	130.1	<0.001*
Positive	28 (96.6)	25 (86.2)	21 (72.4)	0 (0)		

(X^2) Chi-square test. (F-test) one way ANOVA. (NS) non-significant. (S) Significant.(*) Highly significant.

Table 2 shows that there were no statistically significant differences between the four studied groups regarding weight (kg), height (m) and BMI (P>0.05).

Table (2): Anthropometric measures of the four studied groups:

Variable	Uncontrolled group (n=29)	Partially controlled group (n=29)	Well controlled group (n=29)	Control group (n=29)	Tests	
					F-test	P value
Weight (kg)						
Mean ± SD	33.8 ± 6.6	28.7 ± 7.4	33.2 ± 8.2	31.2 ± 5.86	1.1	0.3
Range	(18-60)	(17-50)	(20-66)	(19-66)		
Height (cm)						
Mean ± SD	1.35±0.22	1.26±0.15	1.31±0.13	1.35±0.17	1.6	0.2
Range	(1.06-1.68)	(1.06-1.55)	(1.05-1.56)	(1.06-1.65)		(NS)
BMI (wt/m^2)						
Mean ± SD	17.89 ± 3.73	17.54 ± 3.23	18.36 ± 2.56	16.51 ± 2.74	3.2	0.075
Range	(11.03-26.01)	(13.4-24.4)	(14.38-23.6)	(12.8-24.2)		(NS)

(F-test) one way ANOVA. (NS) Non-significant.

Table 3 shows that there were no statistically significant differences between the four studied groups as regard Hb, WBCs count, platelet count and CRP level. However, there was a statistically significant difference (P <0.05) between them as regard esinophilic count, with all asthmatic groups significantly higher than control group.

Table (3): Laboratory data distribution between four studied groups:

Variable	Uncontrolled group (n=29)	Partially controlled group (n=29)	Well controlled group (n=29)	Control group (n=29)	Tests	
					F-test	P
WBC (*10^9/L)					0.27	0.85
Mean ± SD	8.77 ± 1.75	8.67 ± 1.01	8.67 ± 2.21	8.2 ± 2.11		(NS)
HB (gm/dl), Mean ± SD	12.11 ± 0.79	12.14 ± 0.93	12.29 ± 0.75	11.91 ± 0.80	1.02	0.39 (NS)
Platelet (*10^9/L)						0.187
Mean ± SD	277.86 ± 45.88	304.93 ± 54.55	282.9 ± 53.3	286.1 ± 45.4	1.62	(NS)
Esinophils (Mean ± SD)	2125.93 ± 412.3 1260	1953.3 ± 322.6 1200	1738.44 ± 411.3 870	199.0 ± 44.6 151	65.44	<0.001* P1<0.001* P2<0.001* P3<0.001* P4=0.624 P5=0.451 P6=0.963
CRP (mg/L)						0.31
Mean ± SD	1.14 ± 0.39	1.06±0.103	1.15 ± 0.30	1.02 ± 0.063	1.199	(NS)

(F-test) one way ANOVA. (NS) non-significant. (S) Significant. @ kruskal-wallis ANOVA.
P1 =uncontrolled group vs. control group. P2 =partially controlled group vs. control group.

P3 = well controlled group vs. control group. P4 =uncontrolled vs. partially controlled group.
P5 =uncontrolled group vs. well controlled group. P6 = partially controlled group vs. well controlled group

Table 4 shows that there was a statistically significant difference between asthma group and control group regarding genotypes. AA genotype represented the most common type in the control group (48.2%). However, it was the least predominant genotype in the asthmatic group (29.9%).

Table (4): Comparing genotypes between the asthma group and control group

Variable	Asthma group (n=87)	Control group (n=29)	X^2 test P-value
Genotypes			
AA (n=40)	26 (29.9%)	14 (48.2%)	0.044*
AG (n=51)	41 (47.1 %)	10 (34.4 %)	
GG (n=25)	20 (23 %)	5 (17.4%)	

Table 5 shows that there was a statistically significant negative correlation between FEV1 and IgE level. A significant positive correlation has been found between FEV1 and eosinophilic count.

Table (5): Correlation between respiratory function and different parameters in asthmatic patients (n=87).

Variables	FEV1		FVC	
	P-value	R	P-value	R
BMI (wt/m2)	0.303	.112	0.126	.165
WBC ($*10^9$/L)	0.361	-.099	0.473	-.078
HB (gm/dl)	0.745	.035	0.956	-.006
Platelet ($*10^9$/L)	0.748	.035	0.729	-.038
Eosinophils	0.986	-.002	0.876	-.017
CRP (mg/L)	0.880	..016	0.995	.001
ALT (mg/L)	0.996	.089	0.604	-.056
AST (mg/L)	0.365	.098	0.300	-.112
Urea (mg/L)	0.747	.035	0.778	.031
Creat (mg/L)	0.796	-.028	0.845	-.021
IgE	0.005	-.0301	0.144	-.158

DISCUSSION

In our study, we found that there were no statistically significant differences between the four studied groups as regard age (years), sex, weight (kg) and height (m). The majority of asthmatic patients had positive family history while none of controls had positive family history. There was statistically significant difference between the studied group as regard family history.

In contrast, the study by **Hallit *et al.*** [6] included 1276 children aged from 3 to 14 years old (976 healthy and 300 asthmatic children). They reported that there was a statistically significant difference between asthma patients and control group as regard age group, sex, height and weight.

Guo *et al.* [7] agree with our results. They revealed that there were no statistically significant differences between the studied groups regarding age and sex, while there was a high statistically significant difference between the studied groups regarding the family history.

Ding *et al.* [8] also found high statistically significant differences between the studied groups regarding the family history, which was agreed with our results.

However, asthma is not just a genetic disorder. In addition to family history, one of the few other strong risk factors for asthma is living in a developed country. Over the past decade, the International Study of Asthma and Allergic Diseases in Children (ISAAC) has clearly demonstrated the higher prevalence of asthma in more developed countries, which does not reflect simply diagnostic preferences asthma symptoms mirror trends in diagnosis [9].

In our study, there was a statistically significant negative correlation between FEV1 and IgE levels, with increasing the levels of IgE is associated with decrease in FEV1.

In agreement to our results, the study by **Beeh *et al.*** [9] proved that there was a statistically negative correlation between FEV1 and IgE level.

Regarding gene polymorphism between the studied groups, we found that there was a statistically significant difference between asthma group and control group regarding genotypes (P-value 0.044).

AA genotype represented the most common type in the control group (48.2%). However, it was the least predominant genotype in the asthmatic group (29.9%)**.**

In agreement with our results, a study conducted on Taiwanese children, a total of 269 subjects. The mean age of all subjects was 9.55 ± 1.83 years (range, 7−14 years). There was a statistically higher frequency of LT-α-NcoI*1 allele carriers in the subjects with asthma than in controls (P-value 0.031) [11]**.**

Our result was supported by **Guo *et al.*** [7] as they found that the distribution frequency of the three genotypes of LTα rs2844484 was different between the two groups, and the frequency of genotype AA was significantly higher than that of genotype AG and GG ($p<0.01$).

Our results was supported by **Moffatt *et al.*** [12], as they found that asthma was significantly more common in subjects with allele 1 of the LTα polymorphism ($p = 0.005$). Also, a study by **Subbarao *et al.*** [13] the results indicate that the LTα gene

polymorphysm have been associated with an increased risk of clinical asthma in Italian families.

In agreement with our results, the study by **El Hawary and Kamal** [(14)] showed that asthma was significantly more common in subjects with allele A of the LTA*Ncol polymorphism [55%], rather than allele G [45%]. In addition, the severe persistent asthmatic cases were associated with the LTA*Ncol-AA genotype at a frequency of 80%, while the genotype LTA*Ncol-GG are associated with the mildest form of the disease.

In a Japanese study concluded that polymorphisms of the TNF gene family on chromosome 6p21.1, including TNF-α, LT-α and LT-β genes, play an important role in the pathogenesis of asthma. Also, the authors stated that the minor contribution of the LT-α-NcoI polymorphism of the LT-α gene to asthma susceptibility [(15)]**.**

In contrast to our results, the study by **Nadif *et al.*** [(16)] carried out on five-hundred and fifty-six random individuals found that there was no association with LTa NcoI alleles polymorphism and asthma (P-value 0.12).

In contrast to our results, the study by **Randolph *et al.*** [(17)] showed that Lymphotoxin alpha NcoI gene polymorphism not associated with childhood asthma phenotypes (P >0.05).

CONCLUSION

In conclusion, the polymorphism of lymphotoxin alpha A/G (rs2844484) is associated with higher risk of childhood asthma.

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