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ORIGINAL ARTICLE

Forkhead Box p3 Gene Polymorphism in Bronchial Asthma Patients at Aswan University

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ABSTRACT

Background: *The lungs are impacted by diverse chronic illnesses such as asthma. Through genetic polymorphisms and epigenetic pathways, the aetiology of asthma may be connected to the changed activities of the forkhead box P3 gene (FOXP3).* ***Objective****: the aim of this research was to find whether there was any association between FOXP3 polymorphism rs2232365 and asthma and to study the effect of this polymorphism on asthma severity.* ***Methodology:*** *this research is a case control study of 240 Egyptian participants enlisted and divided into two groups: 120 people with asthma and 120 people were not asthmatic.* ***Results:*** *There was a statistically significant reduction in spirometry parameters (FVC%, FEV1%, and FEV1/FVC ratio) in the patient group relative to the control group. The serum IGE and eosinophils count were higher in cases. A statistically significant difference was observed in the various genotypes of FOXP3 polymorphism (rs2232365) and the most common genotype among the patients under investigation was the GG genotype and it was associated with higher IgE and Eosinophils (both in blood and sputum) in comparison to the AA and AG genotypes (p <0.05).****Conclusion****: Since the GG Genotype was the most common genotype among the patients under investigation and it was associated with higher IgE and Eosinophil (both in blood and sputum). The GG genotype of rs2232365 may act as a risk allele for bronchial asthma*

INTRODUCTION

Asthma is a heterogeneous disease, characterized by chronic airway inflammation. It is characterized by the history of respiratory symptoms such as wheezing, shortness of breath, chest tightness and coughing that change in strength over time, together with variable expiratory airflow limitation[1].

In the past few decades, Research into the underlying environmental and immunologic factors of this prevalent disease has increased due to the rising frequency of allergy disorders and asthma in the developed world. Researchers have determined that Th2 cells are essential in allergic asthma since the crucial distinction between CD4+ helper T-cell type 1 (Th1) and Th2 immune responses was initially clarified[2].

The pathophysiology of asthma is significantly influenced by regulatory T-cells. Treg cells suppress unwanted immunological stimulation and preserve lung tolerance. The primary cells in the lung that promote Foxp3+ Treg cells are alveolar macrophages (AMs) and plasmacytoid dendritic cells (pDCs). If Treg cells are exposed to an allergen locally before systemic inflammation occurs, they can help mice develop tolerance to airway allergic inflammation. This happens through antigen specific Treg cell responses after local exposure[3].

The function of Treg cells may be compromised in the setting of persistent asthmatic inflammation, in contrast to their involvement in preserving lung tissue homeostasis. The quantity of Foxp3+ Treg cells is considerably lower in severe asthma than in healthy controls. Additionally, their CCR5 expression, which indicates their suppressive capacity, also drastically dropped. Moreover, Numerous studies have demonstrated that the balance between Th17 and Treg cells controls lung tolerance[4].

Research indicates that CD4+CD25+Foxp3+ regulatory T cells (Tregs), which constitutively express the transcription factor FOXP3, are essential for immune system homeostasis and for suppressing Th2 responses after allergen exposure[5].

Individual phenotypic differences are caused by SNPs, the most prevalent variants in the genome. Although genes' coding sequences are frequently preserved, the presence of genetic mutations or SNPs may be linked to a person's vulnerability to complicated disorders such as Asthma[6].

METHODOLOGY

Study design:

It is a case control study that was accomplished in Aswan University Hospital in the period from February 2023 to August 2024. This study included 120 asthmatic patients diagnosed based on GINA guidelines 2022 with similar age and sex matched controls selected from Aswan University Hospital's Outpatient Clinics.

The study excluded participants with acquired immunodeficiency, autoimmune disorders, cardiovascular diseases, malignant tumours, B-blocker treatment, mental disorders, patients on monoamine oxidase inhibitors or ACE inhibitors, children younger than five years old, patients with severe uncontrolled asthma, and patients undergoing immunotherapy because of its effect on cytokine profiles.

Ethical Approval: All participants provided a written informed consent before beginning the study, which was authorized by Faculty of Medicine's Ethical Committee, Aswan University.

All the following data were collected from participated subjects in this study:

History taking including age, sex, family history of asthma, allergies, medications, and the present symptoms, including nocturnal cough, wheezing, recurrent chest tightness, dyspnea, sleep disturbances, and recurrent chest infections.

Clinical examination:

All participants had a thorough general and chest examination, and the severity of their asthma attacks was assessed using the Global Initiative Guidelines for Asthma.

Standard spirometry:

It was completed using the WinsproPRo PFT machine for every patient. Forced Vital Capacity (FVC), FEV1/FVC%, and Forced Expiratory Volume in First Second (FEV1) were measured. Spirometry was conducted prior to and subsequently repeated following the inhalation of a short-acting β2-agonist (SABA). An elevation in forced expiratory volume in the first second exceeding 200 ml and/or 12% above the pre-bronchodilator FEV1 at the time of evaluation was deemed diagnostic

The Asthma Control Test (ACT):

Comprises five questions designed to evaluate night-time and day-time symptoms, frequency of beta-2 agonist usage as rescue medications, and limitations on daily activities. A score of twenty-five points indicates "complete control," while a score between twenty and twenty-four points suggests "partial control," and a score below twenty points signifies "uncontrolled" asthma.

Complete blood count:

5 ml venous blood samples were taken by vacutainer and put in tubes (Becton Dickinson Vacuum) containing ethylene diaminetetraacetic acid and gently mixed. (Sysmex XE-21N, Kobe, Japan) device was used to measure the total leucocytic count (TLC), lymphocytes and eosinophils percentage within 1-2 hours after blood specimen collection.

Sputum analysis: It was done according to chest department rules. An eosinophil percentage of 3% or more of the total cell count is indicative of airway eosinophilia.

A commercially available sandwich ELISA kit (Biocheck, Biokit, South San Francisco, CA 94080) was utilized to measure serum total IgE levels, with a 5.0 IU/ml sensitivity limit.

Detection of "FoxP3 rs2232365 A/G Polymorphism" in Human Plasma Sample Using TaqMan Assay**:** The FoxP3 rs2232365 A/G Polymorphism was detected in plasma sample using the following kits:

1. DNA extraction kit (e.g., Thermo Fisher PureLink™ Genomic DNA Mini Kit)
2. TaqMan® SNP Genotyping Assay for FoxP3 r52232365 (A/G polymorphism) (Thermo Fisher, Assay ID: C_15881837_10)
3. TaqMan® Genotyping Master Mix (Thermo Fisher, Cat. No: 4371355)

Steps of DNA extraction

Plasma samples were thawed on ice. DNA was extracted from plasma sample using the PureLink™ Genomic DNA Mini Kit, following the manufacturer's instructions precisely. 200 uL of plasma was added to the lysis buffer, incubated at 56°C for 10 minutes, and the DNA bound to the column, washed, and eluted with 50-100 L of elution buffer. The extracted DNA was quantified using a spectrophotometer to ensure the concentration is sufficient for downstream applications which were 2-10 ng/Ul. Then the DNA samples were stored at -20°C until ready for genotyping. The TaqMan Assay Reaction Mix was prepared with final volume of 20 ul as follow [TaqMan® Genotyping Master Mix (10 uL, TaqMand SNP Genotyping Assay (0.5 uL), Genomic DNA "Sng/l" (2 uL), Nuclease-free water (7.5). For negative controls, the genomic DNA was replaced with nuclease-free water. The cycling protocol was adjusted as presented in Table 1.

Table 1: Thermal cycling conditions

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	10 mins	1
Denaturation	95°C	15 mins	40
Annealing/Extension	60°C	1 min	40

The PCR plate sealed with adhesive PCR seal to prevent evaporation and briefly centrifuged 2000 rpm for 1 minute) to remove any air bubbles and ensure the liquid setles at the bottom of the wells. The PCR instrument (Applied Biosystems, ThermoScientific, USA) was set to detect the specific FAM and VIC fluorescent dyes used in the TaqMan SNP genotyping

assay. The FAM dye represents the allele A, and VICT™ dye represents the allele G. Once the PCR run was complete, the data were analyzed using the QuantStudioT™ Design & Analysis Software. The three genotypes were assigned based on the fluorescence signals:

a. Samples with FAMTM fluorescence correspond to A allele.
b. Samples with VICT™ fluorescence, correspond to G allele.
c. Heterozygous samples will show, both FAMTM and VICTM signals

Interpretation of data: The data were reported as genotype and the frequency of alleles

a. AA Genotype: Only FAM signal detected.
b. GG Genotype: Only VIC signal detected.
c. AG Genotype (Heterozygote): Both FAM and VIC signals detected.

Statistical analysis:

The results were evaluated using the Statistical Package for Social Sciences (SPSS-model 25) software.

RESULTS

Table 2: Comparison of the demographic informations of the groups under study

Variables	BA group (n=120)	Control group (n=120)	Test	P-value
Age (years)			**8.91**	**0.001***
Mean± SD	46.96±14.57	35.5±10.11		
Gender			1.21	0.61
Male	36 (30%)	33 (27.5%)		
Female	84 (70%)	87 (72.5%)		
Family History			6.34	**0.001***
Have no family history	14 (11.7%)	120 (100%)		
Have family history	106 (88.3%)	0 (0%)		
Other allergies				
History of allergic rhinitis	60 (50%)	10 (8.3%)	7.35	**0.001***
History of conjunctivitis	2 (1.6%)	0 (0%)	1.1	0.24
History of atopic dermatitis	9 (7.5%)	2 (1.6%)	0.9	1.1
History of food allergy	4 (3.3%)	1	1.32	0.94

This table indicated a significant difference in age between asthmatic patients and controls, with the patient group exhibiting higher age and history of allergic rhinitis (p-value =0.001). The difference in sex distribution was statistically insignificant, with 36 males (30%) and 84 females (70%) in the patient group, compared to 33 males (27.5%) and 87 females (72.5%) in the control group.

Table 3: Characteristics of the asthmatic patients in the current study (n=120)

Variables	No.	%
Degree of asthma		
Mild asthma	19	15.8%
Mild persistent	41	34.2%
Moderate persistent	39	32.5%
Severe persistent	21	17.5%
Level of symptoms control		
Controlled	74	61.7%
Partially controlled	32	26.7%
Not Controlled	14	11.7%
Hospital admission		
Have no hospital admission	92	76.7%
Have hospital admission	28	23.3%
Drugs		
SABA	19	15.8%
SABA+LABA	41	34.2%
Inhaled steroid	39	32.5%
Systemic steroid	21	17.5%

Among our patients with asthma, mild persistent and moderate persistent cases were more prevalent (34.2% and 32.5%, respectively), while severe persistent asthma accounted for 17.5%. Based on symptoms control level, most cases were well controlled (61.7%), while only a minority (11.7%) remained non-controlled.

Table 4: Comparison of laboratory data and spirometry measures among the studied groups.

Variables	BA group	Control group	Test	P-value
Ig E (IU/ml)			**9.32**	**0.001***
Mean± SD	239.33±53.8	66.85±13.5		
Eosinophils in blood			**6.32**	**0.002***
Mean± SD	0.83±0.1	0.15±0.02		
Neutrophils in blood			1.22	1.98
Mean± SD	54.16±18.2	57.27±19.8		
Lymphocytes in blood			1.32	1.32
Mean± SD	2.185±0.24	1.9±0.2		
Eosinophil in sputum			**5.32**	**0.002***
Mean± SD	7.4±0.9	2.1±0.6		
FVC (%) before bronchodilator			**6.34**	**0.001***
Mean± SD	97.8±7.7	111.9±3.6		
FVC (%) after bronchodilator			1.35	0.48
Mean± SD	114.9±6.6	113.5±3.16		
FEV1 before bronchodilator			**5.69**	**0.003***
Mean± SD	71.3±10.96	108.1±3.1		
FEV1 after bronchodilator			**6.97**	**0.002***
Mean± SD	97.02±13.3	109.7±2.3		
FEV1/FVC ratio before bronchodilator			**7.68**	**0.001***
Mean± SD	72.6±8.1	96.48±2.3		
FEV1/FVC ratio after bronchodilator			**5.19**	**0.002***
Mean± SD	84.35±9.9	96.48±2.3		

Test:-The independent t-test

This table illustrated that the patients' group exhibited a statistically significant increase in eosinophil (both in blood and sputum) and serum IgE levels compared to the control group (p-value = 0.001) and there was a statistically significant reduction in spirometry parameters (FVC% (before bronchodilator, FEV1% (before and after bronchodilator, and FEV1/FVC ratio (before and after bronchodilator)) in the patient group relative to the control group (p-value < 0.05). No statistically significant difference was observed between the studied groups concerning Neutrophils and lymphocyte (p-value > 0.05) .

Table 5: Comparison of FOXP3 polymorphisms between studied groups.

FOXP3 polymorphism	BA group	Control group	Test	P-value
rs2232365	**N (%)**	**N (%)**	**3.65**	**0.004***
AA	25 (20.8%)	20 (16.7%)		
AG	35 (29.2%)	15 (12.5%)		
GG	60 (50%)	85 (70.8%)		

Test: - chi-square test

This table demonstrated a statistically significant difference in the various genotypes of FOXP3 polymorphism (rs2232365) when comparing asthmatic individuals to healthy controls (P = 0.004).

Table 6: Descriptive analysis of FOXP3 polymorphisms with relation to the severity of asthma.

Degree of asthma severity	AA (N=25)	AG (N=35)	GG (N=60)	Test	P-value
	N (%)	**N (%)**	**N (%)**	**6.32**	**0.001***
Mild asthma	5 (20%)	1 (2.9%)	13 (21.7%)		
Mild persistent	11 (44%)	17 (48.6%)	8 (15%)		
Moderate persistent	7 (28%)	6 (17.1%)	22 (36.7%)		
Severe persistent	2 (8%)	11 (31.4%)	16 (26.7%)		

Test: - The chi-square test

In this table the genotypic distribution revealed a notable disparity among mild, moderate, and severe persistent cases in rs2232365 (p-value = 0.001), the data of Genotypes were reported as illustrated in figure 1,2,3**.**

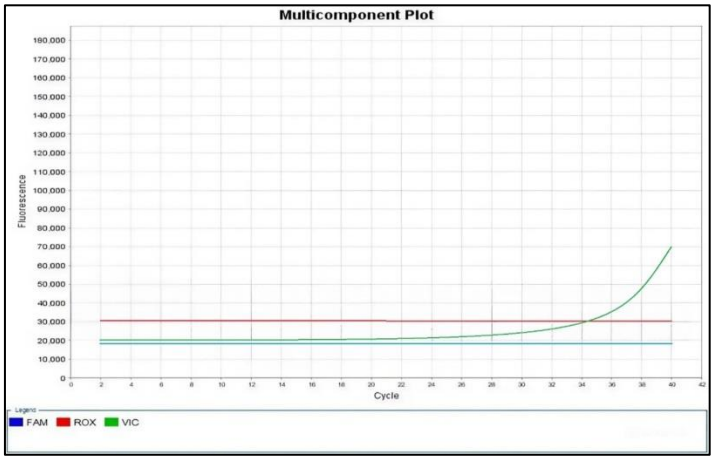

Fig. 1: Multicomponent plot with Homozygous GG genotype for FoxP3 rs2232365 (A/G polymorphism).

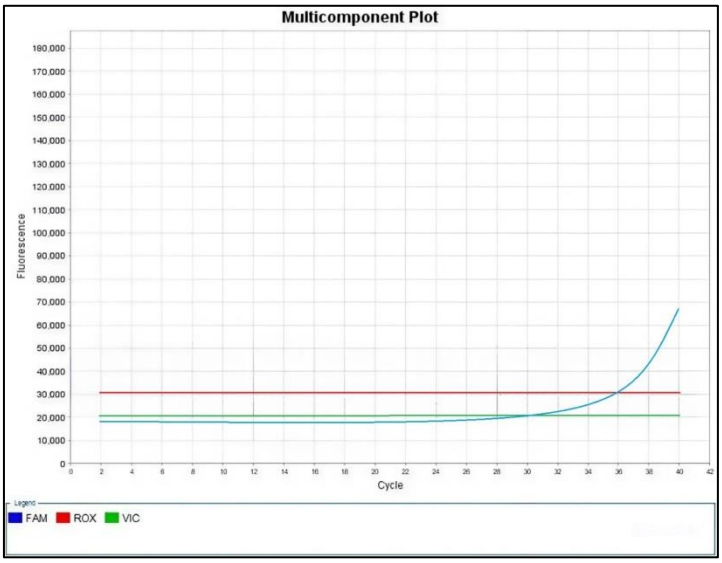

Fig. 2: Multicomponent plot with Homozygous AA genotype for FoxP3 rs2232365 (A/G polymorphism).

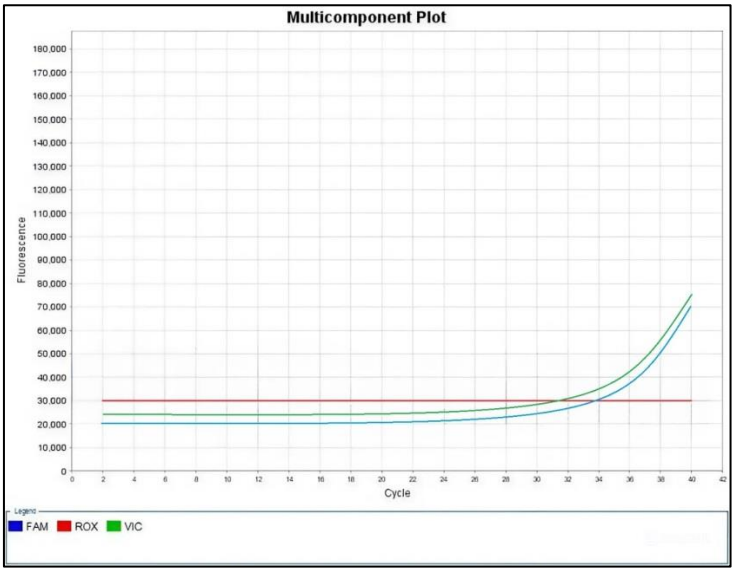

Fig. 3: Multicomponent plot with AG genotype for FoxP3 rs2232365 (A/G polymorphism).

Table 7: Comparisons of FOXP3 polymorphisms regarding asthma control level.

Asthma control level	AA (N=25)	AG (N=35)	GG (N=60)	Test	P-value
	N (%)	N (%)	N (%)		
Controlled	15 (60%)	23 (65.7%)	36 (60%)		
Partially controlled	7 (28%)	7 (20.0%)	20 (33.3%)	1.21	0.86
Uncontrolled	3 (12%)	5 (14.3%)	4 (6.7%)		

Test: -The chi-square test

The genotypic distribution exhibited no significant difference among uncontrolled, partially controlled, and well-controlled patients in rs2232365 (p-value = 0.86), as displayed in this table

Table 8: Relationship between PFTs, laboratory results, and gene polymorphism (rs2232365) among the asthmatic group.

Variables	AA (N=25)	AG (N=35)	GG (N=60)	Test	P-value
Ig E (IU/ml)				**5.61**	**0.002***
Mean± SD	223.91±47.02	238.17±51.32	257.06±57.35		
Eosinophils in blood				**4.64**	**0.004***
Mean± SD	0.78±0.11	0.83±0.06	0.95±0.11		
Neutrophils in blood				1.48	0.35
Mean± SD	132.64±170.04	75.32±118.05	117.6±5.6		
Lymphocytes in blood				0.49	0.49
Mean± SD	2.16±0.22	2.24±0.27	2.2±0.2		
Eosinophil in sputum				**5.62**	**0.001***
Mean± SD	6.89±1.02	7.48±0.63	8.63±1.04		
FVC (%) before bronchodilator				1.03	0.21
Mean± SD	96.93±7.26	96.21±7.76	93.18±7.8		
FVC (%) after bronchodilator				0.84	0.48
Mean± SD	114.41±7.45	114.47±7.41	115.52±5.88		
FEV1 before bronchodilator				1.65	0.92
Mean± SD	72.7±10.32	69.68±13.01	71.62±10.1		
FEV1 after bronchodilator				0.94	0.84
Mean± SD	97.93±7.26	92.68±16.72	90.32±10.35		
FEV1/FVC ratio before bronchodilator				1.26	0.16
Mean± SD	74.7±7.34	72.12±9.84	71.96±7.4		
FEV1/FVC ratio after bronchodilator				0.64	0.26
Mean± SD	84.±9.24	80.76±12.73	80.23±7.8		

The one-way ANOVA test in this table indicated that the GG genotype of rs2232365 polymorphisms was associated with significantly higher IgE and Eosinophils (both in blood and sputum) compared to AA and AG genotypes ($p < 0.05$).

DISCUSSION

Bronchial asthma (BA) and other allergic illnesses exhibit persistent inflammation and share a number of pathogenic pathways. With pertinent clinical results and significant shifts in therapeutic methods, our understanding of these systems has advanced dramatically over the past fifty years[1].

According to some studies, the development of immune-mediated disorders may be influenced by the malfunctioning of regulatory T cells as a result of genetic variants and epigenetic modifications that impact molecules found in regulatory T cells, such as the FOXP3 gene[5].

Our study showed a significant age difference between asthmatic patients and controls with the patient group exhibiting an increase in age compared to controls (p-value < 0.001) and the mean of age was 46.96±14.57. This is in line with Lin et al.[7] who found that the mean age of asthma was 45.31±17.72 years (range, 15–105 years), also Monadi et al.[8] displayed that, in a total of 120 patients with asthma and 115 healthy controls, the mean age of patients and controls were 48±10 and 42.5±10 years old, respectively.

The gender distribution in this study showed a female predominance (70% in the asthma group),

though this was not statistically significant between groups. patients. This agrees with Ismail[9], who determined the demographic and clinical characteristics of bronchial asthma patients in Abbasia Chest Hospital, in which the incidence of asthmatic female patients was 62.7%. However, these results did not match the results of a study by Ezzat[10], who did a study on 140 asthma patients in Cairo to ascertain the prevalence of asthma in adults, finding that male patients had a greater incidence of asthma (55%), compared to female patients.

Our research showed high prevalence of family history (88.3%) in our asthmatic group supports the genetic component of asthma. This agrees with Lababidi et al.[11] who conducted a study on 40 patients, in which 57.5% of them had a family history of asthma. However, this study is not in agreement with a study done by Mostafa.[12] who found that 39% of the studied groups had a positive family history. In harmony, Mabrouk et al.[13] studied bronchial asthma patients in Mahalla Chest Hospital, they reported that 66.98% of the studied cases were female and 60.85% of the studied cases had a positive family history of the disease.

The distribution of asthma severity in our study showed that mild persistent and moderate persistent cases were most prevalent (34.2% and 32.5% respectively), while severe persistent asthma accounted for 17.5%. Based on symptom control levels, most of cases were well controlled (61.7%), while only a minority (11.7%) remained non-controlled. These results agree with Al-Tameemi and Habeeb[14] who noted that among 50 confirmed asthma patients included in their study; 22% of patients showed severe asthma, 42% showed moderate asthma and 36% showed mild asthma, also Mabrouk et al.[13] reported that 28.77% of cases were severe persistent cases, 29.72% were mild persistent cases, and 41.51% were moderate persistent cases.

There was significant elevation in IgE levels and eosinophil counts in asthmatic patients compared to controls ($p<0.001$). the mean of IgE levels were (239.33±53.8 IU/ml) and eosinophil % was (0.83±0.1vs 0.15±0.02). This agrees with sarhan et al.[15] who found high level of eosinophil count with mean 0.69±0.79% in asthmatic groups.

The distribution of FOXP3 rs2232365 polymorphism showed significant differences between asthmatic and control groups ($p=0.004$), with the GG genotype being most prevalent in both groups but showing different distributions (50% vs 70.8%).

This partially agrees with Salah et al.[16] who looked at FOXP3 polymorphisms rs2232365 in children with asthma versus healthy controls found that GG was the most prevalent genotype, followed by AG and AA and There was no discernible difference in the distribution of genotypes between asthmatic patients and the control group ($p >0.05$) .Furthermore, Bottema et al.[17] reported significant associations between FOXP3 SNPs and asthma sensitization ($p< 0.001$), although they studied different polymorphisms (rs3761548). While we examined rs2232365, both studies demonstrate the crucial role of FOXP3 polymorphisms in asthma susceptibility.

Although Raedler et al.[18] reported a greater expression of FOXP3 in asthmatic patients compared to healthy people, Provoost et al.[19] study revealed that FOXP3 protein expression within Treg-cells was significantly decreased among individuals with asthma.

The association between the GG genotype and higher levels of (IgE, eosinophils) provides insight into potential mechanistic pathways. This disagrees with Salah et al.[16] who reported that serum IgE level had no effect on FOXP3 gene polymorphisms, also hassannia et al.[20] found no association of rs2232365 SNP neither the levels of IgE nor peripheral blood eosinophil.

CONCLUSION

Based on the prior findings, we have identified the correlation between the FOXP3 polymorphism rs2232365 and bronchial asthma patients at Aswan University hospital. The distribution of FOXP3 rs2232365 genotype revealed a prominent disparity among mild, moderate, and severe persistent cases. The GG genotype of rs2232365 polymorphisms was associated with significantly higher IgE and Eosinophil (both in blood and sputum) compared to AA and AG genotypes.so The GG genotype of rs2232365 may act as a risk allele for bronchial asthma.

This work has certain limitations, such as sample size and lack of evaluation of additional coding SNPs in the FOXP3 genes

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