Original article

FOXO3a gene polymorphism and bronchial asthma in Egyptian children

***Background**: FOXO3a proteins play multiple crucial roles in immune response. FOXO3 inhibits T cell proliferation, induces T cell apoptosis via upregulation of proapoptotic proteins and it suppresses T cell activation preventing autoimmunity. The role of FOXO3a gene in the pathogenesis of bronchial asthma has been studied in few ethnic groups and revealed its implication in asthma pathogenesis. **Objectives**: The aim of the current study is to detect the association between single nucleotide polymorphism of the FOXO3a gene (rs13217795) and bronchial asthma, atopy and asthma severity in Egyptian children. **Methods**: The current cross-sectional case-control study was performed on 75 asthmatic children aged 2 to 12 years following up in the pulmonology outpatient clinic in Children's hospital, Cairo University and 75 age and sex matched healthy controls. Candidates were subjected to clinical evaluation in addition to genotyping for the FOXO3a gene polymorphism using PCR-RFLP technique. **Results**: The highest frequency was for the heterozygous type CT in both cases and controls groups. The genotype frequencies of mutant type TT for cases and controls were 12 % and 16% respectively, and the T allele frequencies were 37.2% in cases and 46.7% in the control group while CC genotype was present in 37.3% of asthmatic patients and 22.6% in the controls and the C allele was detected in 62.8% and 53.3% for cases and controls respectively. No statistically significant differences were observed between asthmatic patients and controls regarding the different genotypes of the FOXO3a gene polymorphism (p=0.161). No significant association was detected between the different genotypes of the FOXO3a gene polymorphism and the atopic status (p=0.536) or the different grades of asthma severity (p= 0.545). **Conclusions**: The study of FOXO3a gene polymorphism (rs13217795) in asthmatic Egyptian children revealed low frequency of the mutant TT genotype among cases and controls. In the current study, FOXO3a polymorphism has no role in the pathogenesis of asthma or atopy. Moreover, it has no relation to degree of disease severity.*

Keywords: Asthma, FOXO3a, gene, children, Egyptian, polymorphism.

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INTRODUCTION

Bronchial asthma is the most prevalent chronic immunological disorder in childhood period. It is characterized by airways inflammation and bronchial hyper-responsiveness where complex gene-environment interactions play a significant role in its pathogenesis. [1,2] Asthma is a disorder whose primary cause can probably be linked to the disturbed immunoregulatory mechanisms at the lymphocyte level.[3] Inflammation in asthma occurs due to excessive infiltration of cells predominantly T lymphocytes, eosinophils, neutrophils, macrophages and mast cells along with elevated levels of cytokines and tumor necrosis factor-α.[4]

FOX (Forkhead box) proteins are a family of transcription factors that bind condensed chromatin during cell differentiation and play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity.[5] FOXO3 is a member of the forkhead box class O (FOXO) subfamily[6]. The role of FOXO family members in inflammation is a complex role involving wide range of target cells and genes.

FOXO3 inhibits T cell proliferation, induces T cell apoptosis via upregulation of proapoptotic proteins such as Puma and Bim and it suppresses T cell activation preventing autoimmunity.[7] Moreover, FOXO3 inhibits the capacity of dendritic cells to produce IL-6 so it can control the magnitude of T cell in immune responses.[8] FOXO3 also promotes B cell apoptosis via upregulation of proapoptotic genes and antiproliferative genes.[9]

Mice studies revealed that FOXO3a deficiency leads to T cell hyper activation, inflammation, increased activity of proinflammatory genes; IL2 and IFN-γ and increased activity of pro-inflammatory transcription factor NF-κB.[10]

Moreover, inhibiting FOXO3a gene leads to promotion of autoaggressive T cells in mouse model of multiple sclerosis.[11]

FOXO3 also regulates Treg cells generation through affecting FOXp3 expression.[12] Treg cells are responsible for keeping proper immunological tolerance.[13] Loss of FOXP3 expression may lead to maintaining a state of chronic inflammation through loss of Treg cell functions and or numbers.[14] Moreover, FOXO3a initiates TGF-1 dependent pathway in monocytes through which it reduces proinflammatory cytokines and promotes the anti-inflammatory cytokines production.[15]

FOXO3a alteration has been implicated in the pathogenesis of different disease conditions such as idiopathic pulmonary fibrosis, lung cancer and bronchiolitis.[16-18] Few studies were conducted on bronchial asthma in different ethnic groups. However, studies in our Egyptian population are still lacking. Recently, a significant association between the FOXO3a single-nucleotide polymorphism (rs13217795) and susceptibility to asthma and was described by Barkund et al. (2015) and Amarin et al. (2017).[19,20] Furthermore, the study by Barkund et al. (2015) revealed that FOXO3a SNP (rs13217795) is significantly associated with asthmatics plausibly contributing to the hyperactivity of T cells, neutrophils, and mast cells, increased production of proinflammatory cytokines, and downregulation of anti-inflammatory cytokines[19].

Therefore, the current study aimed to detect if there is an association between single nucleotide polymorphism (rs13217795) of the FOXO3a gene and bronchial asthma, atopy and asthma severity among Egyptian children.

METHODS

Study design and subjects

The present cross-sectional case-control study was conducted at the University Children's Hospital, Faculty of Medicine, Cairo University on 75 asthmatic patients following up at the allergy and pulmonology outpatient clinic. Patient diagnosis and assessment of severity classification (mild, moderate and severe persistent) were done according to GINA guidelines for asthma.[1] Patients' age ranged from 2 to 12 years. Seventy-five age and sex matched healthy children with no history or symptoms of bronchial asthma, pulmonary diseases, allergy, or atopic dermatitis and no first – degree relative suffering from bronchial asthma or atopic dermatitis were included as controls. Patients were excluded if having other causes of wheezy chest: bronchiolitis obliterans, cystic fibrosis, autoimmune disorders, immunocompromised patients or patients with gastro-esophageal reflux disease.

All patients were subjected to a complete clinical study (thorough history and physical examination) upon study inclusion, with emphasis on chest symptoms, other atopic manifestations and severity of disease. FOXO3a gene polymorphism using PCR-RFLP technique was performed for all patients and controls.

Detection of FOXO3a Polymorphism using PCR-RFLP

Genomic DNA of included subjects was isolated from venous blood collected on ethylene diamine tetra-acetic acid vacutainer tubes using G-spinTM total DNA extraction kit (iNtRON biotechnology, Korea). Genotyping of FOXO3a polymorphism was performed using PCR-RFLP technique. The primer sequences used were as follows: Forward Primer 5′-CTC CTT GGT CAG TTT GGT G 3′; Reverse Primer: 5′-ATG AGT GAA GAT GGA AGT AAG C -3′[19]. Amplification was performed using 2X PCR master mix solution (iTaqTM) (iNtRON biotechnology, Korea) in total volume of 20µl. For PCR amplification, an initial denaturation at 95ºC for 5 min was followed by 35 cycles consisting of 30 sec of denaturation at 95 ℃, 30 sec of annealing at 62 ℃ and extension for 1 min at 72 ℃ and then a final extension at 72 ℃ for 5 min. Amplification products were subjected to restriction digestion by the enzyme PagI (Thermoscientific, USA). Fragments were separated on 2% agarose gel and bands were visualized by ethidium bromide staining under ultraviolet light. This reaction yielded one fragment of 667 bp indicating a homozygous wild genotype (CC), or two fragments of 391 and 275 bp indicating a homozygous mutant genotype (TT) while the presence of 677,391 and 275 bp products indicated heterozygous genotype (CT), Figure (1).

Ethical considerations

The aim and nature of the study was explained for each parent before inclusion. An informed written consent was obtained from parents / surrogates before enrollment. The ethical committee of the Pediatrics department, Faculty of Medicine, Cairo University approved the work and it conforms to the provisions of the Declaration of Helsinki in 1964 and its later amendments or comparable ethical standards.

Sample size

Sample size calculation was performed using Power and Sample Size Calculator program version 3.0.43. It was based on the following inputs: Power of 80%, type I error of 0.05, equal number of candidates in both cases and controls, true

difference in means between groups of 0.12 and standard deviation of 0.26. It was found to be 73 children in each group (total = 146).

Statistical analysis

Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies and percentages when appropriate. Comparison of numerical variables between the study groups was done using Kruskal Wallis test. For comparing categorical data, Chi-square (±2) test was performed. Exact test was used instead when the expected frequency is less than 5. Odds ratio (OR) and its 95% confidence interval (95% CI) was calculated for genotypic frequencies between cases and controls. P values less than 0.05 were considered statistically significant. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

RESULTS

Demographic data

The study included 75 asthmatic children; 37 (49.3%) experienced mild asthma, 17 (22.7%) had moderate asthma and 21 (28%) showed severe persistent asthma. Patient diagnosis and assessment of severity classification were done according to GINA guidelines for asthma.[1] Patients were aged 2-12 years with a mean age of 6.3 ±1.9 years. Forty-two (56 %) were males and 33 (44 %) were females. A positive family history of allergic disease as conjunctivitis, allergic rhinitis, skin allergy or bronchial asthma was present in 93.3%. Atopic manifestations were found in 55 cases (73%). Seventy-five age and sex matched healthy children with no previous history of atopic disorders were include as controls. Table (1) shows the basic demographic data of asthmatic patients.

FOXO3a gene polymorphism

Genotypic distribution and allele frequencies of the FOXO3a gene polymorphism did not show statistically significant differences between cases and controls as shown in Table (2). Generally, CT genotype represented the most frequent genotype in cases and controls (50.7% and 61.4% respectively) followed by CC genotype (37.3% and 22.7% respectively) and then TT genotype (12% and 16% respectively).C allele was more prevalent in both cases and controls (62.8% and 53.3% respectively) while the T allele was detected in 37.3% of cases and 46.7% of the control group.

Asthmatic patients were subdivided into 3 groups according to disease severity; mild, moderate and severe persistent asthma. Genotypic distribution and allele frequencies did not show a significant difference between mild, moderate and severe persistent cases (p-value = 0.545, p-value 0.961 respectively) (Table 3). CT genotype was the most frequent genotype in the 3 groups (60%, 47% and 48% respectively). Regarding allelic frequencies, the C allele was the most prevalent in the 3 groups (62%, 65% and 62% respectively).

Comparison between asthmatic patients having atopic manifestations (73%) and those without atopic manifestations (27%) revealed no statistically significant difference regarding the genotypic distribution (p-value= 0.536) or the allelic frequencies (p-value= 0.722) as demonstrated in Table (4).

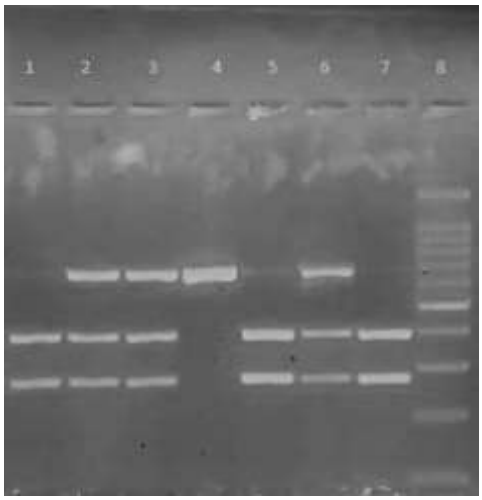

Figure 1. PCR-RFLP products of FOXO3A gene polymorphism.

Lanes 1, 5 and 7 represent RFLP pattern for homozygous mutant (TT). Lanes 2, 3 and 6 represent RFLP pattern for heterozygous (CT). Lane 4 represents RFLP pattern for homozygous wild type (CC). Lane 8 consists of the 100 bp DNA marker.

Table 1. Basic demographic data of asthmatic patients

Variable	Asthmatic patients (N=75) N(%)
Sex	
• Male	42 (56%)
• Female	33 (44%)
Residence	
• Urban	41(54.7%)
• Rural	34 (45.3%)
Family history for allergic conditions	70 (93.3%)
Asthma severity	
• Mild persistent	37 (49.3%)
• Moderate persistent	17 (22.7%)
• Severe persistent	
Atopic manifestations	
• Present	21 (28%)
• Absent	55 (73%)

Table 2. Genotypic distribution and allele frequencies of FOXO3a gene polymorphism in asthmatic patients and controls

Variable	Patients (N=75) No. (%)	Controls (N=75) No. (%)	[a]P -value	Odds ratio	(95% [b]CI) Lower	Upper
CC genotype	28 (37.3%)	17 (22.7%)				
CT genotype	38 (50.7%)	46 (61.3%)	0.067	0.502	0.239	1.051
TT genotype	9 (12%)	12 (16%)	0.143	0.455	0.159	1.306
C allele	94 (62.8%)	80 (53.3%)				
T allele	56 (37.2%)	70 (46.7%)	0.102	0.681	0.430	1.080

[a]P value less than 0.05 is statistically significant; [b]CI= Confidence interval

Table 3. Relation between genotypic distribution and allelic frequencies of FOXO3 gene polymorphism and different grades of asthma severity.

Variable	Mild Persistent (N= 37) No. (%)	Moderate Persistent (N= 17) No. (%)	Severe Persist (N=21) No.(%)	[a]P –value
CC genotype	12 (32%)	7 (41%)	9 (42%)	0.545
CT genotype	22 (60%)	8 (47%)	8 (48%)	
TT genotype	3 (8%)	2 (12%)	4 (20%)	
C allele	46 (62%)	22 (65%)	26 (62%)	0.961
T allele	28 (38%)	12 (35%)	16 (38%)	

[a]P value less than 0.05 is statistically significant

Table 4. Relation between genotypic distribution and allelic frequencies of FOXO3a gene polymorphism and atopic manifestations

Variable	Atopic (N = 55) No. (%)	Non atopic (N= 20) No. (%)	[a]P –value
CC genotype	19 (34%)	9 (45%)	
CT genotype	30 (55%)	8 (40%)	0.536
TT genotype	6 (11%)	3 (15%)	
C allele	68 (61%)	26 (65%)	
T allele	42 (39%)	14 (35%)	0.722

[a]P value less than 0.05 is statistically significant

DISCUSSION

Genetics play a significant role in the development of asthma and allergy as demonstrated in family and twin studies[21]. Genetic researches; genome-wide linkage studies and case–control studies, have identified several genomic regions and more than 100 genes associated with allergy and asthma in different populations[22].

FOXO transcriptional factors exert crucial roles in immunoregulation as FOXO members are predominately expressed in the peripheral lymphoid organs. FOXO3a deficiency has been associated with spontaneous lymphoid proliferation, inflammation in different organs and increased hyperactivated T helper cells.[10]

The association between FOXO3a polymorphisms and different types of inflammatory diseases as idiopathic pulmonary fibrosis, lung cancer, bronchiolitis, Crohn's disease, rheumatoid arthritis and Sjogren's syndrome has been demonstrated in previous studies.[16-18,23,24] To our knowledge, the association between FOXO3a gene polymorphism and asthma has only been explored in two previous studies[19, 20]. Herein, we focused on exploring the association between FOXO3a gene polymorphism and asthma, atopy and asthma severity in Egyptian population.

In this study, the CT, CC and TT genotypes of the FOXO3a gene polymorphism didn't show any statistically significant difference in distribution between asthmatic patients and controls. Generally, CT genotype represented the highest prevalent genotype in both patients and controls while the TT genotype was the least. On the other hand, Barkund et al. (2015) determined that in their asthmatic patients, the TT mutant genotype had the highest frequency (51.75%) and the T mutant allele showed the highest prevalence (71%).[19] Similar results were shown by Amarin et al. (2017) where the frequency of mutant TT genotype was the highest (49%) and the mutant T allele was present in 64% of asthmatics.[20]

Regarding the controls, a previous study showed a near similar frequency compared to our study, with the heterozygous CT genotype showing the highest frequency (57 %) and the TT genotype the lowest (22 %) with the C and T allele frequencies were nearly the same (49% and 51% respectively).[20] Similarly, another study stated that the CC genotype represented the highest prevalence (50.7%) in controls while the TT genotype had the least (12.7%) and the C allele was more prevalent (69%) than the mutant T allele.[19] The similarities in the frequency of the gene distribution in controls in our study and the previous two studies in addition to the discrepancy in the distribution in asthmatic patients especially for the mutant TT genotype suggests that there may be no association between FOXO3a gene polymorphism and asthma in the Egyptian population. Genotypic distribution and allele frequencies of FOXO3a gene polymorphism did not show a significant difference between different grades of asthma severity; mild, moderate and sever persistent groups (p-value= 0.545, p-value= 0.961 respectively). Furthermore, asthmatic patients having atopic manifestations did not differ significantly from those without atopic manifestations as regards the genotypic distribution (p-value= 0.536) or the allelic frequencies of FOXO3a polymorphism (p-value= 0.722). To date, no studies are available addressing the association between the FOXO3a gene polymorphism and atopy or asthma severity.

FOX family members' role in inflammation is highly cell and context specific. As some of FOX family transcription factors maintain the naïve T cells and prevent autoimmunity. As a result, loss of activity of one of these factors may lead to chronic inflammation. On the other hand, some of the FOX family members promote chronic inflammation by maintaining survival of inflammatory cells such as neutrophils.[14] Chronic inflammation pathogenesis is a complex process of interactions between different inflammatory cells, transcriptional factors and inflammatory genes. Moreover, FOXO3a activity is regulated through posttranslational modification; acetylation, phosphorylation, and ubiquitination which may affect the protein level after transcription.[25]

In conclusion, comparison of our results with other studies indicates that FOXO3a gene polymorphism is not universally associated with asthma, atopy or disease severity. The genetic basis of asthma may differ between different ethnic groups. Future large-scale studies should be conducted focusing on the presence or absence of a role of the FOXO3a gene polymorphism and estimation of FOXO3a protein level in relation to asthma, atopy and asthma severity in different ethnic groups.

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