Association Study of FOXO3a Single-Nucleotide Polymorphism and Bronchial Asthma in Egyptian Children

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Asthma is the most common chronic illness in children and is a leading cause of childhood hospitalization and school absenteeism. Asthma presents with different phenotypes depending on age, gender, genetic background, environmental exposures and epigenetic factors. Forkhead box O3 (*FOXO3*) is a transcription factor involved in the pathogenesis of a number of inflammatory and respiratory diseases. The study aims to investigate the association between the SNP rs13217795 in *FOXO3* gene and pediatric onset asthma in the Egyptian population. Ninety asthmatics and 160 healthy controls were subjected to genotyping of FOXO3 SNP (rs13217795) using the PCR–RFLP method. The proportion of homozygous (CC) and heterozygous (CT) genotypes was lower in the asthmatic group compared to the control group but statistically insignificant; P> 0.05. On the other hand the proportion of the mutant homozygous (TT) genotype in asthmatic group was higher; 30 (33.3%) than the control group; 28(17.5%), the difference was significant in Recessive model of disease penetrance with Odds ratio OR (95% CI) of 2.4(1 – 5.49) and P=0.039. This association was more pronounced in male gender; OR and 95% CI of 5.3 (1.4- 19.3) and P=0.01. In conclusions, Egyptian children carrying the mutant (TT) genotype were at higher risk to develop asthma with a higher risk in male gender.

Asthma is an inflammatory disorder, characterized by infiltration of the airway wall by eosinophils, mast cells, and T-helper lymphocytes type 2 (Th2) [1]. Both increased recruitment and decreased cell apoptosis lead to accumulation of T-lymphocytes in the airways of asthmatics, while eosinophils are recruited to the airways of asthmatics by chemotactic agents then induce epithelial damage and remodeling [2]. Allergens stimulate airway epithelial cells to secrete cytokines such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 that release Th2 cytokines principally IL-5 and IL-13 [3,4]. The prevalence of asthma in Egypt increased from 2.2% (two decades ago) to 6.3–7.2% in recent epidemiological studies among school students. This reflects the effect of environmental factors on gene expression [5,6].

FOXO proteins are transcription factors that belong to the Forkhead box family; they are the mammalian homolog of DAF-16, identified in *C. elegans* as a major regulator of lifespan and stress resistance [7]. In mammals, the FOXO subclass includes four members: FOXO1, FOXO3, FOXO4 and FOXO6 (8]. FOXO1 and FOXO3 are the main isoforms expressed in the immune system [7].

The protein kinase B (Akt) is a primary mediator of phosphorylation of FOXO3a that maintain this transcription factors in the cytoplasm, block its translocation to the nucleus hence transcription of target genes [9-11]. Mice deficient for FOXO3a suffer from lymphoproliferation, organ inflammation, and increased activity of helper T cells, indicating the protecting role

of FOXO3a from T cell hyperactivity [12]. In clinical studies, phosphorylation and loss of functional FOXO3a was documented in T lymphocytes from patients with osteoarthritis and rheumatoid arthritis [13]. As the regulation of immune system activity depends partly on apoptotic pathways, FOXO proteins may modulate Fas signaling to get rid of activated T cells [14]. FOXO3a was also linked to caspase-induced apoptotic death [15,16], human longevity [17,18], Crohn's disease and rheumatoid arthritis [19]. Moreover, it acts downstream of many of the complex signaling pathways in the pathogenesis of bronchial asthma, for example Notch, [20], Wnt/β-catenin [21], STAT3 [22] and NF-κB signaling pathways [23]. Many of these pathways are involved in inflammation and tissue remodeling of the airway of asthmatics. Based on these findings the polymorphism of FOXO3a gene was suggested to modulate hyperactivity of T cells and inflammatory cytokines in asthma patients.

Materials and Methods

The study included 90 children with bronchial asthma and 160 age and sex matched controls. The patients group consisted of 54 males and 36 females with a median, interquartile range (IQR) age of 5 (1-14) years. All patients were subjected to full history taking including age, sex, duration of illness, family history of allergic diseases, consanguinity and possible risk factors. All patients were diagnosed and classified into intermittent, mild, moderate and severe asthma according to Global Initiative for Asthma (GINA) (2015) guidelines [24]. Thorough clinical examination including liver, spleen and lymph node examination was done as well. Whereas, 160 healthy control volunteers were also recruited from a similar confined population to determine background population allele frequencies. None of the control subjects had a history of allergic diseases, or history of asthma or other pulmonary diseases. The study was permitted by the local ethical committee and in accordance with Helsinki declaration of Bioethics and its later amendments. Informed consent was obtained from all participants' caregivers.

Detection of rs13217795 SNPs in the human FOXO3a gene by PCR-RFLP

Peripheral blood samples were withdrawn from patients as well as the healthy volunteers in sterile EDTA vaccutainers and stored in -20^{0}C till DNA extraction. Genomic DNA was extracted using Thermo Scientific Gene Jet Whole Blood Genomic DNA purification mini kit (Cat.K0781), according to the manufacturer's instructions. A volume of 5 µl Genomic DNA was added to a PCR reaction mixture of 25 µl including 12.5 µl ready to use My Tag™ Red PCR master mix (2X) (Bioline), 1 µl of 25 pmol of each of forward and reverse specific primers of gene and 5.5 µl of Nuclease free distilled water. The genotype was determined by restriction fragment length polymorphism (RFLP) method after PCR amplification of the FOXO3a region containing the polymorphism for rs13217795 C>T with primers 5′-CTCCTTGGTCAGTTTGGTG-3′ (forward) and 5′-ATGAGTGAAGATGGAAGC-3′(reverse).
Amplification conditions were: 15 min at 94 ^{0}C for pre-denaturation followed by 45 cycles of 20 s at 94 ^{0}C, 30 s at 56 ^{0}C and 60 s at 72 ^{0}C and a single cycle of 72^{0}C for 8 min using the thermal cycler Applied Biosystems2720.The PCR amplicons for rs13217795 C>T were digested with PagI (BioLabs) for 15 minutes at37, and then separated by 2% agarose gel electrophoresis containing ethidium bromide, then visualized using UV transilluminator. The size of the PCR-fragments was estimated using a 50bp DNA ladder (GeneRuler™). For rs13217795 genotyping: heterozygous wild genotype CT produced 3 bands at 667bp, 321bp, and 275bp, homozygous wild genotype CC produced one band at 667bp, and mutant genotype TT produced two bands at 321bp and 275bp (Figure 1). Direct sequencing of 5% of samples was conducted for confirmation of the genotypes obtained by RFLP method and results were 100% concordance.

Statistical Analysis

Statistical analysis was done using statistical package for social sciences (SPSS), computer software (version 22), IBM software, USA. Data were described in the form of median (IQR) for quantitative data, and frequency and proportions for qualitative data. A *P* value <0.05 was considered statistically significant. Differences were analyzed between the groups by non parametric tests. Chi-square (χ2) test was used to calculate the statistical

difference between genotype and allelic frequencies in asthmatic and control children. Calculation of Odds ratio with 95% confidence interval (CI) was used to determine the association between asthmatic children and control's genotype and allele frequencies. Microsoft Excel Sheet was used to analyze whether the distribution of genotypes was in agreement with Hardy-Weinberg equilibrium.

Figure 1.Agarose gel electrophoresis of PCR product. Lanes 1& 7 (CC genotype 667bp), Lane2 (TT genotype 321bp, and 275bp) Lanes 4-6 (CT genotype 667bp, 321bp, and 275bp).

Results

Our study population consisted of 160 controls and 90 asthmatics. The demographic characteristics of the study groups are shown in Table 1. The median age (range) of controls and asthmatics patients was 7 (2-14) years and 5 (1-14) years, respectively. The median age of asthmatic patients shows no significant different from the median age of controls ($P > 0.05$). The proportion of female controls and asthmatics patients was 50% and 40 % respectively. There was no statistically significant association between gender and case–control status ($P > 0.05$).

Table 1. Comparison between the study groups as regarding demographic data.

	Group	Cases	Control	*P* value
Age (years)	Cases	5(1-14)	7(2-14)	NS
Weight (kg)	Cases	19(10-50)	24.5 (5-45)	0.02
Height (cm)	Cases	109(60-140)	121.5 (80-140)	0.001
Sex	Male	54(60%)	80(50%)	NS
	Female	36(40%)	80 (50%)	
Rhinitis	Yes	57 (65%)		
	No	33 (35%)		

P>0.05 is not significant (NS).

The results of the frequencies distribution of rs13217795 C>T genotype variants in both control and study groups show no deviation from Hardy–Weinberg equilibrium ($P = 0.37$ and 0.43, respectively). Genotype-based associations were investigated under the general, recessive, and dominant models of disease penetrance. The results of these analyses are presented in Table 2. The control group comprised 32 (20%) homozygous wild genotype (CC), 100 (62%) heterozygous (CT), and 28 (17.5%) homozygous mutant genotype (TT). The proportion of wild homozygous and heterozygous genotypes were lower in the asthmatic group compared to the control group but statistically insignificant; 12 (13.3%) and 48 (53.3%) respectively, (P>0.05). On the other hand the proportion of the mutant homozygous genotype in asthmatic group was statistically significant higher; 30 (33.3% vs), in the recessive model of disease penetrance (P= 0.039). The odds ratio of asthma mutant genotype was 2.46 (1.05 to 5.8) times higher, in reference to the pooled count of heterozygous and wild allele homozygous. The general and dominant models of disease penetrance were not statistically significant (P> 0.05). The frequency of wild allele was 51%, and 40 % in controls and asthmatics respectively. Both case–control allele-based associations were statistically insignificant ($P = 0.08$), the results are presented in Table 2.

Table 2. Analysis of genotype and allele frequencies of FOXO3 (rs13217795) SNP in asthmatic children and controls

Model	Genotype	Asthma	controls	OR (95% CI)	*P*-value
General	CC	12(13.3%)	32 (20%)	1.00	NS
	CT	48 (53.3%)	100 (62.5%)	1.38 (0.73-2.61)	
	TT	30 (33.3%)	28 (17.5%)	1.35 (0.61-3.00)	
Recessive	TT	30 (33.3%)	28 (17.5%)	2.46 (1.05-5.80)	0.039
	CC-CT	60 (66.7%)	132 (82.5%)		
Dominant	TT-CT	78 (86.7%)	128 (80%)	1.64 (0.59-4.57)	NS
	CC	12 (13.3%)	32 (20%)		
Allele	C	72(40%)	164(51%)	0.6(0.4- 1)	NS
	T	108(60%)	156(49%)	1.6(0.9-2.9)	

P>0.05 is not significant (NS).

The study populations were further stratified by gender and it was observed that in asthma group males had a higher mutant type (TT) frequency (37%) than females (27.5%) (Table 3). It was also observed that the mutant type (TT) frequency was five times higher in male gender in asthmatics than in controls odds ratio 5.3 (95%CI, 1.4- 19.3) and this association was significant (*P*=0.01).

Table 3. Analysis of genotype and allele frequencies of FOXO3(rs13217795) SNP in study population distributed according to gender.

Gender		Genotype			Allele	
		CC	TT	CT	C	T
Female	Patients (n= 36)	4(11.1%)	10(27.8%)	22(61.1%)	30(41.6%)	42(58.3%)
	Control (n=80	16(20%)	20(25%)	44(55%)	70(43.7%)	90(56.3%)
	OR (95%CI)	0.5 (0.1 – 2.6)	1.3 (0.3- 4)	1.3(0.4- 3.9)	0.9(0.4- 2.03)	
	P value	NS	NS	NS	NS	
Male	Patients (n=54)	8(14.8)	20(37)	26(48.1)	46(42.6)	62(57.4)
	Control (n=80)	16(20%)	8(10%)	56(70%)	88(55%)	72(45%)
	OR (95%CI)	0.7(0.2-2.6)	5.3(1.4 19.3)	0.4(0.1-1)	1.6(0.8-3.3)	
	P value	NS	0.01	NS	NS	

P>0.05 is not significant (NS).

Discussion

Pathogenesis of allergic asthma is complex and results from interplay between multiple environmental and genetic factors that cause allergen-induced airway inflammation mediated by TH2 cells [25], and alternatively activated macrophages [26]. FOXO is a family of transcription factors that translate micro-environmental stimuli including oxidative stress and cytokines into specific genetic expression programs. FOXO3a has been extensively studied in metabolism stress-resistance, regulation of cell proliferation, apoptosis, and longevity

[27]. Currently, it is known to increase autophagy and protect cells from environmental stresses. For example, Li *et al.,* 2018 [28], demonstrated that ethanol-induced phosphorylation of FOXO3 induce inflammatory macrophage apoptosis and alternatively activated macrophages proliferation. A meta-analysis was conducted by Bao *et al.,* 2014 [18] and indicates a significant association of five FOXO3A gene polymorphisms including intronic rs13217795 with longevity, and the effect of rs13217795 was male-specific. FOXO3 (rs13217795) SNP was linked to susceptibility to bronchial asthma in only two studies. We conducted a case control study to investigate the possibility of genetic association between C>T transition SNP and the susceptibility to bronchial asthma in Egyptian children suffering from bronchial asthma.

Results of our study revealed that the single-nucleotide variant under study (rs13217795) was polymorphic in our study population. The minor allele in asthmatics was the wild allele(C), while minor allele in controls was the mutant (T) allele. This data is partially consistent with that obtained by Barkund *et al.* 2015 [29] in the study done on Indian population, however the difference was not significant in our study $P>0.5$. Dissimilarly Amarin *et al.,* 2017 [30] denoted that the wild allele was the minor allele in both asthmatic and control groups based on adult Jordan population. Furthermore, on stratifying study populations according to sex, the effect of homozygous mutant TT allele is more obvious in male gender; males bearing the (TT) genotype have a risk of 5.3 times to develop asthma as compared to females risk that was 1.3 times. In addition the frequency of the mutant type (TT) was significantly higher in asthmatic males than males in the control group ($P= 0.01$). Our results were partially inconsistent with the results of (Barkund *et al.,* 2015) who first reported a statistically significant association between FOXO3a and asthma in Indian population. They stated that asthmatics are 5.54 times more likely to carry a CT genotype and 21.45 times more likely to carry a TT genotype as compared to CC genotype and the effect of "T" allele is more pronounced in females. On the other hand the result of the study of Amarin *et al.,* in Jordan population was to some extent consistent with our results in that the risk of incidence of asthma for TT genotype is 2.46 and this risk was significant in recessive model but was not significant in dominant model of inheritance. We are in agreement with (Amarin *et al.,* 2017) in 2 other theoretical point of view; first is that rs13217795 is an intronic single-nucleotide variants that may influence gene expression but the actual association with the disease is more likely to be indirect through in linkage disequilibrium of this locus with the causal variant. Second, targeting the FOXO3 gene or its upstream or downstream pathways is very important therapeutic alternative in treating asthma and other inflammatory disorders. For example, it was documented that, curcumin increases FOXO3 mediated gene expression up to two fold [31]. Likewise, food and other environmental factors affecting gene expression may explain the discrepancy of results between different studies in different populations. We also attributed the discrepancy between current results and that of previous studies to different ethnic groups, age groups (as the mean age of participants in the previous 2 studies was over 40 years and in our study was 6.3 years), and the contributions of other genetics or epigenetic factors that also affect

gene expression and pathologic process severity and duration.

The main limitation of our study is the small sample size so larger samples and multi-centers population are recommended. In addition, effect of environmental factors on FOXO3 expression and linkage disequilibrium of rs13217795 with other functional polymorphisms should be studied

In conclusion, A higher risk of children carrying the mutant (TT) genotype to develop asthma may be present in Egyptian pediatric population with OR (95% CI) of 2.4(1 – 5.49) and p=.039 with a higher risk in male gender.

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