Original Research Article

The Egyptian Journal of Immunology
Volume 29 (4), 2022: 25–32.
www.Ejimmunology.org

Association of Forkhead Box O3a (FOXO3a) single - nucleotide polymorphism with bronchial asthma in Egyptian population

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Abstract

Asthma is a common chronic inflammatory condition with a highly complex genetic predisposition and environmental factors which play an important role in its development. Polymorphism in FOXO3a transcription factor has been linked to a number of inflammatory and respiratory diseases such as bronchiolitis and idiopathic pulmonary fibrosis suggesting that it may be implicated in the pathogenesis of asthma. This study aimed to investigate FOXO3a SNP (rs13217795) association with bronchial asthma and its degree of severity in adult Egyptian population. This case control study included 60 asthmatic patients and 40 apparently healthy controls. Peripheral blood samples were collected from all participants. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The study revealed high frequency of the mutant TT genotype of FOXO3a gene in asthma patients (51.7%) than controls (12.5%) with OR= 7.48 & 95% CI (2.58-21.71) (P<0.05) and in severe cases (41.9%) compared to mild and moderate cases (25.8% and12.5%, respectively). T allele frequency showed significant statistical association with asthma, OR= 12.40, 95% CI (5.65-27.19) (P<0.05). However, there was no association between T allele and disease severity. The high frequency of the mutant TT genotype among patients and sever cases may indicates that FOXO3a rs13217795 C>T single nucleotide polymorphism can be considered as a risk factor in development and severity of asthma.

Keywords: Bronchial asthma, FOXO3a, SNP, PCR- RFLP.

Date received: 27 May 2022; **accepted:** 15 July 2022

Introduction

Bronchial asthma is a complex clinical syndrome of chronic respiratory tract inflammation involving many cells and mediators. It is a type I hypersensitivity reaction, that leads to immediate exaggerated immune reaction.[1] Asthma is characterized by bronchial hyper-reactivity, which causes airways narrowing in response to various stimuli and a variable degree of recurrent and reversible airway obstruction. Asthmatic patients suffer from episodes of coughing, wheezing, chest tightness

and shortness of breath which vary in frequency and severity between patients.[2]

According to the Center for Disease Control and Prevention (CDC), bronchial asthma is a low fatality major non-communicable disease, affects 1 in each 13 people.[3] In Egypt, asthma is estimated to be 8.2% and 6.7% between children and adults respectively.[4] Asthma predominantly begins in childhood but involves all age groups, its prevalence is higher in children than in adults. It is the leading chronic illness in childhood, higher in boys than girls and higher in adult females than adult males.[3]

No significant cause has been identified for asthma, a combination of genetic predisposition and environmental factors seem to play significant roles in its development. Asthma is caused by multiple interacting genes, some having a protective effect and others contributing to the disease pathogenesis, with each gene having its own tendency to be influenced by the environment via epigenetic and transcriptional factors.[5]

The Forkhead proteins act as transcription factors, characterized by the presence of a conserved winged helix DNA-binding domain (the 'Forkhead box', or FOX) and hence the name FOX proteins. They are more than 100 in human classified from FOXA to FOXR based on their sequence similarity. They participate in very diverse functions by regulating the expression of genes involved in cell growth, proliferation and differentiation.[6]

FOXO subgroup members regulate gene expression that co-ordinate cellular metabolism, proliferation, oxidative-stress resistance, regulatory T cell development and apoptosis. Their activity is controlled by post-translational modifications, including phosphorylation, methylation, acetylation, ubiquitination and microRNA (miRNA) binding.[7]

FOXO3a regulates gene transcription in the nucleus in a non-phosphorylated form, its phosphorylation via the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway rendering it inactive, stops the transcriptional activation of its target genes and excludes it from the nucleus.[8]

FOXO3a had a key role in immune regulation through suppression of inflammatory cytokine production by dendritic cells and initiation of TGFβ-1 dependent pathway in monocytes reducing the production of pro-inflammatory cytokines including TNF-α, IL-4, and IL-13 and increasing the production of anti-inflammatory cytokine IL-10. [9]

FOXO3 inhibits T cell proliferation and induces T cell apoptosis. FOXO3 induces T cells apoptosis through up regulation of pro-apoptotic genes such as Puma and Bim. FOXO3 also restrains the magnitude of T cell in immune responses by inhibiting the capacity of dendritic cells to produce IL-6.[10]

FOXO3 also regulates FOXp3 expression that is needed to generate regulatory T cells.[11] Regulatory T cells play an important role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive immune responses harmful to the host. FOXO3 deficiency results in defective TGF-β-driven FOXp3 induction and so deficiency in regulatory T cells formation. [12] FOXO3a deficiency has been also associated with spontaneous lymphoid proliferation, inflammation in different organs and increased hyper-activated T helper cells.[13]

FOXO3a inhibits NF-kB activation, whose over-activity was responsible for T cell hyperactivity in Foxo3a deficient mice. Thus, Foxo3a regulates helper T cell activation and tolerance by inhibiting NF-kB activity.[13]

There is a reported association between polymorphisms in FOXO3a and a number of inflammatory and autoimmune diseases such as chronic obstructive pulmonary diseases, inflammatory bowel's diseases, rheumatoid arthritis and Hashimoto thyroiditis.[14-16] The aim of the study was to determine the correlation between FOXO3a rs13217795 C>T single nucleotide polymorphism, and bronchial asthma and its severity in adult Egyptian population.

Materials and Methods

The study protocol was reviewed and approved by the Research Ethics Committee, Faculty of Medicine, Benha University (dated August 2021). A written informed consent was obtained from all study subjects.

Study design and subjects

This case-control study was carried out at Microbiology and Immunology Department, Faculty of Medicine, Benha University. The study included 60 adult asthmatic patients attending the Chest Department and chest outpatient clinic during the period from August 2021 to December 2021. Their ages ranged from 18 to 65 years. Cases were diagnosed by chest physician according to GINA guidelines 2018 [17] after history taking of variable respiratory symptoms; wheezes, cough, shortness of breath and chest tightness that resolves spontaneously or in response to medications. Patients with other obstructive or restrictive lung disease e.g., chronic obstructive pulmonary disease (COPD) or interstitial lung disease (ILD) and those with other causes of wheezy chest (e.g., cardiac asthma) were excluded. The study also included 40 subjects matched in age and sex as controls. The control subjects were non-smokers, had no history or symptoms of asthma, pulmonary diseases, or allergy.

Sample collection and DNA extraction

Two mls of venous blood were collected in tubes containing EDTA from each asthmatic patient and control subjects. Genomic DNA was extracted using a commercial kit (G-spin™ Total DNA Extraction Mini Kit, iNtRON, Biotechnology, Korea) according to the manufacturer's instructions. The extracted DNA was stored at −20◦C until used.

DNA amplification and detection of the FOXO3a gene

The extracted DNA was used for amplification of the FOXO3a gene. The amplification was performed using FOXO3a sequence specific primers; forward primer [5'-CTCCTTGGTCAGTTTGGTG-3'] and reverse primer [5'-ATGAGTGAAGATGGAAGTAAGC-3'].[18] The total polymerase chain reaction (PCR) volume was 50µl, consisted of 5µl extracted DNA template, 25µl 2×EasyTaq® PCR SuperMix (TransGen Biotech Co, China), 1µl forward primer, 1µl reverse primer and 18µl nuclease free water. Thermal cycling conditions were an initial denaturation step at 95°C for 5 min, followed by 35 cycles each of a denaturation at 95°C for 30s, annealing at 62°C for 30s and extension at 72°C for 1 min, and final extension step at 72°C for 10 min.

The amplified products were separated on 1.5% agarose gel, and bands were stained by ethidium bromide staining, and visualized under UV light on the UV transilluminator. A 100bp DNA marker (Thermo Scientific, USA) was used as a molecular weight size standard on each gel and the FOXO3a gene observed at 667pb. (Figure 1).

Restriction Fragment Length Polymorphism (RFLP) for detection of different genotypes

The amplified PCR products of FOXO3a gene were treated with PagI restriction enzyme (Catalog number: ER1281, Thermo Scientific, USA). RFLP reaction comprised of 10µl PCR product, 18µl nuclease-free water, 2µl 10X Buffer O and 1µl restriction enzyme PagI, the mixture was incubated at 37◦C for 4 hours. The restricted fragments were separated on 2% agarose gel and analyzed for determination of genotype frequencies. The homozygous wild genotype (CC) was indicated by uncut band at 667pb, the heterozygous wild genotype (CT) was indicated by bands at 667bp, 392bp and 275bp while the homozygous mutant genotype was indicated by bands at 392 and 275bp. (Figure 2).

Statistical analysis

Data were analyzed by SPSS statistical software (IBM SPSS: version 21). Comparing data was calculated using Chi-square (χ^2) test, *P*-value of less than 0.05 was considered significant. Odds ratio (OR) with 95% confidence interval (CI) were calculated to determine the association between genotypic and allelic frequencies in asthmatics and controls for evaluating the risk.

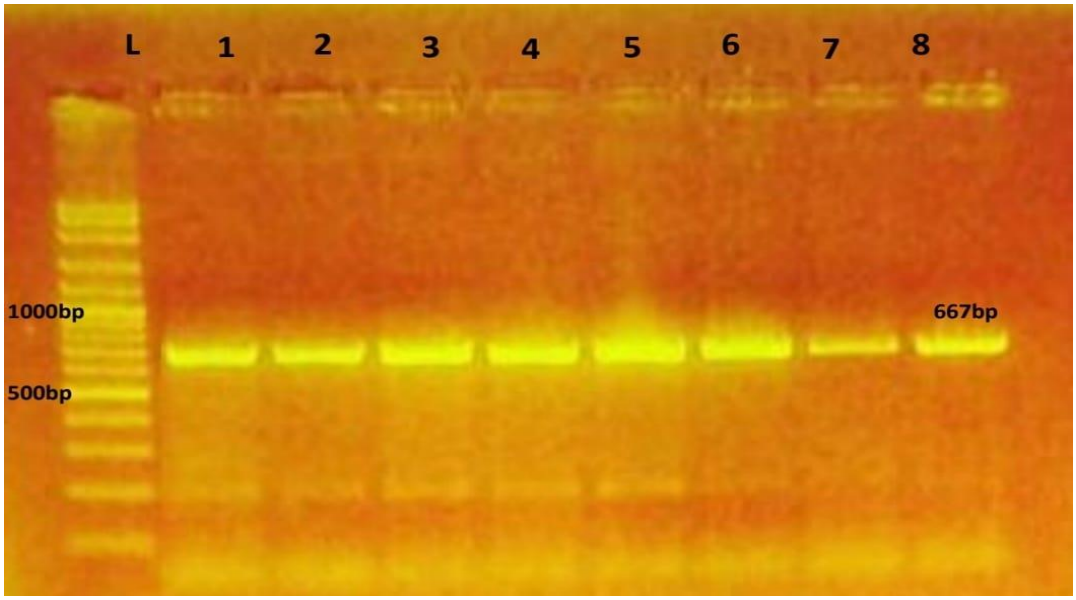

Figure 1. Ethidium bromide stained 1.5% agarose gel of the amplified DNA products shows FOXO3a gene at 667pb using a 100pb DNA marker (L) as a size standard for gel lanes.

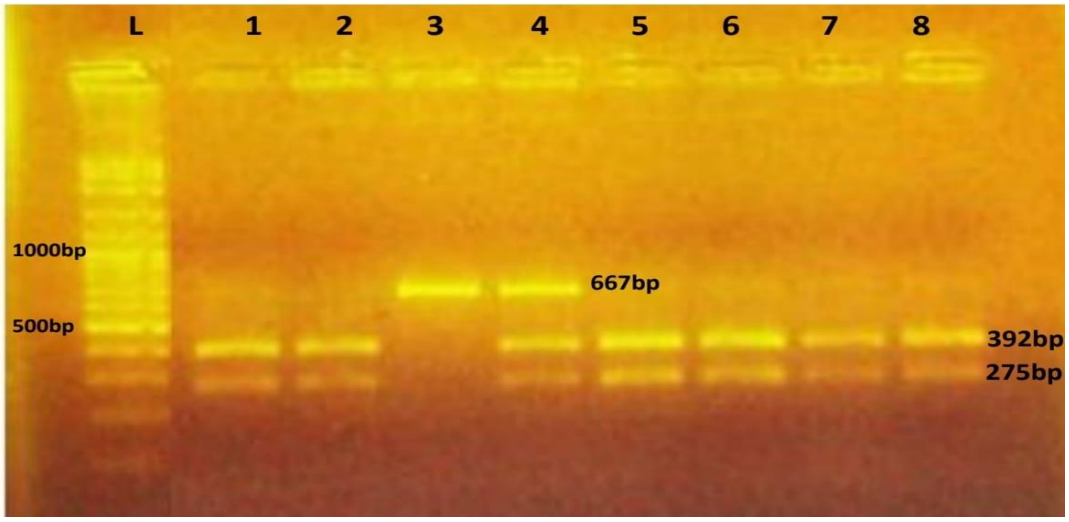

Figure 2. PCR-RFLP analysis of FOXO3a polymorphism shows, (rs13217795) genotypes after digestion with PagI. Lanes 1,2,5,6,7 and 8 show the homozygous mutant (TT) genotype, lane 3 shows the homozygous wild (CC) genotype and lane 4 shows the heterozygous wild (CT) genotype.

Results

In the present study FOXO3a rs13217795 C>T single nucleotide polymorphism was genotyped using PCR-RFLP technique in a total of 100 subjects, including 60 asthma patients and 40 controls.

Comparing genotypes frequencies between cases and controls showed that the mutant type (TT) had a higher frequency (51.7%) in patients than in controls (12.5%), OR = 7.48, 95% CI (2.58-21.71) (P<0.0001). The homozygous wild type (CC) had significantly lower frequency (10.0%) in patients than its frequency (52.5%) in controls, OR = 0.10, 95% CI (0.04- 0.29)

(*P*<0.0001). The heterozygous wild type (CT) had a frequency of 38.3% in patients which was not different than the frequency in controls (35.0%), OR = 0.87, 95% CI (0.38- 1.99) (Table 1).

Calculation of the allelic frequencies using genotype frequencies indicated that in study patients the frequency of the mutant T allele was higher (70.8%) than that of the wild C allele (29.2%), OR=12.40, 95% CI (5.65-27.19) (*P*<0.0001). In control group the frequency of C allele was higher (70.0%) than that of T allele (30.0%), OR = 0.100, 95% CI (0.05-0.22) (*P*<0.0001) (Table 1).

Table 1. Distribution of FOXO3a genotypes and alleles frequencies among the studied cases and control groups

	Patients No (%)	*Control* No (%)	*OR (95% CI)*	*P-value*
CC genotype	6 (10.0%)	21 (52.5%)	0.10 (0.04-0.29)	0.000
CT genotype	23 (38.3%)	14 (35.0%)	0.87 (0.38-1.99)	NS
TT genotype	31 (51.7%)	5 (12.5%)	7.48 (2.58-21.71)	0.000
Allele- type C	35 (29.2%)	56 (70.0%)	0.100 (0.05-0.22)	0.000
Allele- type T	85 (70.8%)	24 (30.0%)	12.40 (5.65-27.19)	0.000

-Odds ratio (OR) estimates the associations between genotypes, allele-types and risk of asthma.
-95% confidence interval (95% CI), - *P* ≥ 0.05 is not significant (NS).

Asthmatic patients were classified according to disease severity into three categories (mild, moderate, and severe). Genotype frequencies showed significant difference between the three categories (*P*<0.0001). The mutant TT genotype had a higher frequency among moderate and severe cases (12.5% and 41.9% respectively). Allelic frequencies showed no association of T allele with asthma severity (*P*=0.080). Its frequency was 36.5% and 34.1% in moderate and severe cases, respectively (Table 2).

Table 2. Frequency distribution of FOXO3a SNP (rs13217795) genotypes and allele-types among the studied cases according to severity.

	Mild 20 (33.3%)	*Moderate* 23 (38.3%)	*Severe* 17 (28.3%)	*P-value*
CC genotype	3 (50.0%)	2 (33.3%)	1 (1.70%)	0.000
CT genotype	9 (39.1%)	11 (47.8%)	3 (13.1%)	
TT genotype	8 (25.8%)	10 (12.5%)	13 (41.9%)	
Allele- type C	15 (42.8%)	15 (42.8%)	5 (14.3%)	NS
Allele- type T	25 (29.4%)	31 (36.5%)	29 (34.1%)	

P ≥ 0.05 is not significant (NS)

Discussion

In this study we tried to explore the association between FOXO3a rs13217795 C>T single nucleotide polymorphism and bronchial asthma and its severity in adult Egyptian population.

The study findings showed that the mutant type (TT) had a higher frequency in patients than in controls (*P*<0.0001). While the homozygous wild type (CC) had lower frequency in patients than in controls (*P*<0.0001). The heterozygous wild type (CT) showed no association between patients and controls. Allele frequencies showed that in patients the frequency of the mutant T allele was significantly higher than that of the wild C allele (*P*<0.0001) indicating that T allele may be risk factor for development of asthma.

Our findings are in parallel with those of a study by Barkund et al., 2015,[18] who determined that in their asthmatic patients, the TT mutant genotype had the highest frequency 51.75% (*P*<0.0001), and the T mutant allele showed the highest prevalence 71% (*P*<0.0001). Similar results were shown by Amarin et al., 2017 [19] where the frequency of mutant TT genotype was the highest 49% (*P*<0.005) and the mutant T allele was present in 64% of asthmatics (*P*<0.006).

A study by Goodi and ALSaadi (2018)[20] in Iraq, reported that (TT) genotype frequency in the patients group showed significant difference between the patient and control groups (*P*<0.0002). On the other hand, CC genotype showed no difference between patient and control groups (22% versus 20% respectively). The mutant T allele was present in 53% of patients while the wild type allele C was present in 60% of the controls.

On the other hand, the study by El Rifai et al., 2019[21] in Egypt, reported that the highest frequency genotype was for the heterozygous CT genotype in both cases and control 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 the 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. However, there was no difference observed between asthmatic patients and controls regarding the different genotypes of the FOXO3a gene polymorphism.

The difference in results between our study and the study by El Rifai et al., 2019[21] may be attributed to the difference in age of the studied groups as they performed their study on asthmatic children. The possible explanation is that patients may acquire mutations in older age.

In the present study asthmatic patients were classified according to disease severity. The mutant TT genotype had the higher frequency among moderate and severe cases (*P*>0.0001). However, the study by El Rifai et al., 2019[21] reported different findings than our study, as they observed no association between the different genotypes of the FOXO3a gene polymorphism and the different grades of asthma severity. It could be suggested that age is an important factor in developing mutation that leads to occurrence of asthma and its severity in the predisposed subjects. Heretofore, based on our information, no other studies were done to explore the association between FOXO3a gene polymorphism and the different grades of asthma severity.

In conclusion, the present study shed light on the association between FOXO3a rs13217795 C>T single nucleotide polymorphism and asthma and its severity. High frequency of the mutant TT genotype among patients and sever cases may indicate that this genotype could be considered as a risk factor in development and severity of asthma.

Author Contributions

MSE and AMA contributed to the design and implementation of the research, performed the laboratory work, and helped shape the research, supervised the findings of this work, discussed the results, read, and approved the final manuscript. MSS; contributed to the design and implementation of the research, aided in choosing the patients and helped shape the research, supervised the findings of this work, discussed the results, read, and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee, Faculty of Medicine, Benha University (dated August 2021).

Informed consent

A written informed consent was obtained from all study subjects.

References

1. Madore, A. and Laprise, C. (2010). Immunological and genetic aspects of asthma and allergy. Journal of Asthma and Allergy; 3: 107–121.
2. Mims, J.W. (2015). Asthma: definitions and pathophysiology. Int Forum Allergy Rhinol; 5: S2–S6.
3. CDC. gov. (2019). CDC - Asthma. [online] Available at: https://www.cdc.gov/asthma/default.htm [Accessed 13 June 2019].
4. Ishak, S.R., Abd El Sayed, S.T.K., Wahba, N.S. (2020). Prevalence of common sensitizing aeroallergens in Egyptian asthmatic patients. World Allergy Organization Journal; 13:100115 http://doi.org/10.1016/j.waojou.2020.100115.
5. Thomsen, S. F. (2015). "Genetics of asthma: an introduction for the clinician" European Clinical Respiratory Journal; 2: 24643.
6. Tuteja, G., Kaestner, K.H. (2007). "Forkhead transcription factors II". Cell; 131 (1): 192–192.e1. doi:10.1016/j.cell.2007.09.016. PMID 17923097. S2CID 322449.
7. Ma, J., Matkar, S., He, X., et al. (2018). FOXO family in regulating cancer and metabolism. Semin Cancer Biol.; 50: 32–41.
8. Stefanetti, R.J., Voisin S., Russell A., et al. (2018). Recent advances in understanding the role of FOXO3. F1000Research, 7(F1000 Faculty Rev):1372 (doi: 10.12688/f1000research. 15258. 1).
9. Conti, V., Corbi, G., Manzo, V., et al. (2015). Sirtuin 1 and aging theory for chronic obstructive pulmonary disease. Analytical Cellular Pathology. Article ID 897327. doi:10.1155/2015/897327.
10. Wang, Y., Zhou, Y. and Graves, D.T. (2014). FOXO Transcription Factors: Their Clinical Significance and Regulation. BioMed Research International; Article ID 925350, 13 pages, 2014. http://dx.doi.org/10.1155/2014/925350.
11. Harada, Y., Harada, Y., Elly, C., et al. (2010). Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. J Exp Med; 207, (7): 1381–91.
12. Kerdiles, Y.M., Stone, E.L., Beisner, D.L., et al., (2010). Foxo transcription factors control regulatory T cell development and function. Immunity; 33, (6): 890–904.
13. Lin, L., Hron, J.D. and Peng, S.L. (2004). Regulation of NF kappa B, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity; 21(2):203-13.
14. Kalemci, S., Edgunlu, T.G., Turkcu, U.Ö., et al. (2014). FOXO3a gene polymorphism and serum FOXO3a levels in patients with chronic obstructive pulmonary disease and healthy controls: Effects of genetic polymorphism in chronic obstructive pulmonary disease. Polish journal of thoracic and cardiovascular surgery; 11(3): 306-310. https://doi.org/10.5114/kitp.2014.45682.
15. Lee, J., Espi, M., Anderson, C.A., et al. (2013). Human SNP Links Differential Outcomes in Inflammatory and Infectious Disease to a FOXO3-Regulated Pathway. Cell; 155: 57–69.
16. Roehlen, N., Doering, C., Hansmann, M.L., et al. (2018). Vitamin D, FOXO3a, and Sirtuin1 in Hashimoto's Thyroiditis and Differentiated Thyroid Cancer. Front. Endocrinol; 9:527. doi: 10.3389/fendo.2018.00527.
17. Global Initiative for Asthma. (2018). Global strategy for asthma management and prevention. GINA; 2:28.
18. Barkund, S., Shah, T., Ambatkar, N., et al. (2015). FOXO3a gene polymorphism associated with asthma in Indian population. Mol Biol Int; Article ID 638515. https://doi.org/10.1155/2015/638515.
19. Amarin, J.Z., Naffa, R.G., Suradi, H.H., et al. (2017). An intronic single-nucleotide polymorphism (rs13217795) in FOXO3 is associated with asthma and allergic rhinitis: a case–case–control study. BMC Med Genet; 18: 32.
20. Goodi, G.A. and AL-Saadi, B.Q. (2018). Polymorphism of FOXO3a Gene and Its

Association with Incidence of Asthma in Iraqi Patients. Iraqi Journal of Biotechnology; 17 (3): 67-77.

21. El Rifai, N.M., Al-Wakeel, H., Osman, H.M., et al. (2019). FOXO3a gene polymorphism and bronchial asthma in Egyptian children. Egypt J Pediatr Allergy Immunol; 17(1):31-36.