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Polymorphism of *FOXO3a* Gene and Its Association with Incidence of Asthma in Iraqi Patients

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Abstract: Asthma is common polygenic multifactorial inflammatory disorder influenced by genetic and environmental factors characterized by inflammation of airways leading to obstruction, coughing and wheezing in response to an allergen or inorganic pollutions. *FOXO3*a (Forkhead Box O3) is a gene plays a vital role in the etiology of asthma disease. The objective of this study is to investigate an association of single nucleotide polymorphism SNP of the *FOXO3a* (rs 13217795, C>T transition) gene with asthma susceptibility in adults of some Iraqi patients. study consists of two groups: fifty asthma patients (28 female and 22 male) and twenty five apparently healthy as a control group (9 female and 16 male). The age of the samples ranged from 20-60 years old. Recruited from the Alzahra'a Center for Asthma Allergy in Baghdad during the period extended from October /2017 to last February /2018. The DNA purity ranged between 1.8-1.9 and the concentration of DNA between 40-120 ng / µl. Then fragments targeted of *FOXO3a* gene (rs13217795 C>T) were amplified by polymerase chain reaction (PCR). Then Restriction Fragment Length Polymorphism (RFLP) technique was used to determined SNP (rs13217795) polymorphism by using *Cci I* restriction enzyme. Samples from control and patients group were sent for DNA analysis sequencing. TT not appeared in the sequencing.The highest rate of asthma was in the 40 to 60 age group. As for gender, the females ratio was higher than the males. Most patients have a family history of asthma. Most patients with asthma were in the autumn. The results were revealed that SNP (rs 13217795) of *FOXO3a* gene with Polymorphism of CT genotype in the control group was significantly higher than in patients group (80 % versus 50%, respectively). And persons with the CT genotype in the patient group were significantly higher than those with the CC genotype. While, the percentage of those with TT Polymorphism genotype in the patient's group were showed highly significant difference as compared with control group (28 % versus 0 %, respectively). The Allele frequency C in the patients and control group shows 47.00% versus 60.00%, respectively. While Allele frequency T in the patients and control group shows 53.00% versus 40.00%, respectively. These results indicated that the polymorphism of C>T (rs 13217795) of *FOXO3a* gene was associated with the developing Asthma in this studied sample. In conclusion, the T allele variant of *FOXO3a* gene (rs 13217795) polymorphism may be associated with increased susceptibility of the development of asthma in Iraqi patients.

Keywords: Polymorphism, *FOXO3a* Gene, Asthma in Iraqi.

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Introduction:

Asthma is known as a heterogeneous disease, usually characterized by inflammation of airway; it is defined by the respiratory symptoms history like shortness of breath, wheeze, cough that vary over time and in intensity and chest tightness, together with changing of expiratory limitation of airflow (1). The occurrence of asthma result from multiple genes interaction with environmental factors, several genetic epidemiology studies have mentioned the correlating of genes of cytokine with asthma among different people, nevertheless, the results are maladjusted

and indecisive (2). According to the World Health Organization, asthma occurs in people of all ages, asthma is the most common chronic disease among children and it currently affects nearly 235 million persons in the world (3). According to the latest World Health Organization data published in 2017 deaths of asthma in Iraq about 566 or 0.32% of total deaths, the age-adjusted mortality rate is 3.33 per 100,000 populations in Iraq (4). There was a molecular study of asthma in Iraq with IL-4 −590 (C>T) gene especially in Iraqi patients with asthma, the results showed no significant differences between patients with asthma and healthy subjects (5). Several risk factors responsible for the onset of asthma have been identified in adulthood, varying from environmental sensitizers to respiratory infections, obesity, hormonal factors and stress (6). (*FOXO3*) a brief for Forkhead box O3 is a protein coding gene located on chromosome six q21, forkhead box protein O3 (FOXO3) product is a member of the forkhead family of transcription factors. These transcription factors play an important role in immune homeostasis; like: function loss different may be linked with chronic inflammatory processes (7). As to our knowledge, this is the first study about the genetic aspect of asthma disease in genetic engineering and biotechnology institute so that: The present study aims to increase knowledge about the occurrence of Asthma in adults in Iraq due to previous rare studies on this part and determining the association of *FOXO3a* gene polymorphism (rs 13217795, C<T) and incidence of asthma in some Iraqi patients by using PCR- RFLP.

Subjects, Materials and Methods:

Study consist of two groups, fifty asthama patients (28 female and 22 male) and twenty five as apparently healthy subjects (control) and personal information such as: age, smoking, season, gender, family history, onset of disease, other diseases, the samples were admitting the Alzahra'a Center for Asthma Allergy in Baghdad during the period between October /2017 to last February /2018. The study design was approved by the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad. Writing informed consents were obtained from all patients and apparently healthy control group; all patients were diagnosed according to clinical examination by a chest physician and selected according to the criteria of the global initiate of asthma, spirometry was performed to measure FEV1(forced expiratory volume in 1 second) and FVC (forced vital capacity).

Genomic DNA Extraction:

Three ml of peripheral venous blood samples were collected from the asthma patients and apparently healthy controls using disposable latex gloves and syringes. Then, blood was kept in EDTA anticoagulant tubes in freezer at -20 to be a source for DNA extraction as described by (8). The DNA was extracted from the samples of blood of asthma patients and apparently healthy subjects by using WizPrep™ gDNA Mini kit (blood) company of Wizbio, (Korea). Then, DNA concentration and purity were measured by nanodrop. The acceptable purity of samples of DNA is range between 1.7-1.9. DNA bands

were visualized using UV light after electrophoresis in a 1% (w/v) agarose gel (9).

PCR Primers for *FOXO3a* Gene:

The sequence of this primer specific for SNP of the *FOXO3a* (rs 13217795, C>T transition) is listed Forward 5'-CTCCTTGGTCAGTTTGGTG -3', Reverse 5'-ATGAGTGAAGATGGAAGTAAGC -3' (10).

Restriction Enzymes:

The restriction enzyme *Cci I* is used in this study, this restriction enzyme was taken from (*SibEnzyme,* Russia)

Detection of *FOXO3a* SNP (rs 13217795), C>T Transition:

Table (1): The reaction components of PCR.

Component	Quantity (µl)
PCR pre Mix: dNTPs, *Taq* DNA polymerase , MgCl2 and reaction buffer (Ready to use) (PH 8.5)	10
Forward primer	1(10 pmol)
Reverse primer	1(10 pmol)
DNA template	2
Nuclease free water	6
Total volume	20

PCR Program:

Optimal annealing temperature of PCR reaction was found out after several try to 55˚C with a total volume of 20 µl. The reaction components are described in table 2.

Table (2): PCR Program.

Steps	Temperature (˚C)	Time	No. of Cycles
Initial denaturation	95	5min.	1
First loop:			
Denaturation	95	30 sec.	35
Annealing	55	30 sec.	
Extension	72	1min.	
Final extension	72	5min.	1

PCR-RFLP for*FOXO3a* gene (rs13217795, C>T) Transition:

By using specific primers, *FOXO3a* gene SNP (rs 13217795), C>T Transition fragment is amplificated, (10 µl) of PCR products are digested by restriction enzyme *Cci I* at 55˚C for 3 hours. The total volume reaction 15µl and the component reaction are described in the table (3). (10 µl) of digested products were moved on (3%) agarose gel for 90 minute. Then, agarose gel was visualized under UV light using ultraviolet transilluminater. DNA ladder (100-3000 bp) was used, and the gel was photographed by a digital camera.

The presence of polymorphism CT heterozygote yielded 667, 392 and 275 bp fragments ,while the polymorphism CC homozygote yielded 667 bp uncut fragment only, while TT mutant yielded 275 and 392 bp (10).

Table (3): Reaction Component for Restriction Enzyme *CciI*.

Component	Component of one sample (µl)
PCR product	10
Enzyme	1
SE Buffer PH 8.5 (10mM Tris-HCL, 150mM NaCl, 10mM $MgCl_2$, 1mM DTT)	0.5
Bovine Serum Albumin (BSA)	2
D.W	1.5
Total volume	15

Frozen PCR products with a total volume (20µl) were sent for sequence analysis.

DNA sequencing:

Sequencing identification for three samples was selected from patients and two samples from apparent healthy control group to confirm the positive results of PCR-RFLP in this study. Frozen PCR products with a total volume (20µl) were sent for sequence analysis to Macrogen Company in Korea. PCR products of 5 samples from each groups that include all samples with mutant type (rs 13217795, C>T Transtition SNP) and some Homozygous normal were sent for sequence analysis by the National Center for Biotechnology Information (NCBI) site and Bioedit system.

Statistical analysis:

According to (11). SAS Program was used to influence of various factors in gender, age, season, family history, and smoker. Chi square test was also used to significant compare between the percentage and determine the odds ratio which used to determine the disease risk in this study, the odd ratio can be range from less than 1 (negative association) to more than 1 (positive association).

Results and Discussion:

Distribution of Asthma patients according to Age:

With regard to age, the age of asthma patients and control range from 20-60 year, the highest percentage of the asthma cases was found in the third age group (40-60 years old) which reached to 48% of total patients, followed by 28% for the first age group (less than 30 years old) and 24% for the second age group (30-40 years old), as shown in table (4).

According to (12) mentioned that young children are highest exacerbations with asthma, but decrease with age; the children are the most frequent reason for childhood hospitalization, about 640 000 child visit emergency department (ED) annually.

With an increase in age, elderly diseases will have an increasing prevalence, although asthma is usually considered a disease of younger persons, mortality of asthma is the largest in the 55-year-old age group, emergency presentations and symptoms for health -care offerings caused by are a major burden on the quality of life of people over 55 years of age due to asthma (13).

Table (4): The percentage of patient's distribution according to age.

Age group	Asthma patient %
Less than 30	28%
30-40	24%
40-50	48%

Distribution of Asthma patients according to Gender:

The number of males and females in the patients group are 20 (40%) and 30 (60%) respectively; therefore, the females percentage was higher than males percentage, while in the control group their respective numbers were 16 and 9 individuals in the present study as shown in Table (5).

According to (14) disagree with present study found that asthma in children has higher in male 177 (63.4%) than female 102 (36.6%) in America.

The Centers for Disease Control and Prevention (15) agree with present study estimated that the rate for females 9.5% while males was 7.0% among adult asthma in 2012.

Table (5): Distribution according to gender in patients asthma and control.

Gender	Asthma patient no. (%)	Control no. %
male	20 (40 %)	16 (64%)
female	30 (60%)	9 (36%)
total	50 (100%)	25 (100%)

Distribution of Asthma patients according to smoking, family history and season:

With regard to smoking, The number of smoker and non-smoker in asthma patients 8 (16%) and 42 (84%) respectively, as well in control group 6 (24%) and patients group 19 (76%) respectively, there is no significant differences between control group and patients group in the present study as shown in table (6). According to (16) Agree with present study shown that no relationship between asthma and smoking in adults has been established. According to (17) disagree with present study reported that active smoking is associated with the onset of asthma in adults and adolescents in a number of studies.

The distribution of patients according to family history of disease, (Table 6), revealed that 30 (60%) of 50 patients have positive family history of asthma and 20 (40%) of patients have no family history. The family history of asthma was more common in the cases than in the controls, the result showed patients with no family history was 150 (58.1%) while patients with family history was108 (41.9%) (14). The asthma is inherited about 79% while the remaining 21% were due to environmental effects (18).

The highest percentage of the asthma cases was found in autumn season which reached to 68% of total patients, followed by 32% for winter season (Table 6). Although the collection of samples extended from October /2017 to last February /2018 but most of the research indicated that the severity of asthma increased in autumn. The asthma peak in the emergency department and outpatient

office visits was affected by children and adults. In adults, the asthma peak was from September to October, while in children the peak asthma was only in September (19, 20).

Table (6): Distribution of sample study according to difference factors in patients asthma and control

Factors		Patients No. = 50	Control No. = 25	P-value
Smoking	Smoker	8 (16.00%)	6 (24.00%)	0.0001 **
	Non-Smoker	42 (84.00%)	19 (76.00%)	
Family history	Yes	30 (60.00%)	-	0.0001 **
	No	20 (40.00%)	-	
Season	Winter	16 (32.00%)	-	0.0001 **
	Autumn	34 (68.00%)	-	

**** (P<0.01).**

DNA Isolation:

The DNA was extracted from frozen blood of asthma patients and control samples yielded enough DNA concentration for PCR technique (Figure 1).

The quantity measurement indicated that the concentration of DNA ranged between (40-120 *ng* / µl) and the purity of DNA range was between (1.8 – 1.9) by using Nano drop devise. The DNA procedure isolation was showed very sharp band and efficient.

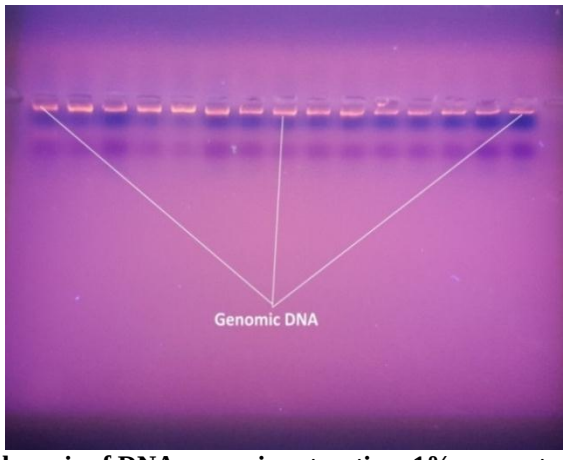

Figure (1): Gel electrophoresis of DNA genomic extraction .1% concentration of Agarose gel at 5 volt/cm^2 for 30 min. then staining with Ethidium BromideAnd visualized under U.V light.

Molecular Identification of *FOXO3a* (rs 13217795) C>T Polymorphism:

The molecular portion of this study has been accomplished by DNA extraction form asthma patients and control groups and then used specific primers for PCR amplification.

PCR Analysis:

In figure 2 shown Polymerase Chain Reaction (PCR) technique amplified regions, the molecular weight (667 bp), represent the region of the *FOXO3a* gene that involve (rs 13217795) SNP.

This technique was used specific primer for *FOXO3a* gene fragment

according to (9, 29), The size of PCR product was revealed by using (100-3000) DNA ladder, and photographed of gel by using a digital camera.

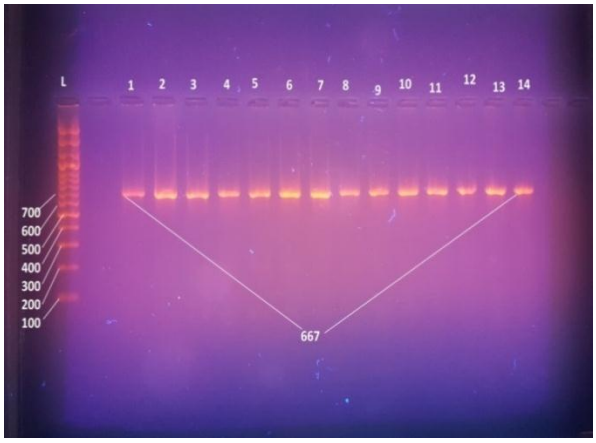

Figure (2): The size of PCR products 667 bp. The PCR product was electrophoresis on 2% concentration agarose gel at 70 voltage for 90 minute. stain with Ethidium Bromide then visualized under U.V light, L: DNA ladder (100-3000), Lane (1-14) PCR products of the *FOXO3a* gene.

Restriction fragment length polymorphism (RFLP):

In Figure (3): The product of PCR was treated with restriction enzyme *Cci I* as follow at

1- Homozygous wild type CC showed No cleavage of the whole 667 bp segment.
2- Heterozygous wild type CT showed three fragments in Agarose gel electrophoresis (667 bp, 392 bp and 275bp).
3- Mutant type TT showed two fragments in Agarose gel electrophoresis fragments of 392 and 275 bp.

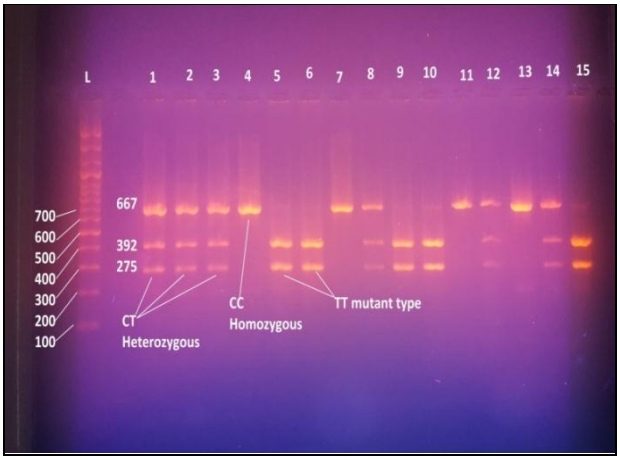

Figure (3): PCR product treated with *Cci I* electrophoresis on 3% concentration. Agarose gel at 70 voltages for 90 minute. The product of (RFLP) were Visualized under U.V light after stain with Ethidium Bromide، L: DNA ladder (100-3000). Lane1**,** 2, 3, 8, 12 and 14**:** Heterozygous wild type CT 667, 392 and 275 bp**,** Lane 4, 7, 11 and 13 Homozygous CC 667 bp., lane 5, 6, 9, 10 and 15 Mutant type TT 392 and 275.

Sequencing of C>T(rs 13217795) SNP:

The analyses of sequence of C>T (rs 13217795) SNP segment of *FOXO3a* gene of the studied samples were compared to C>T (rs 13217795) SNP fragment reference sequence (GenBank) accession no. 000006.12. Figure (4) clarify the sequencing information, presents the electropherogram depicting the position and its flanks. Five samples from the patients and control were sent for sequence analysis to Macrogen Company in Korea (forward and reverse). The results of sequencing showed as not matching with results of RFLP, TT allele not appeared in the sequencing but TC appeared. This means that the mutation is in one base not in two bases.

According to the information on NCBI, the location of *FOXO3a* gene is on chromosome 6q21 NC_000006.12 (108559823..108684769).

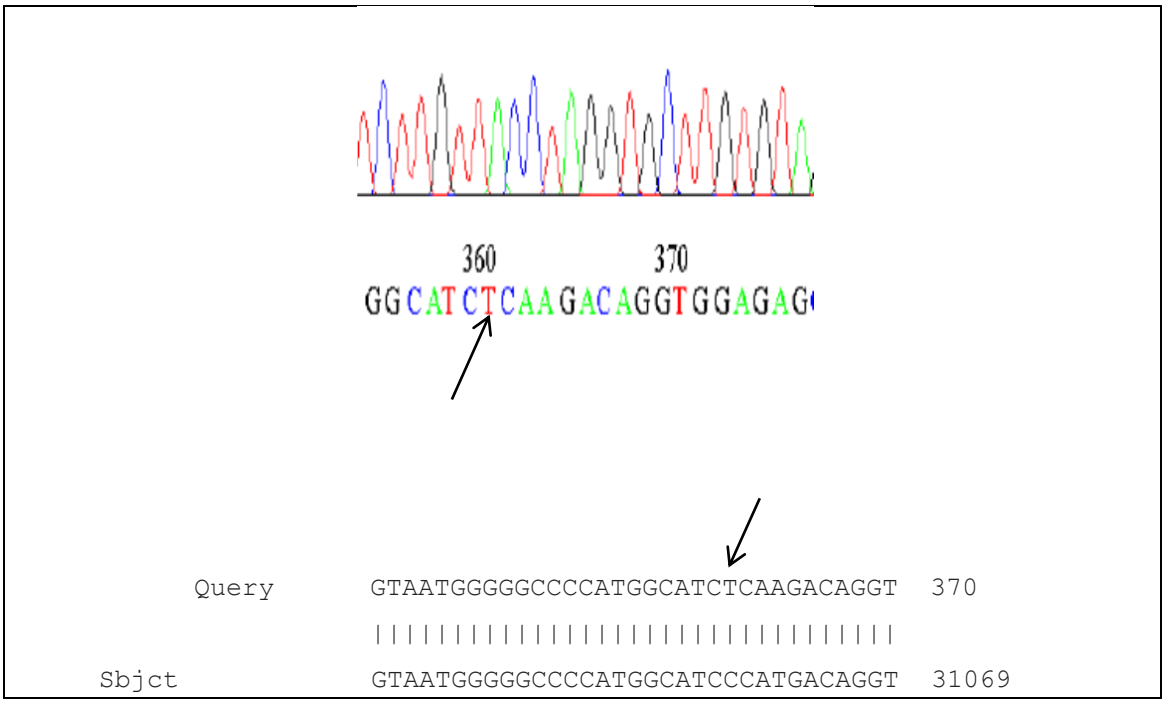

Figure (4): Sequencing of C>T (rs13217795) SNP

Association of *FOXO3a* Gene Polymorphism C>T (rs 13217795) SNP with Asthma:

In table (7) the significant of CT Polymorphism genotype in the control group and patients group was higher than that in the patients group (80 % *versus* 50%, respectively).

The CC genotype frequency shows non-significant difference in the patient group compare with control group (22 % *versus* 20 %, respectively).

While TT Polymorphism genotype frequency in the patients group shows highly significant difference as compared with control group (28 % *versus* 0 %, respectively).

Allele frequency C in the patients and control group shows (47.00% *versus* 60.00%, respectively). While Allele frequency T in the patients and

control group shows (53.00% *versus* 40.00%, respectively).

The odd ratio is more than one of C>T (rs 13217795) SNP polymorphism genotype is highly significantly associated with the risk of developing of asthma in this studied sample, the results of the present study that compatible with (10, 21) that found an association between C>T (rs 13217795) SNP and risk of developing asthma.

Table (7): Genotype distribution and allele frequency of (rs13217795) SNP in patients and control

Genotype	Patients		Control		Chi-Square	O.R. (CI)
	No.	%	No.	%		
CC	11	22.00	5	20.00	0.723 NS	0.006 (0.88-1.62)
CT	25	50.00	20	80.00	9.250 **	0.00 (0.90-1.60)
TT	14	28.00	0	0.00	9.071 **	1.194 (0.91-1.62)
Total No.	50	100%	25	100%	---	---
P-vale	---	0.0002 **	---	0.0001 **	---	---
Allele Frequency						
C	47.00%		60.00%		---	---
T	53.00%		40.00%		---	---

**** (P<0.01), NS: Non-Significant.**

In table (8) Distribution of the rs 13217795 C>T polymorphism of asthma group according to gender, CC genotype frequency in male is no significantly with female (26.32% *versus* 20.69 %, respectively).

CT genotype (dominant mode) frequency in male is no significantly with female of asthma patients (52.63% *versus* 51.72%, respectively).

The frequency TT (mutant type) in male is significantly with female (21.05% *versus* 27.59%, respectively).

Table (8): Distribution of the rs 13217795 C>T polymorphism of asthma group according to gender.

Genotype	Gender				P-value
	Male		Female		
	No.	%	No.	%	
CC	5	26.32	6	20.69	0.072 NS
CT	10	52.63	15	51.72	0.688 NS
TT	4	21.05	8	27.59	0.049 *
Total	19	100 %	29	100 %	---
P-value	---	0.0001 **	---	0.0001 **	---

*** (P<0.05), ** (P<0.01), NS: Non-Significant.**

In table (9) Distribution of sample study according to Age with C>T (rs 13217795) SNP Genotype, the frequency CC genotype in age less than 30 is high significantly with age (30-40) and age more than 40 (30.77% , 15.38%, 20.83%, respectively).

The frequency CT genotype in age less than 30 is high significantly with age (30-40) and age more than 40 (46.15%, 69.23%, 41.67%, respectively).

The frequency TT genotype in age less than 30 is high significantly with age (30-40) and age more than 40 (23.08%, 15.38%, 37.50%, respectively).

Table (9): Distribution of sample study according to age with C>T (rs 13217795) SNP genotype.

Genotype	Age group (year)						P-value
	Less than 30		30-40		More than 40		
	No.	%	No.	%	No.	%	
CC	4	30.77	2	15.38	5	20.83	0.0094 **
CT	6	46.15	9	69.23	10	41.67	0.0024 **
TT	3	23.08	2	15.38	9	37.50	0.0052 **
Total	13	100 %	13	100 %	24	100 %	---
P-value	---	0.0004 **	---	0.0001 **	---	0.0027 **	---

**** (P<0.01).**

In table (10) Distribution of sample study according to family history with C>T (rs 13217795) SNP Genotype, the frequency CC genotype in patients have family history is high significantly with patients have no family history (16.67% *versus* 30.00%, respectively).

The frequency CT genotype in patients have family history is significantly with patients have no family history (46.67% versus 55.00%, respectively).

The frequency TT genotype in patients have family history is high significantly with patients have no family history (36.67% versus 15.00%, respectively).

Table (10): Distribution of sample study according to family history with C>T (rs13217795) SNP genotype.

Genotype	Family history				P-value
	Yes		No		
	No.	%	No.	%	
CC	5	16.67	6	30.00	0.0096 **
CT	14	46.67	11	55.00	0.0398 *
TT	11	36.67	3	15.00	0.0086 **
Total	30	100 %	20	100 %	---
P-value	---	0.0001 **	---	0.0001 **	---

*** (P<0.05), ** (P<0.01), NS: Non-Significant.**

Conclusions:

The most prominent findings of the present work are:

Asthma risk affected by genetic and other environment factors (such as age, gender, season, and smoking etc.).The prevalence of asthma was high in (41-60) years old and in females more than males. The polymorphism of rs 13217795 C>T SNP in the *FOXO3a* gene was associated with developing asthma disease. T allele was more frequency in asthma patient than C allele.

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