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Study of the correlation between total immunoglobulin-E levels and Interleukin-4 polymorphism in asthmatic children

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ABSTRACT

The aim of this study is to analyze the possible correlation between the single nucleotide polymorphisms (SNP) IL-4-590C/T with specific parameters in asthmatic children (blood eosinophils, total serum immunoglobulin-E (TSIgE) levels, and asthma severity). This study includes one hundred asthmatic patients as well as one hundred healthy unrelated age-matched controls from the same locality of Iraq. DNA is extracted and processed by the allele specific-PCR technique for characterization of genetic variants of *IL*-4-590 *C*>*T* polymorphisms. TSIgE levels are determined by ELISA technique while blood eosinophils are determined by blood film staining. Iraqi cases with asthma show a higher frequency of the *IL*-4-590 CC homozygous genotype in comparison to controls (66% versus 7%) with a lower CT heterozygous genotype (17% versus 90%) respectively. *IL*-4-590 shows significantly positive associations with asthma in the dominant, co-dominant, and over-dominant models of inheritance. On the other hand, comparing genotypes of subgroups related to gender, asthma severity shows a non-significant difference ($p > 0.05$). Homozygous genotypes (IL-4-590 CC) can be considered as risk factors, while the homozygous wild types (-590 TT) might be regarded as protective of asthma and there is no association between TSIgE and IL-4-590 SNP.

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INTRODUCTION

Asthma is a complex chronic disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person (World Health Organization, 2018), in which many cells and cellular elements play an important role, particularly mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells (Guill, 2004). The manifestation of asthma includes wheezing, chest tightness, shortness of breath and nighttime or early morning coughing (Thorax, 2014). Asthma in children is a common illness and continues to lead to chronic diseases among children in many countries (Herzog R, 2011; Asher and Pearce, 2014). Two major types of asthma are recognized in childhood which is transient wheeze, that happens in early childhood and persistent wheezing which usually happens in older children and continues in later childhood (Shin *et al.,* 2009). Asthma is considered a type one (Type-I) hypersensitivity reaction where it is produced from a combination of allergens with immunocytes, that releases special proteins as cytokines and antibodies (Abs) as immunoglobulin - E(IgE) that results in bronchitis and asthma symptoms (Djukanovic R, 1990). In Iraq, asthma prevalence is about (15.8%) in younger children than

five years and (16.4%) in primary school children (Salem, 2002). Among the immune cells that play an important role in allergic diseases and asthma, processes are blood eosinophils (McBrien and Menzies-G., 2017). Blood eosinophils are shared with IgE and others leukocytes in the immune response of asthma (Froidure *et al.,* 2015). The rise in total serum immunoglobulin-E (TSIgE) levels appears in patients with atopic diseases such as asthma and some invasive parasitic diseases (Ito *et al.,* 2011). TSIgE levels can be utilized to distinguish between non-allergic and immune-reactions before the determination of allergenic IgE. It is also useful in predicting severity and controlling response to treatment (Owen CE, 2007). In the immune response, many proteins (especially the cytokines) participate in the pathogenesis of asthma, which plays an essential role in transferring the signal and communicates between cells of the immune system (Lambrecht and Hammad, 2015). These cytokines are important and play a key role in regulating the chronic inflammation and changes of the pulmonary tract like asthma and have involved in the evolution of new curative strategies in these diseases (Barnes, 2008). Interleukins (ILs) are a group of cytokines that are expressed by many leukocytes like T-cell, ILs including pro-inflammatory cytokines (Dinarello, 2014). The most important interleukins that play a major role in many allergic diseases are Interleukin-4 (IL-4) and Interleukin-13 (IL-13), which are chemical mediators that have been implicated in the cascade of events that contribute to asthma and many chronic diseases (Sudha S. Deo, 2010). These ILs have pleiotropic functions and the differential functions of interleukins in the asthmatic lung are that IL-13 may be produced at higher quantities under allergic inflammatory conditions (Hong-Erh Liang and Locksley, 2011). IL-4 is a major cytokine in the development of allergic disease that is associated with the induction of the class switching of IgE secreted by B lymphocytes (Rivas, 2016). The activation of the IgE-derived mast cell that is caused by IL-4 has a pivotal role in the development of immediate allergic reactions (Akdis, 2014). The mechanism which is contributed by IL-4 interception of the airway in asthma is through activation of gene expression and then hypersecretion of mucus (Dabbagh K, 1999). IL-13 is an important protein for humans, that is encoded by the IL13 gene (Minty *et al.,* 1993) and the secondary structural features of IL-13 are similar to that of IL-4 (Popovic *et al.,* 2017). IL-13 is a central regulator in IgE synthesis, goblet cell hyperplasia, mucus hypersecretion, airway hyperresponsiveness and fibrosis. https://en.wikipedia.org/wiki/Interleukin_13 - cite_note-ma831-7 It is a mediator of allergic inflammation and different diseases including asthma (Rael and Lockey, 2011). Asthma occurs through the release of cytokines and intermediates like (IL) -13 and IL-4 gene variation that has been reported to contain multiple functional forms. IL-13 is synthesized in elevated levels by eosinophils, T helper-2 (Th2) cells, mast cells, basophils and natural killer cell (NKC) (Anwar J Almzaiel 2017). The genetic variations in IL-4 (IL-4 590) and IL-13 (IL-13 1112) are thought to play a role in the expression of inflammation and airway dysfunctions (Hassan R AL-Rikabi 2017). Those variations in interleukins are called single nucleotide polymorphism (SNP) that is related to asthma and other serious diseases such as type 2 diabetes mellitus (T2DM) (Afaf Alsaid and Mohammad T., 2013). SNPs is utilized as genetic markers in the risk factor of asthma (Haijun *et al.,* 2016; Saddam H.Jaber, 2017). This study is conducted to a better understanding of the development of asthma from the immunological and genetic perspective. This research addresses specific aims of the analytical study which includes asthmatic and non-asthmatic children.

SUBJECTS AND METHODS

This is a case-controlled study that is conducted on one hundred asthmatic, diabetic patients as well as one hundred healthy controls recruited during the time period from December 2017 to June 2018. Patients are recruited from the Karbala Teaching Hospital for Pediatrics (KTHP) including (57%) males and (43%) females of age 8.1years (SD = 4.08). All patients had a diagnosis of established asthma on the basis of the criteria developed by the criteria for determination of asthma in children (NAEPP, 2007). One hundred health unrelated subjects with a mean age of 8.09 years (SD = 4.09) from the same locality are used as the controls. Informed consent is taken from all the participants before the study. In addition, approval is obtained from the ethical and scientific committees of Karbala health service ethical review committee on research involving human subjects and permission. Whole blood and sera are collected from each participant as the whole blood is used for eosinophil determination and DNA extraction while the sera are used to determine TSIgE levels. DNA extractions are used to detect SNPs in IL-4 and IL-13 (IL-4-590C/T, IL-13-1112C/T) by the use of allele-specific PCR (AS-PCR) and the data are statistically analyzed by SPSS version 25 (Chicago, UA).

Genotyping of IL-4-590 C/T Polymorphisms

DNA is isolated from the whole blood according to the gSYNC™ DNA Extraction Kit, GS100 (100 Preparation Kit). Detection of the IL- 4-590 C/T gene polymorphism (rs2243250) is done by the allele-specific PCR (AS-PCR) technique as described by

(Howell *et al.,* 2003). Beta-actin gene is used as internal control and is done by the PCR technique as described by (Kafita, 2015). The sequences of primers and conditions are shown in the tables below.

The products of PCR are resolved on 1.5% agarose gels and the DNA 100 bp ladder (Bioneer, Korea) is also loaded on the agarose gel. The gel electrophoresis is performed by using 70 V for one hour and stained with Ethidium bromide (Biotech, lot: K901R0KGA) for fifteen minutes and the gel is documented with the gel documentation system (Cleaver, Scientific).

RESULTS

The results for patients is 57% males versus (vs) 43% females while for healthy control it is 55% males vs 45% females. Age group of patients and control are (43%, 30% and 37%) for (<5 year, 5-10 year and >10 year) of patients respectively, vs (31%, 42%, 1nd 27%) for healthy respectively. The age by used mean ± standard deviation (SD) is (8.1±4.08) for patients, while (8.09±4.09) for healthy control as for the weight of patients it is (29.7±14.7) and (26.6±13.9) for healthy control. Body Mass Index (BMI) is (18.04±3.986) of patients, while (17.54±3.802) for healthy control. The major characteristics of asthmatic patients are as follows; 59% are mild vs 41% are moderate in severity and 69% are well controlled vs 31% are not well controlled. Regarding the type of treatment, 75% are montelukast and 25% receive ICS. Highly significant ($p<0.001$) differences are reported in TSIgE levels between patients and healthy control. No significant difference ($p > 0.05$) is reported in blood eosinophils between patients and healthy control. There is a highly significant difference in the distribution of the SNPs between asthmatic patients and the healthy control group (p <0.001). Majority of the patients (66%) are found to carry the CC type whereas the majority of the healthy controls (90%) are found to carry the CT genotype, whereas this genotype appears in only 7% of the healthy controls. The CT genotype seen is 17% of the patients. Collectively, the results of this study indicate that CC is the major genotype in the asthmatic patients. In addition, the finding of this study indicates that CT genotype is outmost genotype (major) in a healthy individual (non-asthmatic). The analysis of demographic data shows a significant association *(p <0.05)* between gender and severity of asthma, whereas among females the proportion of moderate asthma is slightly higher than mild asthma. The majority of male patients (68.4%) are found to have mild asthma and there is no significant difference (p >0.05) between asthma severity and demographic data (age, weight and height). Higher eosinophils percentage and TSIgE levels are seen in moderate asthma in comparison to mild asthma. However, these differences are not statistically significant. Regarding the distribution of IL-4-590 C/T SNPs, it can be seen that the genotypes CC and TT are seen in the nearly double proportion of mild asthma compared to moderate asthma. However, these apparent differences are statistically insignificant (p > 0.05). IL-4-590 C/T SNPs significant difference is reported in the distribution of genotype between the well-controlled and not well-controlled asthmatic patients. The IL-4-590 CC and TT genotype are regarded in higher proportions of well-controlled than not well-controlled asthmatic patients.

A statistically significant difference of the well-controlled and the not well- controlled asthmatic patients (p >0.05) where TT genotype is found to be associated with well-controlled asthmatic patients. In the distribution of types of treatment with the level of control and severity of asthma for asthmatic patients, there is a highly significant with montelukast and level of control ($p<0.001$), while there is no significant difference ($p>0.05$) with ICS treatment. In addition, there is a significant difference ($p<0.05$) between two treatments and the severity of asthma. Hardy-Weinberg equilibrium (HWE) has used it for the allele of the IL-4 gene that has no significant differences between observed and expected genotype frequencies. HWE children met for the IL-4 allele (p-*value* <0.05) and the test for deviation HWE for healthy control and asthmatic children and the test for association (CI: 95% confidence interval), according to wild-type (W.t.), Heterozygous (Het) and Homozygous (Hom.) with high significance of ($p< 0.01$) for cases as for controls it is IL-4-590 C/T.

Polymerase chain reaction (PCR) based detection of SNPs

In this study, *beta-actin* (a housekeeping gene) is used as internal PCR control. Specific primers are used to amplify 200bp segments of the *beta-actin* gene to ensure the efficiency of DNA extraction procedure as shown in figure 1 of 200 bp that are successfully amplified from all DNA extracts. Patient and control subject. In the first PCR reaction, primers specific for C allele are used, whereas in the second reaction primers specific to T allele are used. The PCR amplification products for each of C and T alleles are 224bp in size. PCR products are visualized by gel electrophoresis using 1.5% agarose gel concentration, seventy volts for one hour. Presence of one band from the two reactions has indicated homozygosity (CC or TT, according to the PCR reaction used), if both reactions have yielded bands, this indicates heterozygosity (CT) as shown in figure (3.3) C/T gene, the resulting bands in 224 bp line are compared with the ladder100 used (L).

Table 1: Distribution of Primers sequences for beta actin, IL-4 alleles

Primer	Sequence	Product size (bp)	References
IL-4 −590(C/T)	5′-ACACTAAACTTGGGAGAACATT-GTT-3′	224	(Howell et al., 2003),
T allele	5′-ACACTAAACTTGGGAGAACATT-	224	(NCBI,2018)
C allele	GTC-3′		
Reverse	5′-GAATTTGTTAGTAATGCAG-TCCTCC-3′		
Beta Actin (Internal con-trol)	Forward primer: 5‘-GCCATGTAC-GTTGCTATCC-3‘	200	(Kafita, 2015)
	Reverse primer: 5‘-CCGCGCTCGGTGAGGATC-3‘		

Table 2: PCR mix reaction for genotyping of IL-4 SNP

Component	Volume (µl)	Final concentration
Forward T primer	1 or	10 pico.mol/ µl
Forward C primer	1 and	10 pico.mol/ µl
Reverse primer	1	10 pico.mol/ µl
DNA template	2	100 ng
Deionized H_2O	16	

Table 3: PCR mix reaction for beta – actine primers

Component	Volume (µl)	Final concentration
Forward primer	1 and	10 pico.mol/ µl
Reverse primer	1	10 pico.mol/ µl
DNA template	2	100 ng
Deionized H_2O	16	

Table 4: Conditions of PCR for beta-actin

Steps	Cycles	Temperature/C°	Times / second
Denature template	1	95	30
Initial denaturation	35	95	30
Annealing		55	60
Extension		72	60
Incubation		4	5 minutes

Table 5: PCR conditions for genotyping of IL-4 gene position 590 (C/T)

Steps	Cycles	Temperature/C°	Times / second
First Denature template	1	96	60
First initial denaturation	10	95	15
First annealing		65	50
First extension		72	40
Second Denature template	20	95	50
Second initial denaturation		59	50
Second annealing		72	50
Final extension	1	72	7 minutes
Incubation		4	5 minutes

Table 6: Distribution of biomarkers in patients and healthy control

Biomarkers	Patients	Healthy control	*P* value
Blood eosinophil	1.91 ± 0.842	1.62 ± 0.693	(0.148)
TSIgE (IU/ml)	259.84±187.609*	73.980±39.7886	(<0.0001)

Data presented by mean±S.D; * Highly significant, ($p<0.001$) as compared with control

Table 7: Distribution of IL-4-590C/T gene polymorphism between patients and healthy control children

Subject	IL-4-590 C/T		
	CC (%)	CT (%)	TT (%)
Healthy Controls (N=100)	(7%)	(90%)	(3%)
Patients (N=100)	(66%)*	(17%)*	(17%)*
p-value	0.000	0.000	0.006

= highly significant, (p<0.01) as compared with control*

Table 8: Severity of Asthmatic patients associated with their biomarkers

Biomarkers			Severity of asthma		*P value*
			Mild N=59	Moderate N=41	
Data presented by mean±S.D.	Blood eosinophil		1.75 ±0.751	2.12±0.916	0.103
	TSIgE (IU/ml)		224.17.53±153.421	286.59±188.146	0.348
Data presented by percentage	IL-4-590 C/T	CC	41 (69.4%)	25 (60.9%)	0.536
		CT	7 (11.8%)	10 (24.3%)	0.068
		TT	11 (18.6%)	6 (14.6%)	0.526
	IL-13-1112 C/T	CC	10 (16.9%)	3 (7.3%)	0.059
		CT	44 (74.5%)	34 (82.9%)	0.634
		TT	5 (8.4%)	4 (9.7%)	0.652

Table 9: Hardy-Weinberg Equilibrium for the IL-4 -590(C/T) genotype in patients and controls

Gene	Geno-type	Observed asthma patients number (%)	Expected asthma patients number (%)	P value	Observed controls number (%)	Expected controls number (%)	P value
IL-4 -590 (C/T)	CC	(66 %)	(55.5%)	0.429	(7%)*	(27.04%)	0.002
	CT	(17 %)*	(38%)	0.013	(90%)*	(49.92%)	0.007
	TT	(17 %)**	(6.5%)	0.0001	(3%)	(23.04%)	0.001

*=Significant difference (p < 0.05) as coparesed with expected number; **=Highly Significant difference (p < 0.001) as coparesed with expected number

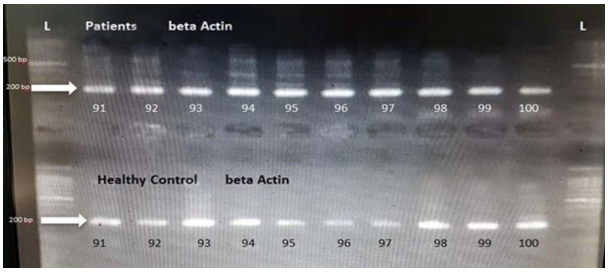

Figure 1: PCR amplification Beta Actin gene segments from DNA samples extracted from patients and healthy controls

(Samples from 99 to 100 in white are patients and black samples are healthy). Gel electrophoresis is done by using 1.5% agarose gel concentration, 70 volts for 60 minutes. L: Ladder.

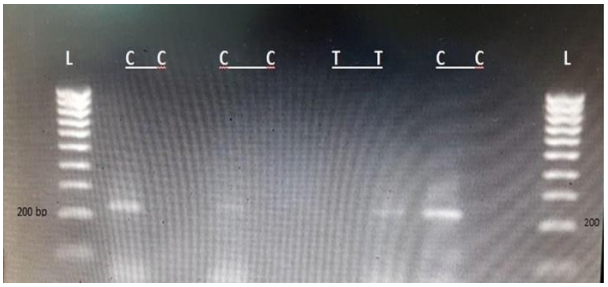

Figure 2: PCR amplification of IL-4 gene -590 C/T showing the C and T alleles (both alleles are 224 bp in size).

PCR products from patients. Tube PCR runs are performed, the first has used primers specific for C allele, whereas the second has used primers specific to T allele. (PCR products are visualized by gel electrophoresis using 1.5% agarose gel concentration, 70 volts for 1 hour (CS electrophoresis MP-250V). L: ladder, Presence of one band for C lane and absence of this band for T (224 bp) lane refer to the genotype homogenous CC Ladder 100(L).

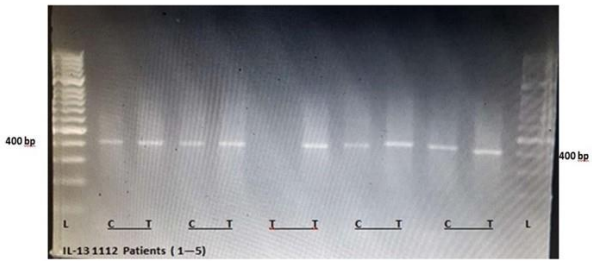

Figure 3: PCR amplification of IL-13 gene -1112 C/T showing the C and T alleles (both alleles are 396 bp in size).

PCR products from patients, tube PCR runs are performed, the first has used primers specific for C allele, whereas the second has used primers specific to T allele. (PCR products are visualized by gel electrophoresis using 1.5% agarose gel concentration, 70 volts for one hour (CS electrophoresis MP-

250V). L: ladder, Presence of one band for C lane and absence of this band for T (396 bp) lane refer to the genotype homogenous CC Ladder 100(L).

DISCUSSION

Asthma has multi-factors with immunological, environmental and genetic factors, all contributing to disease manifestation and progression (Toskala, 2015). In the genome-wide association studies, it has been proposed that genetic variations in the genes of the immunological pathways such as IL-4/IL-13 might be associated with the disease phenotype. However, contradictory reports exist regarding the association of C-590T with allergy susceptibility (Cui *et al.,* 2003). Polymorphisms in the IL-4 R, IL-4 and the IL-13 loci have been reported to be involved with various immune disorders as well as the regulation of serum immunoglobulin levels (Zaiman *et al.,* 2001). Therefore, it is interesting to study the genetic polymorphisms of the IL-4 gene that are mainly involved in the asthma immune response pattern. In this study, it is found that asthma in Iraqi patients is mainly positively associated with the homozygous CC variants of the IL-4- 590, that is conforming to the codominant and overdominant models of inheritance. On the other hand, the homozygous variants IL-4-590 TT genotypes seem to be low-risk ones. They might have significant differences between asthmatic patients and healthy control. The CC allele with asthma in this study is higher than healthy controlled (66% vs 7%). Ihsan Hussein and Saddam H. Jaber have found that the increased risk of asthma is associated with Interleukin-4 SNP especially in genotype CC allele (87.5% vs 32%) (Saddam H.Jaber, 2017) and several local and international studies have confirmed these findings regarding interleukin 4 and asthma. This gene is an important risk factor for asthma susceptibility and severity, with the implications for asthma healthcare management (Berenguer *et al.,* 2014) while in some studies the frequency of CC genotype is (51% vs 71.6%) case vs control respectively (De Guia, 2010) whereas another study has reported (1%.19%,88%) of (CC, CT, TT) respectively (Chiang *et al.,*2007). Z. Hijazi M.Z. Haidar has not found that association between the genotypes of C590T promoter polymorphism of the IL-4 gene and the clinical onset of asthma in the Arabic population of Kuwait (Hijazi, Z., 2000). In this study the C allele for case vs control is (84% vs 97%) respectively, while of Zhang, J. H. et al. study it is (36% vs 67%) (Zhang, J. H., *et al.,* 2016). T allale frequency in control is higher than cases (case vs control) which is (34% vs 93%) respectively. The T allel is a risk allele in the previous study (79.75% vs 79.95%) respectively (HUA, Li *et al.,* 2016). Roldan M.de. Has found that the -590C/T IL4 polymorphism is a potential risk factor as well and correlates with the atopic allergy (Roldan M. de.,2010). Noguchi, E. *et al.,* has found that the -590C/T IL4 polymorphism is associated with the development of asthma children (Noguchi, E. et al.1998).

Asthma tends to be more common in children with insulin-dependent diabetes mellitus (IDDM) than in children without the disease. This indicates that the T helper 1 (T_H1) and TH2 diseases can coexist, showing a common environmental denominator behind the disease processes (Kero *et al.,* 2001).

Severity is a significant difference between male and female patients as mild severity is higher than the moderate severity. Mani Kant has found that in childhood, the moderate is more than mild (33.3 vs 12.3) respectively (Kumar, 2014). Meghan E. et al. have found that obesity is associated with bronchodilator unresponsiveness among children and adolescents with asthma (Meghan E. *et al.,* 2015). Erick et al. have found that obesity is associated with airway dysanapsis in children. Dysanapsis is associated with increased morbidity among obese children with asthma and may partly explain their reduced response to inhaled corticosteroids (Erick *et al.,* 2017).

Cristine S. Rosario et al. have found that there is no significance between eosinophils count and asthma severity, but there is an association between serum IgE and eosinophil counts (Rosario, 2017). Isabela C. and his colleagues have found that polymorphisms might be involved in the modulation of asthma severity (Isabel C. J. de Faria 2008). Wanda and colleagues have found that suggesting a component of corticosteroid non-responsive pathobiology in adults with severe asthma may differ in children (Wanda, *et al.,* 2017).

The relationship between the severity of asthma (mild and moderate) and the Biomarkers of this study show that there is no significance ($p > 0.05$) with eosinophils and TSIgE level means and also has no significance ($p > 0.05$) between severity of asthma and percentage for (IL-4 and IL-13) genes. Daniël A. and colleagues have found that blood eosinophils and IgE have moderate diagnostic accuracy. Their usage of a single surrogate marker for airway eosinophilia in patients with asthma leads to a substantial number of false positives or false negatives (Daniël A. *et al.,* 2015). Matthew S. et al. has found that high-titer IgE antibodies to allergens are strongly associated with the diagnosis, severity and persistence of asthma, as to the large proportion of patients with asthma (Matthew S. *et al.,* 2016). Amina Hamed and colleagues have found that moderate asthma is higher than mild asthma as Serum IgE level is predictive in asthma

and it may be used to differentiate between asthmatic and non-asthmatic individuals in conjunction with other biomarkers. Specific immune therapy reduced serum total IgE level in 36% of patients with asthma (Amina Hamed *et al.,* 2008).

Conflict of Interests The authors declare the complete freedom of any issue concerning conflict of interests related to this work.

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