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Impact of IL-4R (rs1805011) Gene Polymorphism on IL-4 Serum Level in Iraqi Allergic Asthma Patients

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Abstract: Allergic asthma is a chronic inflammatory disease of the airway in which exposure to allergens leads to a complex interaction between genetic and environmental factors while many cytokines are involved in the immune mechanism of the disease.IL-4R play a vital role in the pathogenesis of asthma. The subject of this study is design to investigate an association of single nucleotide polymorphism SNP of the (rs1805011, A>C) gene and its serum level with allergic asthma susceptible in some Iraqi patients. Study consists of two groups: first group consist of sixty asthma patients (34 female and 26 male) and second group contain sixty apparently healthy as a control group (30 male and 20 female). The age of the participants ranged from 15-55 years old. Recruited from the Alzahra'a Center for Asthma Allergy in Baghdad during the period extended from January to April /2022, all information's of the study groups were collected in questionnaire forma such as age, gender, family history, season, smoker and non-smoker and severity of the disease were also included. The level of IL-4R in the blood serum which detected by ELISA- Enzyme-linked-immune-sorbent assay, while (real-time PCR-HRM) technique was used to investigate the Single nucleotide polymorphism. The result of the questionnaire for the group of patients and apparently healthy people was as follows: the highest incidence of asthma cases was in the age group (15-30 years = 50%). The percentage of family history of the disease among patients was (91.67%), while the percentage (8.33%) for those without a family history. The percentage of smokers was higher among patients (63.33%) compared to non-smokers (36.67%), and it was observed that most cases of severe asthma (73.33%) followed by mild asthma (26.67%), and patients who were sensitive to various allergens (68.33%) while (31.67%) have no sensitivity. The laboratory results showed an elevated in mean level of IL-4R in asthmatic patients compared to the control group, and the mean level was (22.99 ng/l) in patients, while it was in control (13.98 ng/l) with significant differences (p=0.0001). The ROC curve analysis of IL-4R described an AUC of 0.887. The results of the allele and genotype of SNP (rs1805011 A>C) also showed clear differences between patients and the control group, the frequency of the C allele was higher in patients compared to the control group (39.2%vs.30.0%) respectively with a significant difference (P=0.044), the frequency of the CC genotype was significantly higher (p=0.034) than in the control group (26.6% vs.10.0%) respectively. In conclusion, genetic and environmental factors such as smoking, family history of asthma, disease severity, allergens, increased level of IL-4R in serum, presence of the C allele and CC genotype in IL-4R gene. They may be considered as important factors in the pathogenesis of allergic asthma in Iraqi patients.

Keywords: Polymorphism, IL-4R Gene, Allergic Asthma, real time- PCR-HRM.

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Introduction

Allergic asthma is a highly heterogeneous airway disease induced by exposure to environmental triggers and is characterized by airway inflammation, airway hyper-responsiveness, elevated immunoglobulin (Ig) E level, and

airway remodeling accompanied by clinical symptoms such as wheezing, shortness of breath, chest tightness, cough, and restricted airflow (1) .The World Health Organization (WHO) estimates in 2020 that approximately 339 million people were affected by asthma and that most deaths occur in older adults , as the Global Initiative for Asthma (GINA) reports in 2020, asthma affects 1%–18% of populations in different countries and its prevalence has been increasing around the world (2-3).

Asthma is more prevalent in developed countries, a study of conduct in the adult population of Tehran, Iran in 2017 showed the prevalence of asthma as (8.9%), (4). While in the Eastern Mediterranean Region (EMR) showed Kuwait and Qatar to have one of the highest prevalence rates for asthma (25.9%) and (19.8%), alongside Saudi Arabia (17.6%) compared with other EMR countries (5). In Iraq, there are many previous researches studied, Abbas (2020) indicated the percentage of asthmatic patients appeared in the age group (31-45) years old of studied participants, which reached to (40%) and that many attributed to their allergen exposure (6). In the development of allergic asthma, both genetic predisposition and environmental factors like exposure to air pollution and allergens play a role (7). Cytokines are a vast group of proteins, peptides or glycoproteins secreted by specific cells of the immune system. These are extracellular signaling molecules that have the ability to mediate and regulate immune response, inflammation and hematopoiesis. In addition, cytokines can have autocrine or paracrine effects (8). IL-4 receptor (IL-4R) play an important role in the establishment and maintenance of type 2 immune responses. It is constitutes a receptor subunit for both IL-4 and IL-13 signaling. The type 1 IL-4R is composed of IL-4Ra and common gamma chain and the type 2 IL-4R consists of IL-4Ra and IL-13Ra1 (9). Furthermore, up to our knowledge no previous research has been performed to measure the level of IL-4R in the serum of patients with allergic asthma, and no study has been conducted to investigate the SNP (1805011 A>C) of *IL-4R* gene in allergic asthma for the Iraqi population and this study is considered the first of its kind at the Iraqi people level. This study aim to Investigate the genetic polymorphisms (rs1805011 A>C) SNP in *IL-4R* gene and its serum level in a sample of Iraqi patients with allergic asthma.

Materials and methods

This study was carried out during the period January – June 2022. the samples were admitted to the Alzahra'a Center for Asthma Allergy in Baghdad. The study design was approved by the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad. A total of 120 exacerbations asthmatic patients and control were included in the study. sixty patients (26 male and 34 female) as first group and sixty apparently healthy individuals (30 male and 30 female) as second group. Personal information such as: age, gender, smoking state, family history of disease, sensitive allergen, incidence intensity (mild and severe disease), season, and period of disease was collected from each participant through questioner form. Writing informed consents were obtained from all patients and

apparently healthy control group, all patients were diagnosed by specialized physicians in respiratory and chest diseases, according to the international criteria based on the clinical details of the medical history, physical examination and medicines.

Enzyme-linked immunosorbent assay (ELISA)

A volume of 3 mL of peripheral blood was dispensed from the asthma patients and apparently healthy controls, the blood was collected in a gel tube then left to clot for about 15 minutes at room temperature (20-25 °C), and then the tube was centrifuged (3000 rpm) for 15 minutes in temperature-controlled centrifuge (4 °C). The separated serum was divided into aliquots (0.25 mL) using 0.5mL Eppendorf tubes, which were kept frozen at -20 °C until using for ELISA assay.

Determinations the IL-4R serum level

Investigation the level of IL-4R in nanogram per liter (ng/l) by ELISA through Sunlong Kit (China). This kit was based on sandwich Enzyme linked immune sorbent assay technology. The wells of the microplate were pre-coated with specific anti-marker antibody (Capture antibody: antibody specific to IL-4R). Upon adding of standards or serum samples to the appropriate wells, a reaction occurs with the specific antibody. This step is followed by adding horseradish peroxidase (HRP)-conjugated with specific antibody. After a period of incubation that is followed by a washing step, the TMB substrate solution (3,3′,5,5′-tetramethylbenzidine) is added to each well. At this point, a blue color is developed in the wells, and after adding the stop solution, the color turns to yellow. The density of the color is proportional to the level of IL-4R. This density (optical density: OD) is measured spectrophotometrically at a wavelength of 450 nm. The levels of IL-4R was calculated from standard curve and expressed in ng/l.

Genomic DNA extraction:

Two ml of peripheral venous blood samples were collected from the asthma patients and apparently healthy controls. Then, blood was kept in Ethylene Diamine Tetracetic Acid (EDTA) anticoagulant tubes in freezer at -20 to be a source for DNA extraction. The DNA was extracted from the samples of blood of asthma patients and apparently healthy subjects by using *EasyPure*®Genomic DNA Kit (Transgenbiotech Company). After genomic DNA was extracted, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA. Then, DNA concentration and purity were measured by nanodrop.

Primer design

IL-4R gene SNP (rs1805011A>C) was determined in the present sample of asthma patients. Primers used in the study were designed according to their reference sequence in the database of National Center for Biotechnology Information (NCBI), as shown in (Table 1).

Table (1): Designed Primers used in the present study

Primer	Sequence (5′→3′ direction)	Primer size bp	Product size (bp)	Ta °C
***IL-4R* rs1805011 A>C**				
Forward	AGAGCAGCAGGGATGACTTC	20	61	58
Reverse	CTCTCTGTTAGCCGGGCC	18		
***IL-13* rs1295685 A>G**				
Forward	CGTTGGGGATTGGGGAAG	18	63	58
Reverse	CCCTGAGTCTCTGAACCCTT	20		

Primer sequence matching

Detecting Primer prepared genes IL4R was assessed for SNPs which included (rs1805011 A>C) for IL4R primers sequences were designed according to their reference sequence (rs) in the database of NCBI (National Center for Biotechnology Information). Primers sequences were matched by the bioinformatics programs NCBI, (Table 2).

Table (2): Matching of the primer sequences using NCBI for IL4R gene

IL4R gene (rs1805011 A>C)
61 bp region
Homo sapiens chromosome 16, GRCh38.p14 Primary Assembly
NCBI Reference sequence: NC_000016.10

CTTCTGTGCATCGCCTGAGAGCAGCAGGGATGACTTCCAGGAGGGAAGGGCGGGCATTGT
>>>>>>>>>>>>>>>>>>>>
GGCCCGGCTAACAGAGAGCCTGTTCCT
<<<<<<<<<<<<<<<<<<<

Primer preparation

The primers for *IL4R* working solution were prepared from the lyophilized sample after dissolving by nuclease free water according to the manufacture to make a stock solution with a concentration of 100 µM for primer were stored at -23C. A working solution with a concentration of 10µM was prepared by diluting 10µL of primers and stock solution in 90µL of nuclease free water and stored at -20°C until use.

High resolution melting (HRM) analysis

The HRM analysis starts with PCR amplification of the region of interest in the presence of a dsDNA binding dye. This binding dye has a high fluorescence when bound to dsDNA and low fluorescence in the unbound state. Amplification is followed by a high-resolution melting step using instrumentation capable of capturing a large number of fluorescent data points per change in temperature, with high precision. When the dsDNA dissociates (or melts) into single strands, the dye is released, causing a change in fluorescence. The result is a melt curve profile characteristic of the amplicon. A Rotor gene Q Real-time PCR System (QIAGEN) was used to perform PCR-HRM followed by an HRM analysis with 0.2 °C scaling from 55 to 95 °C. The 2xTransStart® Tip Green qPCR Super Mix Synthetic SNP sequences were evaluated using duplicates, and used master mixes were containing EVA-Green. To identify allelic differences, real time PCR-HRM was used on triplicate synthetic controls, and normalized melting curves (NMC) and

differential curves (DC) were constructed using the HRM Tool and the program shown in (Table 3).

Table (3): Thermal profile of HRM genotyping (*IL-4R rs1805011 A>C*)

Step	Temperature	Duration	Cycles
Enzyme activation	94ºC	60 sec	1
Denature	94ºC	5 sec	40
Annealing	ºC58	15 sec	
Extension	72ºC	20 sec	
HRM	55-95	0.2sec for 1 degree	

Statistical analysis

The Statistical Analysis System-SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) and T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimate of correlation coefficient between variables. Estimate of Odd ratio and CI in this study. (10).

Result and discussion

Asthma patients distribution by age groups

The age of the patients who enrolled in this study ranged from 15 to 55 years. and they were divided into three groups, the first group of the patients with age ranges (15-30) years, the second group with age range (31-45) years, and the third group with age (>46) years. It was found that the highest asthma rate appeared in the first age group which reached to (50 %) followed by second age group (27%), while third group represented (23%) had the lowest percentage (Figure 1)

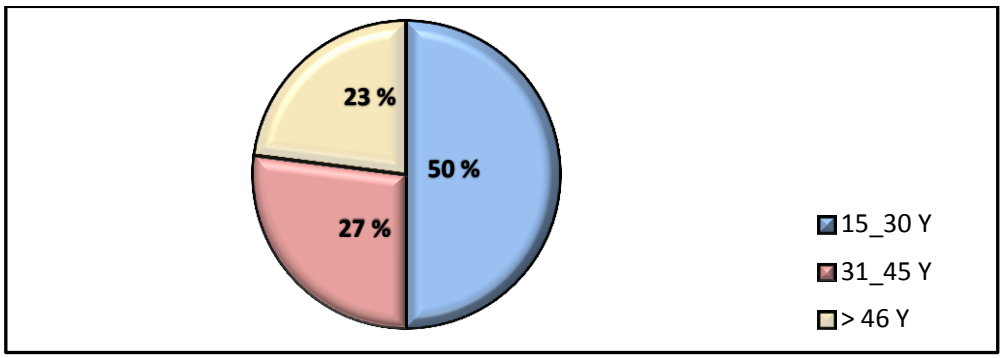

Figure (1): Percentage of distribution patients according to age group.

This study indicate that allergic asthma is high in younger age and gradually decreased in older age. similar to previous researches (11). The reason for the decline in the incidence of allergic asthma with increasing age at asthma diagnosis may be related to atopic allergy often begins in childhood and early adulthood while non-allergic asthma may be related to cumulative exposure to irritating factors such as occupational exposures and smoking and thereby becomes evident only later in life span after sufficient exposure times (12). The present study parallel with some studies of asthma in Iraq,

such as Abbas (6) who found the asthmatic patient's group of (15-45) years constituted high percentage reached (70 %).

Asthma patients and control distribution by gender

In this study, females and males were similarly distributed among asthma patients (56.67 and 43.33%, respectively) and controls (50 and 50%, respectively), and there was no significant difference between their frequencies (p-value = 0.081) as shown in (Table 4). The presence of asthma cases in females in this study are more percentage than male. Possibly, fluctuations in sex hormone levels during puberty, the menstrual cycle and pregnancy are associated with asthma pathogenesis (13).

Table (4): Distribution of the asthma patients and control according to gender

Factors		Patients No. (%)	Control No. (%)	P-value
Gender	Male	26 (43.33%)	30 (50.00%)	0.081
	Female	34 (56.67%)	30 (50.00%)	
NS: Non-Significant.				

Furthermore, before puberty male are more frequently affected, from puberty onwards female predominate, so that, a relationship with male and female hormones has been suggested, testosterone seems to suppress type 2 inflammation (14).

This study was similar to many studies in Iraq, such a recent study by Alsaimay *et al.* (15) who indicated the high percentage of asthma cases was in females and reached (45.31%), where as that of male was (38.68%). Another study by Abdul-Jabbar and Rashid (16) who demonstrated in their results that higher percentage of asthmatic cases was in females (63.3%) compared to males (36.7%).

Association between asthma patients and family history

The distribution of patients according to family history of disease, (Table 5) detected that 55 (91.67%) of patients have a positive family history of asthma and 5 (8.33%) of patients have no family history.

Table (5): Distribution of the Asthma Patients according to Family History

Factors		Patients No. = 60	Percentage (%)	P-value
Family history	Yes	55	91.67	0.0001 **
	No	5	8.33	
** (P<0.01).				

The results of the current study come in parallel with the result of a study conducted by Abdulkareem and AL-Saadi who noticed that (82.67%) of asthmatic patients have a positive family history and (17.33%) have no family history of asthma (17). Another study by Pedersen *et al*. (18) and Alem *et al*. (19) their results showed that patients who had asthma in their family were highly risked to increase severity levels of asthma, suggesting that genetic factors play a central role in increasing asthma severity.

Distribution of asthma patients according to incidence intensity

Most of asthma cases were observed to have severe disease (73.33%), followed by mild asthma (26.67%) there was a significant in variance with *p* value (0.0001), shown in (Table 6).

Table (6): Distribution of Asthma patients according to incidence intensity

Factors		Patients No. =	Percentage (%)	P-value
Incidence intensity	Severe	44	73.33	0.0001 **
	Mild	16	26.67	
** (P≤0.01).				

The current results showed that severe asthma was more common in adult patient, and this agreed with results of Almomani *et al.* (20) who noticed percentage of severity of disease in adult was (67.3%), and mild - moderate was (32.7%) from uncontrolled asthma cases. Furthermore, a study by Rial *et al*. (21) showed that 84% of percentage cases had severe persistent asthma and 16% intermittent or mild persistent asthma.

Distribution of asthma patients and control according to smoking

The distribution of current study asthma patients and control showed high significant difference for smokers in patients which represented (63.33 %) compared to nonsmokers which represented (36.67 %), where the percentage in control was (30%) smoker and (42 %) nonsmoker the significant was (*P* =0.0006) as in (Table 7).

Table (7): Distribution of Asthma Patients and control according to smoking

Factors		Patients No. =	Control No. =	P-value
Smoking	Smoker	38 (63.33%)	18 (30.00%)	0.0006 **
	Non-Smoker	22 (36.67%)	42 (70.00%)	
** (P≤0.01).				

Many researchers were conducted in relationship of asthma and smoking such as Engelkes *et al*. (22) and Tiotiu *et al*. (23) they found cigarette smoking in asthma patients was associated with more severe symptoms; increased healthcare use, costs (unscheduled doctor visits and frequent hospital admissions) and mortality; worse asthma-related quality of life; accelerated decline of lung function; and reduced response to inhaled corticosteroids, the cornerstone of controller treatments in asthma. This results were agreed with results of Sharma and Choudhary (24) who showed smoking was a risk factor amongst (56.7%) percentage of the cases and (22.2%) of the control.

Distribution of asthma patients according to sensitive allergen

The distribution of patients according to sensitivity of disease, (Table 8) detected that 41 (68.33%) of patients have sensitive for, house dust mites, trees pollen, food, animal, perfume, and other environmental factor and 19 (31.67 %) of patients have no sensitive. A significant difference was found between these groups regarding to the sensitivity (p=0.0037).

Table (8): Distribution of Asthma patients according to sensitive allergen

Factors		Patients No. =	Percentage (%)	P-value
Sensitive Allergen	Yes	41	68.33	0.0037 **
	No	19	31.67	
** (P≤0.01).				

This results were agreed with several studies in Iraq, like a study by Abdulkareem and AL-Saadi (17) who detected that 62 (82.67%) of asthma cases have sensitive for drug, food, dust, animal, and other environmental factor and 13 (17.33%) of patients have no sensitive. Similar results by Jebur (25) were they found the common inhaled allergens were mites and trees pollen, also the percentage of allergic asthma patients were sensitive for animal was (82%,) and perfume was (67.3%). The current study believed that sensitivity to numerous environmental allergens may be a risk factor of allergic asthma. Furthermore, Exposure to a range of environmental allergens and irritants are also thought to increase the risk of asthma, including indoor and outdoor air pollution, house dust mites, molds, and occupational exposure to chemicals, fumes or dust (26).

Cytokines measurement

Distribution of human IL-4R serum level

The result of present study showed a highly significant elevation level of the IL-4R in the serum of asthma patients group when compared with control group, IL-4R mean level was 22.99 ng/l in patients, while in control individuals was 13.98 ng/l with (p= 0.0001), (Table 9).

Table (9): Distribution of Human IL-4R serum level in asthma patients and control group

Studies Group	Mean ± SE IL-4R(ng/l)	T. test	*P*. value
Patients	22.99 ±1.80	3.597 **	0.0001
Control	13.98 ±0.24		
** (P≤0.01).			

This study is unique because it is the first study in Iraq to look at the serum levels of IL4R with asthma disease. However, similar results from Hytönen *et al.* (27) who showed significantly higher levels of soluble IL-4R (sIL-4R) in asthma patients compared with controls differences was (P<0.0001). Similar result was shown in a study by Fitch *et al*. (28) who indicated the higher levels of sIL-4R when investigating the bronchoalveolar lavage in children was in the asthmatic cases compared to control group and with a significant differences (P=0.018).

Signaling through the IL-4 receptor (IL-4R) a plays an important role in the establishment and maintenance of type 2 immune responses. Therefore, IL-4R signaling not only regulates the fates of typical type 2 immune cells but also exerts a direct suppressive effect on neutrophils, which further underlines the importance of type 2 immune responses in health and disease (29-9).

Receiver operating characteristic (ROC) analysis of IL-4R

The analysis of ROC revealed that elevated serum level of IL-4R occupied a AUC, which was 0.887 (p-value = 0.001). At a cut-off value of 16.4530

ng/l for serum IL-14R level, the sensitivity and specificity were 73 and 98 %, respectively (Figure 2).

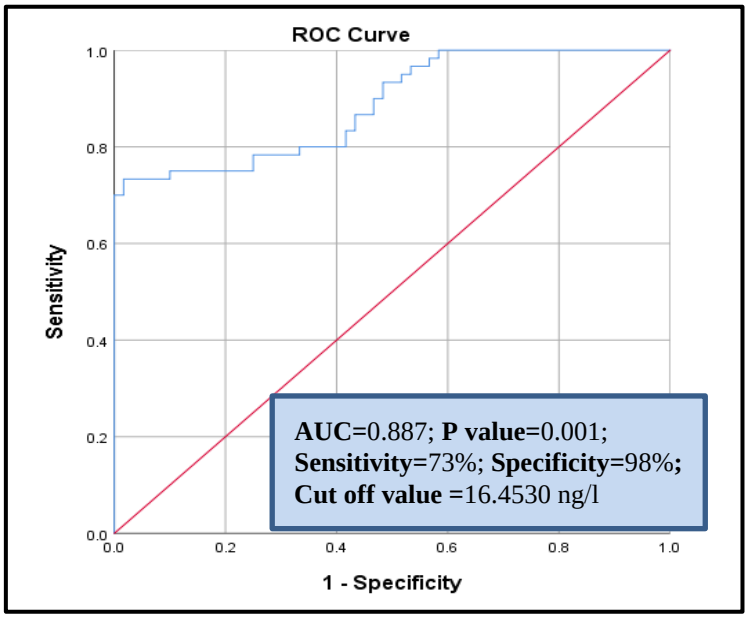

Figure (2): Receiver operating characteristic (ROC) analysis of IL-4R serum level among asthma patients showing area under curve (AUC), *p*-value, sensitivity, specificity and cut-off value.

Molecular analysis

DNA extraction

DNA was extracted from whole blood by using EasyPure® Blood Genomic DNA Kit for all asthma patients and controls. All specimens showed bands, which referred to the genomic DNA on the gel electrophoresis. The purity of DNA was ranged between (1.76- 2.0) and the concentration ranged between (38-62 ng/µl).

Real time-PCR-HRM genotyping

DNA samples of study groups were genotyped of *IL4R* gene (rs1805011A>C) SNP detection was achieved by using real-time PCR-HRM.

Position of (rs1805011 A>C) SNP for *IL4R* gene studied

The position of (rs1805011 A>C) SNP in 3_prime_UTR regain of interleukin 4 receptor gene in chromosome 16: (NC_000016.10).

Genotype and allele frequencies of *IL-4R gene* polymorphism (rs1805011 A> C).

The distribution of genotype and allele frequencies among patient groups compared with the healthy group for the *IL-4R* gene polymorphism (rs1805011 A>C) is shown in (Table10). The genotypic frequencies of asthma patients were 41.6% (n=25) wild type AA and 31.6% (n=19) heterozygous AC. Mutant homozygous was found in CC 26.6% (n=16). In controls, the results demonstrate 50.0% (n=30) wild type AA, 40.0% (n=24) heterozygous AC and mutant homozygous CC 10.0% (n=6).

The result of genotype frequencies of asthma patient's and control analysis reveals that the AA genotype and A wild type allele were taken as reference.

The odds ratio for the CC genotype was 3.2 with (P-value =0.034) indicating that homo mutant genotype CC was at a higher risk of asthma than the wild-type AA. The AC genotype had an odds ratio of 0.9 and (P-value=0.9). The AC+ CC genotype had an odd ration 1.4 and (p-value=0.3). While the odd ratio of CC genotype vis. AA+AC was higher 3.2 with (P-value=0.022). The frequency of the A allele in asthma patients and controls was 60.8% (n=69 and 70.0% (n=84) respectively. While the frequency of the C allele in asthma patients and controls was 39.2% (n=51) and 30.0% (n=36) respectively with a high odd ratio 1.7 and (p-value= 0.044). This indicate that C allele was a high risk of asthma than A allele.

Table (10): Comparison of the genotype and allele frequencies of *IL-4R* gene polymorphism (rs1805011 A> C) between patients and control group.

***IL-4R* polymorphism rs1805011 A> C**	**Frequencies (%)**		**P-value**	**Odds ratio (95% CI)**
	Control group (n=60)	**Patients Group (n=60)**		
Codominant				
AA	50.0% (n=30)	41.6 % (n=25)	---	1.00 (Reference)
AC	40.0% (n=24)	31.8 % (n=19)	0.9	0.9 (0.4-2.1)
CC	10.0% (n=6)	26.6 % (n=16)	0.034*	3.2 (1.0-9.4)
Dominant				
AA	50.0% (n=30)	41.6% (n=25)		1.00 (Reference)
AC+ CC	50.0% (n=30)	58.4% (n=35)	0.3	1.4 (0.6-2.8)
Dominant				
AA+AC	90.0% (n=54)	73.4% (n=44)		1.00 (Reference)
CC	10.0% (n=6)	26.6 % (n=16)	0.022*	3.2 (1.1-9.0)
Allele				
A	70.0% (n=84)	60.8% (n=69)		1.00 (Reference)
C	30.0% (n=36)	39.2% (n=51)	0.044*	1.7 (1.0-2.9)
*** (P≤0.05)**				

Cytokines, having central functions in immunological and inflammatory processes, are expected to play important roles in the pathogenesis of various diseases, such as asthma (30) Therefore, genetic polymorphisms of those cytokine and cytokine receptor genes are the focus of genetic association studies, however, the genetic association of *IL-4R* with asthma might provide valuable insights into the pathogenesis of asthma (31). Mutations in the IL-4R gene are associated with asthma exacerbations and immunopathologic changes in the airways, Also, biologicals targeting IL4Rα show promise in the treatment of persistent asthma (32).

In this study result found that genetic polymorphisms in (rs1805011 A>C) for IL-4R gene was a risk factor for allergic asthma. Similar to a study by Narożna *et al*. (33) and (34) their results indicated that rs1805011 polymorphism of IL4Rα gene seems to influence allergy risk, especially mild asthma and atopic dermatitis predisposition in Polish children, in subgroup analysis, they detected an association of rs1805011 with mild asthma and significant differences was (p =0.00005), with C allele more frequent in patients than in the control group (p=0.029; OR =2.029).

Conclusion

In this study, genetic and environmental factors such as smoking, family history of asthma, disease severity, allergens, increased levels of IL-4R in serum, presence of the C allele and CC genotype in IL4R gene. They may be considered as important factors in the pathogenesis of allergic asthma in Iraqi adult patients. Furthermore, no previous research has been performed to measure the level of IL-4R in the serum of patients with allergic asthma, and no study has been conducted to investigate the SNP (1805011A>C) of IL-4R gene in allergic asthma for the Iraqi population and this study is considered the first of its kind at the Iraqi people level.

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