GENETIC POLYMORPHISM OF ASTHMA IN IRAQ

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ABSTRACT

This case-control study was conducted to evaluate the influence of interleukin (*IL) - 17A* and *-17F* gene polymorphisms on the risk of asthma. In the present study blood samples were collected from 45 asthma patients and 50 healthy controls. Serum levels of cytokines interleukin-17A and interlukin-17F *(IL-17A and IL17F,* respectively) and immunoglobulin E (IgE) were measured using ELISA kit in both subjects. Serum levels of *IL-17A* and IgE were shown a higher significant differences (p< 0.05) while no significance difference (p>0.05)of IL-17F was shown in serum levels in comparison with controls . The frequency of the allele and genotypes in the patient groups and control groups were determined using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP). *IL-17A* gene amplified using specific primers then digested by (*XagI)* restriction enzyme, in order to detect (G197A) (rs2275913) SNP. While *IL-17F* gene amplified using specific primers then digested with (*Nla III)* restriction enzyme to detect (A7488G) (rs763780) SNP. There were no significant differences between asthma patients and wild type GG and G allele and mutant type AA and A allele for *IL-17A* SNP (GG: OR 1.71, P=0.284, G: OR 1.71, P=1.00, AA: OR 1.99, P =0.294, A: OR 0.96, P=1.00) while the mutant type AG for mentioned SNP was determined and tested for their association significantly with asthmatic patients (OR 0.39, P=0.039). Frequencies of *IL-17F* genotype AA and allele A and mutant type AG or GG were found no associated with asthma patients.

Keywards: interlukin-17A, interlukin-17F, polymerase chain reaction, mutation

تعدد الشكل الوراثي للربو في العراق

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المستخلص

أجريت دراسة المرضى ونماذج السيطرة لتقييم تأثير الأشكال الوراثية للإنترلوكين 17A – (IL) والانترلوكين –17 F على مرضى الربو. في هذه الدراسة ، تم جمع عينات الدم من 45 مريضا بالربو و 50 من نماذج السيطرة. وتم قياس مستويات مصل السيتوكينات إنترلوكين –17 A وإنترلوكين –17 F والغلوبولين المناعي E (IgE) باستخدام تقنية الاليزا في كلا المجموعتين. أظهرت مستويات المصل لـ IL– 17A و IgE اختلافات معنوية أعلى (p <0.05)) في حين لم يظهر هنالك فرق معنوي (p <0.05) من IL–17F في مستويات المصل مقارنة مع مجاميع السيطرة. تم تحديد تردد الأليل والأنماط الجينية في مجموعات المرضى ومجموعات السيطره ,باستخدام تقنية تفاعل سلسلة الدنا (PCR–RFLP). ضخم جين IL–17A باستخدام بادئات محددة وتم هضمها بواسطة إنزيم التقييد (*XagI*) ، من أجل االبحث عن النيوكلوتيدة الواحدة متعددة الاشكال الواراثية (rs2275913) (G197A). في حين تم تضخيم جين IL–17F باستخدام بادئات محددة ثم هضمها مع انزيم تقييد (*NlaIII*) للكشف عن النيوكلوتيدة الواحدة متعددة الاشكال الواراثية ((A7488G) (rs763780). لم تكن هناك فروقات ذات دلالة إحصائية بين مرضى الربو والنوع الوراثي GG واليله G والنوع الطافر AA واليله A للانترلوكين 17A للطفرة (rs2275913) (G197A) (AA: OR 1.99, P =0.294, A: OR 0.96, P=1.00) بينما النوع الطافر AG للطفرة المذكورة اظهرت ارتباطا كبيرا مع مرضى الربو (OR 0.39، P = 0.039). كما لم يتم العثور على فروق معنوية لل IL–17F للنمط الوراثي AA واليلهA والنوع الطافر AG أو GG في مرضى الربو مقارنة بنماذج السيطرة.

الكلمات المفتاحية: انترلوكين 17– A، انترلوكين 17–F، تفاعل سلسلة البلمرة، الطفرة الواراثية.

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INTRODUCTION

Asthma is a common disease related to a chronic airway inflammation that is characterized by intense eosinophil and lymphocyte infiltration, mucus hypersecretion airway hyperresponsiveness (AHR), and airway remodelling. Increased disease severity is usually coincided by the additional influx of their cytokines within the pathogenesis of allergic asthma is now well recognized (13). Accumulating evidence indicates that Th2 cells and their derived cytokines like interleukin *IL-4, IL-5,* and *IL-13* play critical roles in orchestrating and amplifying the allergic inflammation in asthma. However, recent studies have shifted attention toward Th17 cells as being liable for the pathological processes (6). Th17 cell is a lineage distinct from Th1, Th2, and Treg cells characterized by the production of IL-17. Until now, the IL-17 family contains six members, noted as IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E, and IL- 17F, according to the order within which they were discovered (11). similarly as a common receptor (IL-17 receptor A/IL-17 receptor C complex) (40), share the identical genes locations on chromosome 6p12, and have similar functions (13). Recent studies have a rise in the expression of IL-17 in chronic inflammatory disorders. An increase in IL-17 levels has been reported in rheumatoid arthritis (24), multiple sclerosis (24), and chronic obstructive pulmonary disease (COPD) (37). In patients with established asthma, IL-17 mRNA and/or proteins were reported to be increased within the lungs, induced sputum, bronchoalveolar lavage (BAL) fluids, or sera (1 , 42) and therefore the levels of IL-17 correlated with the degree of disease severity (42) suggesting the role of this cytokine in airway remodelling. The ability of IL-17A and IL-17F to induce neutrophils migration suggested that they are involved in severe asthma, during which accumulation of neutrophils in the airways is a major characteristic (15). The association between IL-17A and IL-17F and neutrophilic inflammation was also confirmed by murine asthma models (14). However, the direct regulatory effect and mechanism of this cytokine on eosinophilic airway inflammation are somewhat complex to decipher. Studies using murine asthma models showed the role of those two cytokines in enhancing eosinophilic inflammation during the sensitization phase. In fact, mice deficient in IL-17 or IL-17 receptor have a reduced ability to recruit eosinophils into the airways (35) due to their low expression of Th2 cytokines such as IL-4 and IL-5 necessary for eosinophils attraction (26). It appears, therefore, that, at the sensitization phase, IL-17 promotes eosinophilic airway inflammation by mounting allergen-specific Th2 cell responses. These findings were confirmed by the study of Wakashin and colleagues (5) suggesting that high IL-17 levels could inhibit eosinophilic inflammation at the effector phase. These various actions of IL-17 suggest that this cytokine may play different roles in the airways, depending on the context and the timing. The role of IL-17 in eosinophilic inflammation of mild and moderate asthma should be more investigated. Genetic studies using polymorphisms could present the aid in demonstrating links between certain molecules and diseases like asthma, characterized by interaction between several genes in different chromosomes (7)of one SNP in *IL-17A* gene (-197G/A, rs2275913) and SNP in *IL-17F* gene (7488A/G, rs763780) with susceptibility to asthma.

MATERIALS AND METHODS

Study design and subjects: Five microliter of blood samples were taken from patients and controls and distributed in to two tubes one for DNA extraction and the other for cytokine concentrations which allowed to clot at 4°C for 1 h and centrifuged at 2000 g for 10 min. The study included 45 patients with asthma and the control group consisted of 50 healthy subjects with no systemic diseases, matched according to age (years) and gender (women and men). The study protocol was approved by the local Ethics Committee and has a cross-sectional design and is based both in a private setting and in a community hospital. Adults/adolescents patients with doctor diagnosed persistent asthma were selected from the hospital database. Asthma was diagnosed both by history and clinical examination.

Measurement of cytokine concentrations: The obtained serum was stored at –20°C until

analysis. Measurements of IgE, IL-17A and IL-17F in serum samples were performed using ELISA sandwich kits (Monoband Inc., CA92630, USA) following the manufacturer's instructions.

DNA extraction

DNA of all subjects was separated from 2 ml peripheral blood with Reliaprep promega Kit (promega, USA) and the DNA was resuspended in RNAse/DNAse-Free Distilled water after following the manufacturer's instructions.

***IL-17A* and *IL-17F* genotyping**

IL-17A and *IL-17F* gene polymorphisms were analyzed using a polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP). Primer sequences and restriction enzymes for them are demonstrated in Table 1 and 4,

Table 2. PCR Program for IL-17A gene

Steps	Temperature (˚C)	Time	
Initial Denaturation	94	5 min	
Dentura tion	94	45 sec.	
Annealing	60	45 sec.	(35 cycle)
Extension	72	60 sec	
Final Extension Step	72	10 min	

respectively. The PCR amplification was per-formed in a total volume of 25 µl mixtures containing 50-150 ng of genomic DNA, 10 pM of each primer and Promega green Taq (Promega, USA). For *IL-17A* G197A PCR amplification, an initial denaturation at 94°C for 5 min was followed by 35 cycles at 94°C for 45 s, 58°C for 45 s, at 72°C for 1 min, and a final extension at 72°C for 10 min table(2). For *IL-17F* A7488G PCR amplification, an initial denaturation at 94°C for 5 min was followed by 36 cycles at 94°C for 45 s, 60°C for 45 s, at 72°C for 1 min, and a final extension at 72°C for 10 min table (3). The PCR products of *IL-17A* G197A and *IL-17F* A7488G were digested with *XagI* (Fermentas, Lithuania) and *NlaIII* (New England Biolabs, Ipswich, MA, USA) restriction enzymes at 37°C for 5 minutes, respectively. The products were then analyzed in 3% agarose gel stained with ethidium bromide and visualized under UV light.

Table1. Primers sequence of *IL-17A* and *IL-17F*

GENES	Primers sequences
IL-17A	Forward: 5'-AACAAGTAAGAATGAAAAGAGGACATGGT-3' Reverse: 5'-CCCCCAATGAGGTCATAGAAGAATC-3'
IL-17F	Forward:5'-GTTCCCATCCAGCAAGAGAC-3' Reverse:5'-AGCTGGGAATGCAAACAAAC-3'

Table 4. Genes, restriction enzymes, the size of digested fragments that are used for screening by PCR-RFLP for *IL-17A* and *IL-17F* genes polymorphisms

Steps	Temperature (˚C)	Time	
Initial Denaturation	94	5 min	
Denaturation	94	45sec.	
Annealing	58	45 sec.	(35 cycle)
Extension	72	60 sec	
Final Extension Step	72	10	min

Statistical analysis

Hardy-Weinberg equilibrium of genotypes was evaluated with chi-square test. Odds ratios (ORs) and 95% confidence intervals (CIs) for asthma in association with genotypes were calculated with unconditional logistic regression adjusted by age and gender. All data were analyzed using the statistical package SPSS version 17.0 (SPSS, Chicago, IL). p values < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Serum levels of IL-17A, IL-17F and IgE in patients with asthma

Serum levels of IL-17A and IgE were showed a higher significant differences ($p<0.05$) (59.121pg/ml ±55.383 and 210.678 IU/ml ± 146.697,respectively) (mean ± S.D) compared

with healthy controls (7.476pg/ml ±24.796 and 130.837 IU/ml±144.881, respectively) while no significance difference(p> 0.05)of IL-17F was showed in serum level in comparison with controls (148.63pg/ml±17.146 and 143.548pg/ml ±15.710 respectively) . The importance of Th2 in asthma has led .

Table 3. PCR Program for IL-17F gene

Genes	Restriction enzymes	Genotypes	Bands (bp)
IL-17A	***XagI***	**GG**	**68 and 34**
		AA	**102**
		AG	**102, 68 and 34**
IL-17F	***NlaIII***	**GG**	**412**
		AA	**288 and 124**
		AG	**412, 288 and 124**

to classifying asthma into two main subclasses: Th2-high and Th2-low. In Th2-high asthma, amplification in expression of Th2 cytokines can lead to symptoms of asthma such as airway eosinophilia and bronchial hyperresponsiveness. The increase in serum IgE and development and activation of Th2 cells, eosinophils, and mast cells, are the main features of atopic asthma (27). It has been accepted that atopic asthma is associated with activation of Th2 cells in the airways and augmented expression of Th2 cytokines that could lead to symptoms of asthma such as airway eosinophilia and bronchial hyperresponsiveness. Th17 cells are new members of T helper family that produce IL-17A and IL-17F. IL17A has a key role in allergic responses especially in allergic airway inflammation. IL17A can initiate inflammatory processes via stimulating production of various proinflammatory cytokines and chemokines, followed by recruiting neutrophils, enhanced antibody production, and activation of T cell subsets (3,31). The most reviewed function of IL-17A is to drive inflammation through induction of macrophage and neutrophil chemokines and growth factors such as CXCL2 (MIP-2), CXCL8 (IL-8), CCL-2 (MCP-1), granulocyte colony stimulating factor and granulocyte-monocyte colony stimulating factor so that increased production of IL-17A has been indicated in asthma (8). The production of IL-17 is increased in peripheral blood mononuclear cells of asthmatic patients. In sputum, it has been shown to correlate with AHR (2,3). Baghdad reported the highest number of respiratory infections due to its crowded city of people may according to the increase in migration of people from the rural area to the cities so facilities transmission of these RTI and increase average of incidence among them(4) .The elevated serum level of IL-17A is important in immune pathgenesis of allergic asthma and the Th17 immunity can, in addition to Th2 immunity, contribute to asthma occurrence. IL-17A, like IgE, rises in sera of asthmatic patients in a different manner.

Comparison of *IL-17A* and *IL-17F* polymorphisms between asthma patients and healthy controls

A total of 45 asthmatic patients and 50 controls were enrolled in this study. Only Genotype distribution of IL-17A G197A was in Hardy-Weinberg equilibrium ($p<0.05$). In order to study the impact of *IL-17A* G197A and *IL-17F* A7488G polymorphisms on development of asthma patients, we employed PCR-RFLP to assess the distribution of the genotypes in all subjects. *IL-17A* was amplified using the primers shown in table (1). The PCR product was then digested by *XagI* restriction enzyme to produce fragments with 68 and 34 bp bands in the presence of GG genotype and the production of 102 bp bands indicate AA genotype while the production 102, 68 and 34 bp represented AG genotype as shown in table (4). The frequencies of *IL-17A* (197G/A) illustrated in table (5). There were no significant differences between asthma patients and wild type GG and G allele and mutant type AA and A allele for IL-17A SNP (GG: OR 1.71, 95%CI: 0.67-4.34, P=0.284, G:

OR 1.04, 95%CI: 0.56-1.93, P=1.00, AA: OR 1.99, 95%CI: 0.62-6.70, P =0.294, A: OR 0.96, 95%CI: 0.52-1.79, P=1.00) while the mutant type AG for mentioned SNP was determined and tested for their association significantly with asthmatic patients (OR 0.39, 95%CI: 0.16-0.98, P=0.039). The genotype distribution of *IL-17A* gene SNP (197) G > A genotypes in asthmatic patients was not in Hardy-Weinberg equilibrium ($p<0.05$) table (6). To achieve the equilibrium five conditions must be met: population must be very large, population must be isolated from other populations (no immigration or emigration), no alleles mutations (deletion or insertion), random mating (no inbreeding) and no natural selection (i.e. every individual has an equal chance of survival). If the five conditions are not met then evolution occurs and there is a change in allele frequency in the population and Hardy–Weinberg equilibrium is not present (11).Because the mutation occurred in *IL-17A* gene (197) G > A genotypes in this study and inbreeding (increased recessive alleles) may occurred that led to deviate in Hardy – Weinberg equilibrium and the genotypes distribution were not in this equilibrium.

Table 5. Observed numbers and percentage frequencies of SNP (197 G/A) in *IL-17A* gene in asthmatic patients and controls

IL-17A Genotypes		Patients (No.=45)		Controls (No.=50)		Odd Ratio	95% C.I.	p-value
		No.	%	No.	%			
GG (homozygous wild type)		19	42.2	15	30.0	1.71	0.67-4.34	0.284
AG (heterozygous mutant type)		15	30.0	28	56.0	0.39	0.16-0.98	0.039
AA (homozygous mutant type)		11	24.4	7	14.0	1.99	0.62-6.70	0.294
Total		45	100.0	50	100.0	-	-	-
Allele frequency	*G*	53	34.44	58	58.0	1.04	0.56-1.93	1.00
	A	37	65.56	42	42.0	0.96	0.52-1.79	1.00

Table 6. numbers and percentage frequencies (observed and expected) of *IL-17A* gene SNP (197) G > A genotypes and their Hardy-Weinberg equilibrium (HWE) in asthmatic patients compared with control groups

Groups			*IL-17A* genotypes polymorphism					
			Genotypes			HWE	Alleles	
			GG	AG	AA	p – value	G	A
Asthmatic patients (No. = 45)	Observed	No.	19	15	11	0.036	35	37
		%	42.2	30.0	24.4		58.89%	41.11%
	Expected	No.	15.61	21.79	7.61		Not estimated	
		%	34.68	48.42	16.9			
Controls (No. = 50)	Observed	No.	15	28	7	0.290	58	42
		%	30.0	56.0	14.4		58.0%	42.0%
	Expected	No.	16.82	24.36	8.82		Not estimated	
		%	33.64	48.72	17.64			

Genotypes of *IL-17F* gene was amplified under the conditions previously described using primers. The PCR product was then subjected to RFLP analysis using *NlaIII* restriction enzyme to detect the genotypes of *IL-17F* gene to produce fragments with 288 and 124 bp bands in the presence of AA genotype and the production of 412 bp bands indicate GG genotype while the production 412, 288 and 124 bp represented AG genotype as shown in table (7). Frequencies of *IL-17F* genotype AA and allele A and mutant type AG were found no significant association with asthma patients $p>0.05$ while the mutant type GG was not found in our study. The results of genotype and alleles showed (AA genotype: OR 1.99, 95%CI 0.77-5.24 and p=0.135), (A allele: OR 1.73, 95%CI 0.75-4.12 and p=0.183), (AG genotype: OR 0.50, 95%CI 0.19-1.30 and p=0.135) and (G allele: OR 0.58, 95%CI 0.24-1.33and p=0.183)

Table 7. Observed numbers and percentage frequencies of SNP A7488G in *IL-17F* gene in asthmatic patients and controls

IL-17A Genotypes		Patients (No.=45)		Controls (No.=50)		Odd Ratio	95% C.I.	P-Value
		No.	%	No.	%			
AA (homozygous wild type)		33	73.33	29	58.0	1.99	0.77-5.24	0.135
AG (heterozygous mutant type)		12	26.67	21	42.0	0.50	0.19 -1.30	0.135
GG(homozygous mutant type)		0	0.0	0	0.0	-	-	-
Total		45	90.0	50	100.0	-	-	-
Allele frequency	*A*	78	86.66	79	79.0	1.73	0.75 – 4.12	0.183
	G	12	13.33	21	21.0	0.58	0.24 – 1.33	0.183

Table 8. numbers and percentage frequencies (observed and expected) of *IL-17 F* gene SNP (A7488G, rs763780) A > G genotypes and their Hardy-Weinberg equilibrium (HWE) in asthmatic patients compared with control groups

Groups			*IL-17F* genotypes polymorphism					
			Genotypes			HWE	Alleles	
			AA	AG	GG	P- value	A	G
Asthmatic patients (No. = 45)	Observed	No.	33	12	0		78	12
		%	73.33	26.27	0.0	0.302	86.67%	13.33%
	Expected	No.	33.8	10.4	0.8		Not estimated	
		%	75.11	23.11	1.78			
Controls (No. = 50)	Observed	No.	29	21	0	0.060	79	21
		%	58.0	42.0	0.0		79%	21%
	Expected	No.	31.21	16.59	2.2		Not estimated	
		%	62.41	33.18	4.41			

The following figures are representative examples of genotypes of *IL-17A and IL-17F* genes investigated in this study after digested with restriction enzymes.

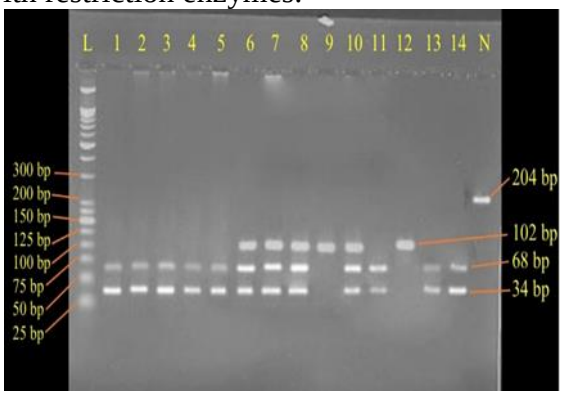

Figure 1. A photograph of 3% agarose gel showing the PCR-RFLP product for *IL-17A* gene after digestion with *XagI* restriction enzyme for patients. Gel viewed under Gel documentation – UV trans-illuminator after being stained with ethidium bromide stain. Lane L: 25/100 bp mixed DNA Marker. Lane N: DNA before cutting. Lane 9 and 12 :one fragment band indicate AA genotype(102)bp. Lane 1-5,11,13 and 14: two fragments (68+ 34) bp bands indicate GG genotype, Lane:6,7,8,10:three fragments bands (102+68+34) indicate AG genotype

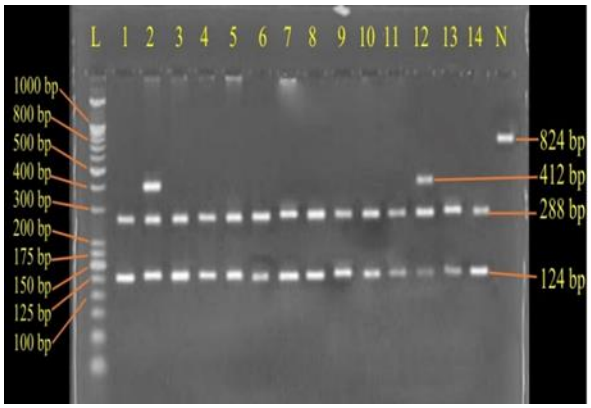

Figure 2. A photograph of 2% agarose gel showing the PCR-RFLP product for *IL-17F* gene after digestion with *NlaIII* restriction enzyme for patients. Gel viewed under Gel documentation – UV trans-illuminator after being stained with ethidium bromide stain. Lane L: 25/100 bp mixed DNA Marker. Lane N: DNA before cutting. Lane 2 and 12:three fragment band indicate AG genotype (412+288+124) ,Lane 1,3-11,13,14: two fragments (288+ 124) bp bands indicate AA genotype in

In the present study, we identified correlations between polymorphisms of *IL-17A* and *IL-17F* genes and asthma development in Iraqi patients in comparison with healthy controls. Our results revealed that *IL-17A* 197AG genotype was associated with asthma, while no significant differences in *IL-17F* A7488G

distribution was found between patients and controls. It suggested that *IL-17A* may play a role in the development of asthma. In line with our findings, a recent study demonstrated that *IL-17A* G197A (rs2275913) AG genotype was related with an increased risk of asthma development. In last ten years, a flurry of emerging evidence has established correlations between *IL-17* polymorphisms and the pathogenesis of a wide range of diseases, such as autoimmune disorders (32). Polymorphisms of IL-17 in asthma patients have been less characterized. We conducted a case-control study during which we found that the frequencies of *IL-17A* G197A , AG genotypes in asthma patients were significantly more than in healthy controls, while there was no statistical difference in other genotypes of *IL-17A* G197A and *IL-17F* A7488G genotypes distribution between asthma patients and healthy controls. Other studies with more patients of more varied racial/ethnic backgrounds should be conducted to verify our results. Our findings may suggest that immunity-mediated cytokines like *IL-17A* play a role in the pathogenesis of various autoimmune diseases (40). Disturbed cytokine profile in asthma is related to loss of immune tolerance. For instance, it has been well established that Th1 polarization is correlated with disease activity in asthma (18), The mechanism involved may be that IL-17A plays a role in inflammation by inducing release of proinflammatory and neutrophil-mobilizing cytokines (25). IL-17 plasma levels were found to be significantly increased in a series of inflammation-related diseases, such as asthma (29), systemic lupus erythematosus (30), allergic rhinitis (39) and HBV-related liver cirrhosis (40). Both IL-17A and IL-17F are produced by Th17 cells and lie immediately adjacent to one another on human chromosome 6 (38). However, our investigation suggests that IL-17A, but not IL-17F, contributes to susceptibility of asthma development in Iraqi patients. We estimated that different ethnic groups or relative small numbers of our subjects contribute to the results. In brief, we suggested that *IL-17A* G197A in mutant type is a candidate gene that confers the genetic susceptibility for asthma development in Iraqi population.

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