Article

Polymorphism of IL13 (rs1295685) Gene and Its Serum Level in a Sample of Iraqi Patients with Allergic Asthma

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ABSTRACT

Interleukin 13 (IL-13) is an immune-regulatory cytokine, primarily secreted by activated T Helper-Type (Th) 2 cells, which inhibits inflammatory cytokine production in allergic asthma. Single nucleotide polymorphism SNP (1295685A>G) of the IL-13 gene and its serum level was included in a case-control study on 60 Iraqi asthmatics and 60 controls. ELISSA-linked immune-sorbent assay was used to estimate blood serum levels. The real-time high-resolution melting polymerase-chain-reaction (Real-time PCR-HRM) method was used to determine this variant. The results showed that the mean level of IL-13 was high in asthmatic patients compared to the control group (22.90 pg/ml vs.13.99 pg/ml), respectively, and significant differences were (p=0.0001). ROC curve analysis of IL-13 described an AUC of 0.882. Regarding the allele and genotype of (A>G 1295685) SNP, there were clear differences between patients and the control group, as the frequency of the GG +AG genotype was significantly higher (p=0.001) in patients compared to the control group (81.7%vs.23.3%) respectively, and the G allele frequency was higher in patients compared to the control group (48.8% vs. 16.6%) respectively, with significant difference (p=0.001). In conclusion, increased levels of IL-13 in serum and the presence of the G allele, GG + AG genotype in the IL13 gene may be associated with the pathogenesis of allergic asthma in Iraqi adult patients.
Keywords: Allergic Asthma, IL-13, SNP, RT-HRM-PCR

INTRODUCTION

Asthma is the most common chronic respiratory disease worldwide, affecting 339 million people. It is a chronic disease involving the airways that carry air in and out of the lungs. In people with asthma, these airways are inflamed, making them very sensitive and often reacting to allergens or irritants [1,2]. Allergic asthma is one of the best-described asthma phenotypes of primary studies. It counts for up to 80% of childhood asthma and more than 50% of adult asthma cases[3,4]. In this type, the cytokines such as interleukin IL4 and IL-13 secreted by immune cells, including innate lymphoid cells (ILC) and T helper cells (Th2), act on multiple effector cell types, including B cells, mast cells, eosinophils, smooth muscle cells, fibroblasts, and epithelial cells, to produce various features of allergic asthma [5]. The pathological mechanism of allergic asthma is not yet completely

clear. It is generally recognized that it starts with the activation and differentiation of allergen-specific T helper 2 cells (TH2). Hence, this is followed by the production of immunoglobulin E (IgE) and the recruitment of eosinophils into the lungs. Eventually, the persistent airway inflammation and asthma symptoms develop [6]. When the invading antigen binds to antigen-specific IgE on mast cells, the mast cells release mediators (degranulation). The released mediators consist of histamine, prostaglandin, leukotriene and several cytokines such as IL-8, IL- 13, tumor necrosis factor (TNF)-a and chemokine ligand (CCL) 2 [7]. These mediators induce mucus production, bronchoconstriction, and the recruitment of leukocytes such as neutrophils, lymphocytes, and eosinophils [8].

IL-13 is a regulatory cytokine mainly secreted by CD4+ Th2 and type 2 innate lymphoid cells and is also produced in lesser quantities by mast cells, eosinophils, basophils, CD8+ Th cells, and natural killer cells. IL-13 target B-cells and induce these lymphocytes to operate an immunoglobulin (Ig) class switch, leading to the synthesis of IgE [9]. IL-13 drives airway goblet cells to secrete excessive mucus, one of the characteristics of airway pathological changes in allergic asthma. During this process, IL-13 activates epithelial autophagy [10]. Nearly 100 asthma-related genes have been identified for asthma susceptibility, and the former gene is IL13 [11]. IL13 gene is located on human chromosome 5q23-q31. It consists of 4 exons and 3 introns encoding the protein containing 132 amino acids [12]. More than 10 papers report an association between single nucleotide polymorphisms (SNP) in IL-13 and the effects on asthma in adults and children in the context of infections, atopy, IgE levels, or risk for asthma [13]. This study aims to evaluate the association of the *IL13* gene (A>G 1295685) SNP and its serum level with the risk of allergic asthma. To the best knowledge of investigators, IL13 (A>G 1295685) SNP has not been investigated in Iraqi allergic asthma patients.

MATERIALS AND METHODS

During January– April 2022, a case-control study was conducted for 60 Iraqi adult Asthmatic patients (*26* male and 34 female), and 60 healthy individuals as the control group (30 male and 30 female) and their age was 15-55 years old. The samples were admitted to the Alzahra'a Center for Asthma Allergy in Baghdad. The Ethics Committee at the Center (Ministry of Health and Environment) approved the study. 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 and left to clot for about 15 minutes at room temperature (20-25 °C). Then, the tube was centrifuged (3000 rpm) for 15 minutes in a 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 used for ELISSA assay.

Determinations of the IL-13 serum level

Investigation of the level of IL-13 by direct ELISA through Sunlong Kit (China). This kit was based on sandwich Enzyme-linked immune sorbent assay technology. The microplate wells were pre-coated with a specific anti-marker antibody (Capture antibody: antibody specific to IL-13). Upon adding 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 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 yellow. The density of the color is proportional to the level of IL-13. This density (optical density: OD) is measured spectrophotometrically at a wavelength of 450 nm. The levels of IL-13 were calculated from the standard curve and expressed in pg/ml.

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 EDTA anticoagulant tubes in a 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 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

IL13 gene SNP (1295685A>G) 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 the National Center for Biotechnology Information (NCBI) and shown in (table 1).

Primer	Sequence (5′→3′ direction)	Band size (bp)
	IL13 rs1295685 A>G	
Forward	**CGTTGGGGATTGGGGAAG**	63
Reverse	**CCCTGAGTCTCTGAACCCTT**	

Table 1. Designed Primers used in the present study

Primer Sequence Matching

Detecting Primer prepared genes IL13 was assessed for SNPs, which included (1295685A>G) for IL13 primers sequences were designed according to their reference sequence (rs) in the database of NCBI (National Center for Biotechnology Information). Primer sequences were matched by the bioinformatics program NCBI (table 2).

IL13 gene (rs1295685 A>G)

63 bp region

Homo sapiens chromosome 5, GRCh38.p14 Primary Assembly

NCBI Reference sequence: NC_000005.10

TAAATGAGTGTGTTTGTCAC CGTTGGGGGATTGGGGAA-
GACTGTGGCTGCTGGCACTTGGA
>>>>>>>>>>>>>>>>>>
GCCAAGGGTTCAGAGACTCAGGGCCCCAGCACTAAAGCAGT
<<<<<<<<<<<<<<<<<<<<<

Table 2: Matching of the primer sequences using NCBI for IL13 gene.

Primer Preparation

The primers for the IL13 working solution were prepared from the lyophilized sample after dissolving by nuclease-free water according to the manufacturer to make a stock solution with a concentration of 100 μM for PrimerPrimer and 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 -23C until use.

Real-Time PCR runs (HRM)

Real-time PCR was performed using the Qiagen Rotor gene Q Real-time PCR System (Germany). Every reaction was done in duplicate and included a non-template control (NTC), non-amplification control (NAC) and non-primer control (NPC) as negative controls. 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. HRM analysis with ramping by 0.2 °C from 65 to 95 °C. Used master mixes were containing EVA-Green. HRM Master Mix Synthetic SNP sequences were tested using duplicates. To detect allelic differences, triplicate synthetic controls were analyzed by qPCR-HRM, and normalized melting curves (NMC) and differential curves (DC) were obtained using the HRM. The cycling protocol was programmed for optimized cycles according to the thermal profile (table 3).

Step	Temperature	Duration	Cycles
Enzyme activation	94ºC	60 sec	1
Denature	94ºC	5 sec	
Annealing	ºC58	15 sec	40
Extention	72ºC	20 sec	
HRM	65-95	0.2sec for 1 degree	

Table 3. Thermal profile of HRM genotyping (IL13 rs1295685 A>G).

Statistical Analysis

The Statistical Analysis IBM SPSS Statistics 26 program [14] was used to detect the effect of different factors on study parameters. One-way ANOVA and T-test were used to compare between means significantly. The chi-square test was used to compare between percentages (0.05 and 0.01 probability) significantly. Logistic regression analysis was used to estimate the odds ratio (OR) and confidence interval (CI) for each SNP in this study. Graph Pad Prism 9 program was used to draw the figures. SPSS program was used to detect the genotyping. Receiver operating characteristic (ROC) curve analysis was performed to determine the area under the curve (AUC) and the optimum cut-off value of serum markers best predicting [15].

RESULT

Detection of IL-13 serum level for asthmatic patients and control group

The statistical analysis of the result showed a highly significant elevation level of IL-13 in the sera of the asthma patients group compared to the control group. IL-13 mean level was 22.90 pg/ml in patients, while in control individuals was 13.99 pg/ml (p= 0.0001) (Table 4).

Studies Group	Mean ± SE IL-13 (pg/ml)	T. test	*P*. value
Patients	22.90 ±2.24	4.479 **	0.0001
Control	13.99 ±0.32		
**** (P≤0.01).**			

Table 4. IL-13 serum level in Asthma patients and control group

Receiver operating characteristic (ROC) analysis of IL-13

The analysis of ROC revealed that elevated serum levels of IL-13 occupied an AUC, which was 0.882 (p-value = 0.001). At a cut-off value of 16.9905 pg/ml for serum IL-13 level, the sensitivity and specificity were 77 and 91 %, respectively (Figure 1).
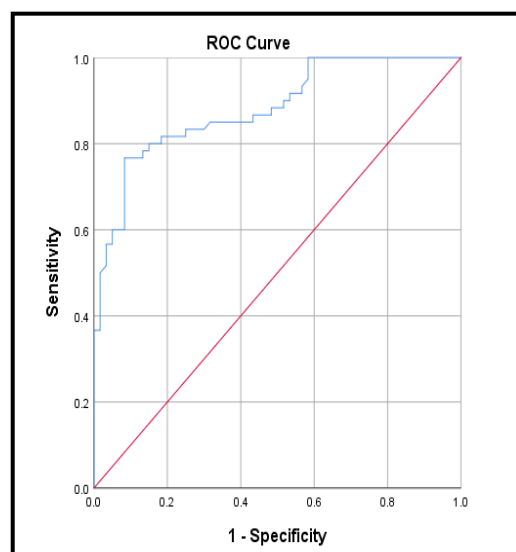
AUC=0.882;   **P value**=0.001;
Sensitivity=77%;   **Specificity**=91%;
Cut-off value =16.9905 pg/ml

Figure 1. Receiver operating characteristic (ROC) analysis of IL-13 serum level among asthma patients showing area under the curve (AUC), *p*-value, sensitivity, specificity and cut-off value.

Molecular Analysis

DNA Extraction

The purity of DNA ranged between (1.76- 2.0), and the concentration ranged between (*38-62* ng/µl).

HRM RT-PCR Genotyping

DNA samples of study groups were genotyped with the IL13 gene (1295685A>G). SNP detection was achieved by using HRM real-time PCR. The resulting output of the thermocycler of genotypes is shown in Figure 2.
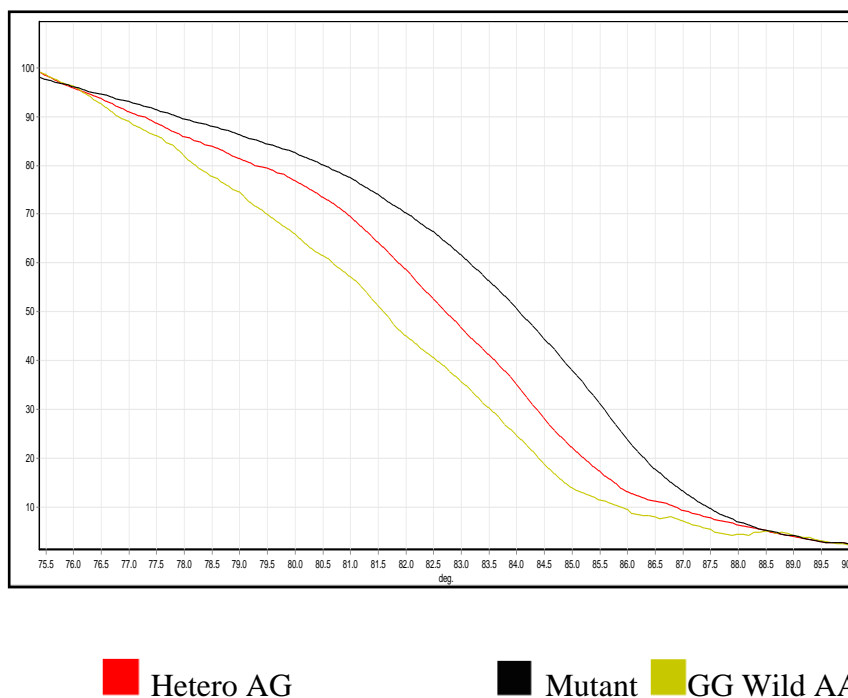

■ Hetero AG   ■ Mutant ■ GG Wild AA

Figure 2. The resulting output of HRM for the genotypes in SNP (rs1295685A>G) for the *IL13* gene

Position of (rs1295685 A>G) SNP for IL13 gene studied

The position of (rs1295685 A>G) SNP in 3_prime_UTR untranslated regain of interleukin 13 gene in chromosome 5: (NC_000005.10).

3.2.4. Genotype and Allele Frequencies of IL13 gene polymorphism (rs1295685 A>G)

The distribution of genotype and allele frequencies among patient groups compared with the healthy group for the IL-13 gene polymorphism (rs1295685) is shown in Table (4- 17). The genotypic frequencies of asthma patients were 18.3% (n=11) normal AA and 65.0% (n=39) heterozygous AG. Mutant homozygous was found in GG 16.6% (n=10). In controls, the results demonstrate 76.7% (n=46) wild-type AA, 13.3% (n=8) heterozygous AG and mutant homozygous GG 10.0% (n=6). The genotype frequencies of asthma patients and control analysis reveal that the wild AA genotype and A wild type allele were taken as reference.

In IL-13 gene polymorphism (rs1295685), the odds ratio for the GG genotype was 6.9 with (P-value =0.001). The AG genotype has a high odds ratio of 20.3

(P-value=0.0001). Also, The AG + GG genotype had a higher risk of asthma than the wild-type AA with an odds ratio of 14.6 (P-value=0.001). At the same time, the GG vis AA+ AG genotype had an odd ratio of 1.8 and (p-value=0.2). The frequency of the A allele in asthma patients and controls was 51.1% (n=61) and 83.4% (n=100), respectively. In comparison, the frequency of the G allele in asthma patients and controls was 48.8% (n=59) and 16.6% (n=20), respectively. This indicates that the G allele was at a higher risk of asthma than the A allele, with an odds ratio of 4.8 and (P-value=0.001).

IL13 polymorphism rs1295685 A>G	Frequencies (%)		P value	Odd ratio (95% CI)
	Control group (n=60)	Patients Group (n=60)		
Codominant				
AA	76.7% (n=46)	18.3% (n=11)	---	**1.00 (Reference)**
AG	13.3% (n=8)	65.0% (n=39)	**0.0001**	**20.3 (0.7-5.5)**
GG	10.0% (n=6)	16.6% (n=10)	**0.001**	**6.9 (0.2-2.3)**
Dominant				
AA	76.7% (n=46)	18.3% (n=11)		**1.00 (Reference)**
AG+GG	23.3% (n=14)	81.7% (n=49)	**0.001**	**14.6 (0.6-3.5)**
Dominant				
AA+AG	90.0% (n=54)	83.4% (n=50)		**1.00 (Reference)**
GG	10.0% (n=6)	16.6% (n=10)	**0.2**	**1.8 (0.6-5.3)**
Allele				
A	83.4% (n=100)	51.1% (n=61)		**1.00 (Reference)**
G	16.6% (n=20)	48.8% (n=59)	**0.001**	**4.8 (2.6-8.8)**

Table 5. Comparison of the Genotype and Allele Frequencies of *IL13* gene polymorphism (rs1295685 A>G) between the Patients Group and Control group

DISCUSSION

The present results agree with other Iraqi studies, such as [16], which found that the percentage of IL-13 serum concentrations was substantially higher in asthmatic patients than in control individuals (Value of Probability = 0.001). Also, the results agreed with the results that a highly significant IL-13 level between asthmatic patients and controls was (P = 0.001[17]. However, IL-13 is an archetypal Th2 cytokine that has already been well-established as a contributor to asthma pathogenesis and is involved in bronchial hyper-responsiveness, goblet cell differentiation, and IgE production. It represents an attractive therapeutic target in

established disease [18]. Patients with T2-high type of asthma have eosinophilia and other signs of type 2 inflammation, including high levels of IL-4 and IL-13[5,19].

This study found that a genetic variant in (rs1295685A>G) for the IL13 gene was a risk factor for adult asthma. Parallel to a study by [20] who observed in the rs1295685 polymorphism of IL13, the OR for the GA +AA versus GG genotypes was 2.2 (95% CI 1.2–3.9), and the P value was (P= 0.007. Another study by Lee et al.(2020) showed that the rs20541 and rs1295685 SNPs in the 3′-untranslated region (UTR) of *IL13* had significant associations with atopic dermatitis ($P <$ .001 and .01, respectively). [21] Therefore, genetic variants in the IL-13 gene may play an essential role in the development of asthma [22].

CONCLUSION

 Increased levels of IL-13 in serum and the presence of the G allele and GG + AG genotype in the IL13 gene may be considered important factors in the pathogenesis of allergic asthma in Iraqi adult patients.

Acknowledgments

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