

Assessment of the Effects of *IL9*, *IL9R*, *IL17A*, and *IL17F* Gene Polymorphisms on Women with Allergic Rhinitis in Shahrekord, Iran

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Abstract

Background: The genes encoding *IL9*, *IL9R*, *IL17A*, and *IL17F* have recently been implicated in the genetic basis of rhinitis and allergy. **Aim:** The purpose of this study was to assess the association of the single nucleotide polymorphisms (SNPs) of *IL9*, *IL9R*, *IL17A*, and *IL17F* and potential interaction of these genes with the determination of IgE levels in women with allergic rhinitis (AR) in Shahrekord, Iran. **Subjects and Methods:** In a case-control study, SNPs from the *IL9*, *IL9R*, *IL17A*, and *IL17F* were genotyped in 394 random samples including 195 AR patients and 199 normal controls. Enzyme-linked immunosorbent assay was performed for the determination of serum total IgE levels. The Student's *t*-test was used to compare the differences. The Chi-square test was performed to compare proportions of cases with different clinical features among cases with different genotypes. The genotype and allele frequencies were obtained by direct counting. Hardy-Weinberg equilibrium was tested between cases and controls separately. The relative risk associated with rare alleles was estimated as an odds ratio with 95% confidence interval. $P \leq 0.05$ was considered statistically significant. **Results:** The rs731476 SNP in the *IL9R* was significantly associated with the AR phenotype in women. No association was found between any of the other SNPs in *IL9*, *IL17A*, and *IL17F* genes and AR. In the gene-gene interaction analysis, we found that *IL9R/IL9* genotype rs731476 T/rs2069885 G conferred a higher risk for AR phenotype development. We also did not find a significant association in terms of IgE levels between cases and controls. **Conclusion:** Our result suggests that the rs731476 SNP located in the *IL9R* is associated with an increased susceptibility to AR in females. In a subsequent gene-gene interaction analysis, the rs731476 T/rs2069885 G-genotype combination (*IL9R/IL9*) has significantly been associated with the development of the AR phenotype.

Keywords: Allergic rhinitis, Gene-gene interaction, *IL17A*, *IL17F*, *IL9*, *IL9R*, Single nucleotide polymorphisms

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Introduction

Allergic rhinitis (AR), which is among the most common allergic disorders, is an inflammatory disorder of the nasal mucosa caused by an IgE-mediated response, following exposure to an allergen. The clinical characteristics of AR include itching, sneezing, rhinorrhea, and nasal congestion or stuffiness that can be reversible either spontaneously or by treatment.^[1] With a prevalence of 9–42 among the general population (and 10–15% in Iran), AR is considered as a global healthcare problem.^[2] It has a considerable impact on quality of life which is negatively affected patients' social life and school performance. AR is also a risk factor for the development of asthma. The exact etiology of AR is currently unknown but could involve a complex interaction between genetic predisposition and environmental exposure to different factors including allergens. Although the disease does have a hereditary component with a 45–60% concordance among monozygotic twins and an estimated 0.33–0.75 of heritability, it does not follow a certain Mendelian hereditary pattern and is widely considered to be complex. Thus, as expected, an array of genetic, epigenetic, and environmental factors should be engaged.^[3,4]

The interleukin-9 receptor gene (*IL9R*), belonging to the hematopoietin receptor superfamily, is located on the pseudoautosomal region of X and Y chromosomes (Xq28 and Yq12).^[5] It is expressed on T cells, macrophages, mast cells, eosinophils, and neutrophils.^[6] Upon IL9 binding to *IL9R*, JAK-STAT signaling pathway is activated.^[7]

In humans, *IL9* is located on chromosome 5 (5q31–35) between *IL3* gene and early growth response-1.^[8] Activated *IL9* receptor plays a key role in immunologic processes such as T cell development.^[9] It is also involved in the prevention of apoptosis.^[10] It can prompt the release of chemotactic factors from bronchial epithelial cells and smooth muscle cells.^[11,12] *IL9/IL9R* can act directly on B lymphocytes and regulate IgE synthesis. Therefore, it may play a major role in the development of allergy.

The *IL17* and *IL17F* genes, mapped on the same chromosome at position 6p12, share a 50%, homology. These genes are expressed by the activated T cells which, in turn, induce the expression of cytokines and chemokines.^[13] The available evidence suggests that *IL17F* is an excellent candidate gene for chronic inflammatory disease including ulcerative colitis,^[14] asthma,^[15] inflammatory bowel disease,^[16] and AR.^[17] *IL17F* is a powerful pro-inflammatory cytokine capable of inducing other cytokines.^[18] Thus, it may be important for neutrophilic inflammation in acute airway inflammation^[19-21] and AR.^[17] There are some reports about the association of single nucleotide polymorphisms (SNPs) with increased risk of AR as discussed below. In this study, we performed an association study on case and control populations to evaluate the possible association of SNPs from *IL9* (rs2069885), *IL9R* (rs731476),

IL17A (rs2275913), and *IL17F* (rs763780) genes, both alone and in combination, with an increased risk of AR.

Subjects and Methods

In a case–control study, AR samples were obtained from nonasthmatic AR patients examined at Kashani Hospital, an educational hospital in Shahrekord University of Medical Science, Shahrekord, Iran, between 2009 and 2010. All samples were selected from Fars ethnic group randomly. Patient's written informed consent was obtained.

We selected women because (1) women would present the allergic-related diseases more than men; (2) the studied genes in *IL9* were located on sexual chromosomes; and (3) there are few studies on women than men in this regard. In this study, SNPs from the *IL9*, *IL9R*, *IL17A*, and *IL17F* were genotyped in 394 random samples including 195 AR patients and 199 normal controls. Enzyme-linked immunosorbent assay (ELISA) was performed for determination of serum total IgE levels. The systematic random sampling was used to select cases and controls using the file numbers. The controls were selected from patients who were referred but did not have AR. The total numbers of cases and controls were obtained using Cochran's sample size formula. Based on the discussion with experts, the questionnaire did not need to verify for reliability and validity due its questions which were only related to general demographic and history questions.

The diagnosis criteria for AR included positive history of disease, positive physical examination (turbinate hypertrophy and submucosal inflammation), ruling out of other causes of nasal obstruction, and not having anatomic disorders. Among those with asthma or atopic dermatitis, patients who might have got AR as secondary symptom were excluded from the study. Patients with positive family history (having at least three persons with AR phenotype in their family) were selected for the study. In total, 195 patients with AR and 199 healthy controls (all women) were included in the study. The normal cases were recruited locally, primarily from medical students and volunteers with no prior history of autoimmune or inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis or inflammatory bowel disease, AR, or asthma. They were matched in terms of gender, ethnicity, and age (with a range of 5-year) with the patient group. All the cases with anatomic problem in their nose, related to cancer, and smokers were also excluded from the study. This study was approved by the Ethics Committee of Shahrekord University of Medical Sciences and was conducted according to the declaration of Helsinki principles. Written informed consents were obtained from all the participants.

Blood samples were drawn by venipuncture in EDTA-containing tubes. Serum total IgE levels were determined by human IgE ELISA kit (BioCheck, USA). A serum IgE level was considered elevated if it exceeded the highest reference value of 150 IU/ml.

DNA extraction and genotyping

Genomic DNA was extracted using a standard phenol/chloroform extraction method. *IL9* rs2069885, *IL9R* rs731476, *IL17A* rs2275913, and *IL17F* rs763780 genotyping were performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism. Primer sequences for *IL17A* G-152A and *IL17F* 161His-Arg were as follows:

Forward: 5'-CAGAAGACCTACATGTTACT-3', Reverse: 5'-GTAGCGCTATCGTCTCTCT-3' for *IL17A*

Forward: 5'-GTTCCCATCCAGCAAGAGAC-3', Reverse: 5'-AGCTGGGAATGCAAACAAAC-3' for *IL17F*

Forward: 5'-CATCATTTGAGTCACTCTGTCCTT-3', Reverse: 5'-TTGCCTCTCATCCCTCTCAT-3' for *IL9*.

The primers for *IL9R* were as follows:

Forward: 5-CTTGTCACCCAACACCTCT-3, Reverse: 5-CTGCATCCGTGAGGTAAAGG-3.

The reverse primer was designed in such a way to create a new restriction digest site for a restriction enzyme. Primers were designed by primer3.^[22] The PCR amplification was performed in a total volume of 25 µl mixture containing: 100-ng genomic DNA, 1.0 µM of each primer, 200 µM of each dNTP, 2.0 µM of MgCl₂ and 1.0 U Taq DNA polymerase and 10 µl Taq buffer (Fermentas, Germany) using the Astec gradient 96 (Astec, Japan). PCR products were digested overnight at 37°C with NlaIII (Fermentas, Germany) for *IL17F* 161His-Arg and *IL9R*rs2069885, XmnI for *IL17A* rs2275913, and HaeIII for *IL9R*rs731476 genotypes. They were resolved on 10% polyacrylamide gel electrophoresis for *IL9R*rs731476. PCR products were shown to be digested into three fragments for all of them except *IL9R* that digested in four fragments [Figure 1]. To confirm the genotyping results, randomly selected PCR samples were tested by DNA sequencing [Figure 2].

Determination of levels of IgE in cases and controls

The levels of IgE were determined using specific ELISA kits (Ray Biotech Inc., Norcross, Georgia, USA).

Statistical methods

The quantitative variables were expressed as mean (standard deviation). The Student's *t*-test was used to compare the differences. The Chi-square test was performed to compare proportions of cases with different clinical features among cases with different genotypes. The genotype and allele frequencies were obtained by direct counting. Hardy-Weinberg equilibrium was tested between cases and controls separately. The relative risk associated with rare alleles was estimated as an odds ratio (OR) with 95% confidence interval (CI). $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

To determine whether interactions between the rs731476SNP, rs2069885 SNP, rs2275913 SNP, and rs763780 SNP affect disease susceptibility, association test was conducted using genotypic combinations of these four SNPs. A logistic regression model was used to evaluate the statistical significance, using age and gender as adjusting covariates. The ORs for each genotypic combination were also obtained from the logistic regression analysis, after the genotypes were subdivided into two groups for each locus (GG and others for rs731476, TT and others for rs2069885, AA and others for rs2275913, and GG and others for rs763780).

Results

The frequencies of the genotypes

A total of four SNPs from the *IL9*, *IL9R*, *IL17A*, and *IL17F* were genotyped from the 394 samples. Information from the SNPs is shown in Table 1, including genomic function, chromosomal position, dbSNP ID, and minor allele frequency. The Fisher exact test indicated that all genotyped SNPs were in Hardy-Weinberg equilibrium. The

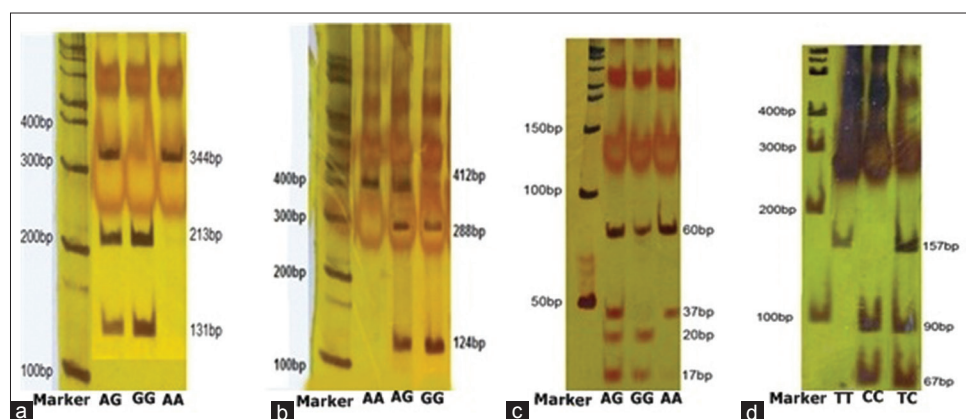


Figure 1: Polymerase chain reaction-restriction fragment length polymorphism polyacrylamide gel electrophoresis of the (a) *IL17A*, (b) *IL17F*, (c) *IL9R*, and (d) *IL9*

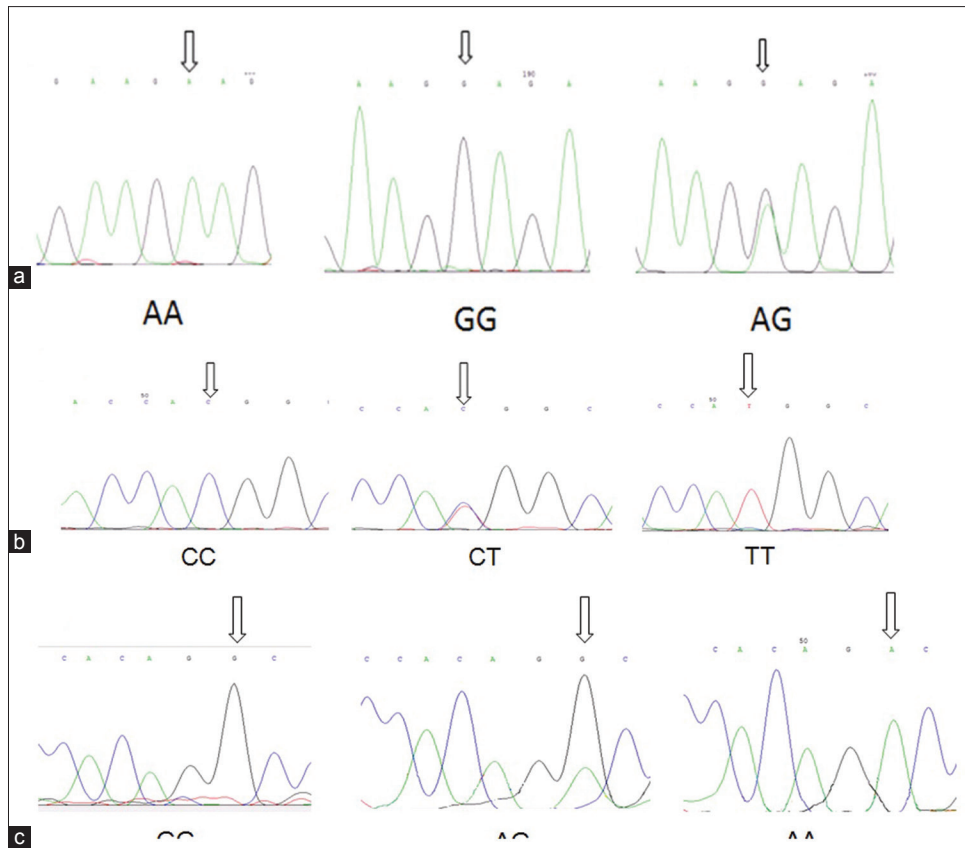


Figure 2: Sequencing analysis to confirm genotypes of the (a) *IL17A*, (b) *IL9*, and (c) *IL9R*

Table 1: Single nucleotide polymorphism markers genotyped for the case-control samples (dbSNP build 126)

Gene	rs number	Chromosomes	Function	MAF*
<i>IL9</i>	rs2069885	Chromosomes 5	Exon 5	0.1436
<i>IL9R</i>	rs731476	Chromosomes X/Y	Intron 1	4974
<i>IL17A</i>	rs2275913	Chromosomes 6	Promoter	0.3538
<i>IL17F</i>	rs763780	Chromosomes 6	Exon 3	0.154

*MAF: Minor allele frequency

distributions of the allelic and genotypic frequencies for the four SNPs were compared among the AR and normal groups. The allelic and genotypic frequencies of these four SNPs are shown in Table 2. The SNP rs731476 from the *IL9R* was genotyped, showing significantly different allelic and genotypic distributions between the AR and normal groups (allelic $P=0.01$; genotypic $P<0.001$). For the allelic test, the resulting OR was 0.5 (95% CI = 0.34–0.77) [Table 2]. No significant allelic or genotypic associations were found between AR and any of the three SNPs from the *IL9*, *IL17A*, and *IL17F* or the rs2069885, rs2275913, and rs763780 SNPs. Polymorphisms in the four genes were also compared with total IgE level between cases and controls to identify any significant correlation with AR; as a result, the SNPs in the *IL9*, *IL9R*, *IL17A*, and *IL17F* genes were observed to have significant associations with these variables.

Gene-gene interaction between *IL9* and *IL9R*, *IL17A*, and *IL17F*

To investigate whether interactions between the rs2069885 and rs731476 SNPs and between rs2275913 and rs763780 affect the disease susceptibility, an association test was conducted using genotype combinations of each of the two SNPs. After analysis, these data showed that two SNP genotype combinations are significantly increased the risk of AR. When interaction between an allele from *IL9* and G allele from *IL9R* was compared between the two AR and normal controls, the resulting OR was 0.67 (95% CI = 0.46–0.98) ($P=0.04$). These results suggest that the combination of the two genotypes from other two genes has no effect on increasing the disease susceptibility [Table 3].

Discussion

AR is largely considered to be a complex disease with poorly understood genetics. Although many genes have been studied, few have been successfully associated with the disease. In the present investigation on four different autoimmune-related genes which were chosen on the basis of candidate gene approach, for the first time, the rs731476 SNP in *IL9R* was found to be significantly associated with AR in the studied women. In addition, the rs731476 T-/rs2069885 G-combination

Table 2: Comparison of allelic and genotypic frequencies between the two allergic rhinitis and normal control groups: (a) rs731476 single nucleotide polymorphism from the *IL9R* gene, (b) rs2069885 single nucleotide polymorphism from the *IL9* gene, (c) rs2275913 single nucleotide polymorphism from the *IL17A* gene, and (d) rs763780 single nucleotide polymorphism from the *IL17F* gene. Age and gender were used as adjusting covariates

Group a	Allele		OR (95% CI)	P
	A	G		
NR	132 (0.6633)	67 (0.4974)	0.51 (0.34-0.77)	0.01
AR	98 (0.5026)	97 (0.4974)		
Group a	Genotype			P
	AA	AG	GG	
	NR	66 (0.3317)	133 (0.6683)	
AR	48 (0.2462)	100 (0.5128)	47 (0.2410)	
Group b	Allele		OR (95% CI)	P
	C	T		
NR	27 (0.1357)	172 (0.8643)	1.07 (0.60-1.89)	0.82
AR	28 (0.1436)	167 (0.8564)		
Group b	Genotype			P
	CC	CT	TT	
	NR	0	53 (0.2663)	
AR	4 (0.02051)	47 (0.2411)	144 (0.7385)	
Group c	Allele		OR (95% CI)	P
	A	G		
NR	76 (0.3819)	123 (0.6181)	0.89 (.59-1.53)	0.56
AR	69 (0.3538)	126 (0.6462)		
Group c	Genotype			P
	AA	AG	GG	
	NR	20 (0.1005)	112 (0.5628)	
AR	24 (0.1231)	89 (0.4564)	82 (0.4205)	
Group d	Allele		OR (95% CI)	P
	A	G		
NR	188 (0.9447)	11 (0.0553)	0.89 (0.38-2.07)	0.79
AR	183 (0.9385)	12 (0.6154)		
Group d	Genotype			P
	AA	AG	GG	
	NR	177 (0.8894)	22 (0.1106)	
AR	171 (0.8769)	24 (0.1231)	0	

NR: Normal control, AR: Allergic rhinitis, OR: Odds ratio, CI: Confidence interval

Table 3: Gene-gene interaction *IL9* and *IL9R*, *IL17A*, and *IL17F*

Interactions	AR versus NR OR (95% CI)	P
rs731476/rs2069885		
A-/C-	1.40 (0.77-2.52)	0.13
G-/C-	0.72 (0.39-1.32)	0.29
T-/A-	1.31 (0.93-1.83)	0.12
G-/T-	0.67 (0.46-0.98)	0.04
rs2275913/rs763780		
A-/A-	1.07 (0.73-1.57)	0.72
A-/G-	0.83 (0.34-2.01)	0.68
G-/A-	1.05 (0.76-1.45)	0.75
G-/G-	0.94 (0.40-2.21)	0.88

NR: Normal control, AR: Allergic rhinitis, OR: Odds ratio, CI: Confidence interval

in the *IL9/IL9R* genes was found significantly increase the risk of developing the AR phenotype (OR = 0.67; 95% CI = 0.46–0.98; $P = 0.04$).

Allelic association between *IL9* and total serum IgE levels has been previously demonstrated.^[23] SNPs from *IL9*, *IL9R*, *IL17A*, and *IL17F* and total serum IgE, in cases ($n = 195$) and controls ($n = 199$), have not been correlated ($P = 0.08$). Both *IL9* and *IL9R* have been implicated in asthma pathogenesis.^[23-26] Although, according to a recent study, *IL9* is produced in larger quantities by Treg than Th2 cells,^[27] most of the present evidence indicates that the physiological function of *IL9* is somehow connected to the Th2 signaling – *IL9* is involved in defense against helminthic infections; it also contributes to allergic reactions in the lung.^[28] Furthermore, *IL9* may mediate the activity of both T cells and Treg cells.^[29] *IL9R* gene polymorphism has been previously associated with AR; according to the study, males with homozygous SNP in *IL9R* are three times more likely to be affected than females. The effect could be assigned to either the presence of different *IL9R* splice variants influencing the *IL9*-binding ability or the involvement of *IL9R* in the early T cell development.^[30]

Aschard *et al.* were able to show a significant effect of rs2069885 of IL9 in males on lung function and polysensitization.^[31] On the other hand, Schuurhof *et al.* suggested that the *IL9* genetic polymorphism (rs2069885) has an opposite effect on the risk of severe respiratory syncytial virus infection bronchiolitis in boys and girls^[32] and Nouri-Aria *et al.* showed that IL9 is upregulated in the nasal mucosa during the pollen season and correlates with tissue infiltration by eosinophils.^[33] Finally, Melén *et al.* showed sex-specific protective effects of *IL9R* haplotypes on childhood wheezing and sensitization. Their results suggest a role for *IL9R* in the pathophysiology of allergic diseases, which may be sex dependent.^[34] Besides, Namkung *et al.* revealed an association between *IL9R* gene polymorphisms and atopic dermatitis. They showed that a genotype combination of *IL9/IL9R* genes significantly increased the risk of developing the AD phenotype.^[8]

The IL17 families of cytokines have been linked to many diseases.^[35,36] Elevation of plasma IL17 levels to >20 pg/mL has been considered an independent risk factor for severe asthma.^[37] *IL17A* and *IL17F* can induce expression of several cytokines that have also been linked to asthma or asthma-related phenotypes.^[38] One significant finding was the lack of an association between asthma and the examined SNPs of *IL17F*, including rs763780 (H161R). This SNP was associated with asthma in a Japanese population as one of its alleles was found to reduce the risk of asthma.^[15] Ramsey *et al.* observed minimal contributions of 5 *IL17F* SNPs to asthma among white females.^[39] These discrepancies could be due to differences in patient demographics, sample size, environmental factors, and genetic background, all of which can affect association studies.

The similar findings for *IL17A* and *IL17F* could well be due to either of the several factors:

- Coordinated chromatin modifications: Since both genes are located on the same chromosome (6p12), their promoters and the conserved noncoding sequence regions might have undergone coordinated chromatin modifications^[40]
- Both genes act as homodimers or heterodimers and share similar biological functions, sequences, and expression patterns and many polymorphisms may influence IL17 expression.^[41]

Therefore, the observed correlation between *IL17F* and *IL17A* genotype combination adds more validity to the results of this study. Recently, Wang *et al.* found an association among polymorphisms in the cytokine genes *IL17A* and *IL17F* and development of AR and severe asthma in Chinese cases.^[17] *IL17A* rs2275913 has shown association with asthma phenotype in Chinese population.^[41] However, another study on the association of *IL17F* rs763780 with asthma in European Americans, African Americans, and Saudi Arabian populations did not find any association.^[39,42]

AR is genetically complex and may be influenced by several genetic and environmental components. *IL9R* belongs to the group of genes with a clear potential role in allergic diseases, and its modest associations with asthma have been reported.^[26,43] *IL9R* is located on the pseudoautosomal region of X and Y chromosomes. Therefore, it seems to be biologically plausible to infer that the variants affect males (XY) and females (XX) differently. As the *IL9* signaling pathway is involved in both airway and immunological processes, a possible role is suggested for the *IL9R* in the pathophysiology of AR. Further studies are warranted to further clarify the issue.

This study has a limitation on the number of participants due to lack of eligible patients in the study. In addition, it was not possible to apply the functional experiments because of impossibility of conducting these methods in our university.

Conclusion

Our data identify an association between the rs731476 SNP in the *IL9R* gene and the AR phenotype in the Iranian women while the combination of rs731476 T/rs2069885 G in the *IL9R/IL9* encoding genes led to a significantly higher risk for developing AR. IgE levels were not different between patients and controls. There are several limitations in our study including the sample size and lack of males group. The latter hampered from comparison of the results between the males within both groups.

As discussed above, determination of patient's allelic state regarding the mentioned SNPs in these genes can improve management of patients' care and quality of life. We recommend further studies with larger sample size in various ethnic groups on more candidate genes and functional studies. We also suggest Genome-wide Association Studies through next generation sequencing method.

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Conflicts of interest

There are no conflicts of interest.

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