Iraqi Journal of Science, 2025, Vol. 66, No. 10, pp: 4292 – 4301
DOI: 10.24996/ijs.2025.66.10.21

ISSN: 0067-2904

Interleukin 9 single nucleotide polymorphisms rs17317275 association with celiac disease in Iraqi Population

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Received: 25/7/2024 Accepted: 9/10/2024 Published: 30/10/2025

Abstract

Celiac disease is a condition in which the small intestine's lining is damaged and the body has trouble absorbing nutrients, typically occurring in individuals who are genetically susceptible and triggered by the consumption of gluten. The pathogenesis of this disease involves the interactions between environmental, genetics, and immunological factors. This research designed to inspect the frequencies of genotypes and alleles of IL-9 rs17317275 in an Iraqi celiac disease population via allele specific primers-polymerase chain reaction technology. The study included fifty patients with celiac disease, with a mean age of 23.5±11.88 years, preforming between November 2021 to February 2022. The results indicated that the AA genotype and *A* allele were significantly more frequent in celiac disease patients compared to the control group (72.0% *vs*. 10.9% OR:20.96, p= 1.98 x 10^{-16} and 84.0% *vs*. 34.0%, OR: 10.25, p= 2.4 x 10^{-8}, respectively). Additionally, within the celiac disease group, the AA genotyping and *A* allele were more frequent in celiac disease group than the other genotypes and allele in the same group (72.0% and 84.0% *vs*. 24.0%, 4.0% and 16.0%, respectively). It can be concluded from the results that the AA genotype and the *A* allele may be related with a greater risk of developing digestive disorders than other genotypes.

Keywords: celiac disease, IL-9, PCR, single nucleotide polymorphisms, rs17317275

التغاير أحادي النيوكليوتيدة للبين إبيضاضي التاسع rs17317275 المرتبط مع مرض حساسية الحنطة في المجتمع العراقي

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الخلاصة

يتميز مرض حساسية الحنطة بإصابة الغشاء المخاطي للأمعاء الدقيقة وسوء امتصاص المغذيات، الذي يميز الأفراد الذين لديهم استعداد وراثي بعد تناول الغلوتين. يتضمن التسبب بالمرض التفاعلات بين العوامل البيئية والوراثية والمناعية. يهدف هذا البحث الى تحديد تردد الأنماط الجينية والأليلات لجين البين إبيضاضي

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التاسع rs17317275 في مجتمع مرضى حساسية الحنطة العراقيين عن طريق تقنية تفاعل البلمرة المتسلسل – البوادئ المحددة الأليل. تضمنت الدراسة الحالية مشاركة خمسين مريضا من مرضى حساسية الحنطة، كان متوسط أعمارهم 23.5 ± 11.88 عاما، خلال الفترة من تشرين الثاني 2021 إلى شباط 2022. أشارات النتائج الحالية الى أن النمط الجيني AA والأليل *A* كانت أكثر ترددا وبشكل ملحوظ في مرضى حساسية الحنطة مقارنة مع مجموعة السيطرة الصحية (72.0% vs. 10.9% OR:20.96, p= 1.98 x 10−16 and 84.0% vs. 34.0%, OR: 10.25, p= 2.4 x 10−8، على التوالي)، كما كان التنميط الجيني AA والأليل *A* أكثر ترددا من الأنماط الجينية الأخرى في مجموعة مرضى حساسية الحنطة (72.0% and 16.0% vs. 24.0%, 4.0%، على التوالي). يمكن الاستنتاج من النتائج أن النمط الجيني AA والأليل *A* قد تكون مرتبطة بخطر الإصابة باضطرابات الجهاز الهضمي بشكل أكبر من الأنماط الجينية الأخرى.

1. Introduction:

Interleukin-9 (IL-9) is a member of the IL-2 cytokine family that is released by the induced CD_4^+ T lymphocytes by tumor growth factor-beta (TGF-β) and IL-4 (T helper-9 (Th-9) pathway) [1, 2]. Interleukin-9 is also secreted by the stimulated Th2 lymphocytes and serves as a growth agent for the mast cells and T lymphocytes that assistance facilitate T helper lymphocytes-9 (Th-9) immune responses to allergic inflammatory diseases [3]. Additionally, IL-9 also can stimulate T helper lymphocytes-17 (Th-17) for differentiation and mediation of autoimmune and inflammatory diseases. Furthermore, IL-9 is produced by Th-17 cells, which primarily secrete and produce interleukin-17A (IL-17A) and interleukin-17F (IL-17F) [3, 4, 5]. Furthermore, IL-9 is produced by various cells, including T helper lymphocytes-2 (Th-2), Th-17, and natural killer cells [6]. IL-9 plays crucial role in triggering pathophysiological processes for several autoimmune disorders, including ankylosing spondylitis (AS), celiac disease (CeD), diabetic mellitus (DM), multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [7, 8, 9]. Furthermore, IL-9 regulates the function of several immune cells such as T and B lymphocytes, mast cell in addition to the epithelial cells, also it activates the signaling pathways such as the signal transducer and activator of transcription1 (STAT1), STAT3 and STAT5 that have roles in many cellular immunity aspects, proliferation, apoptosis and differentiation process [1, 10]. Furthermore, IL-9 promotes the production of chemokine C-C motif ligand 20 (CCL20) that mediates the recruitment of CC chemokine receptor6 (CCR6)-expressing dendritic cells (DCs) and CD_8^+ cytotoxic T lymphocytes (CTLs) [11].

IL-9 gene is positioned at chromosome 5 5q31–35 consisting of 5 exons and 4 introns [12], it is described for the first time as a cytokine that has a pivotal role in the multiple cell lineages development, proliferation, survival, and differentiation of innate and adaptive immunity, that is recognized as a T lymphocytes growth agent [8, 9, 13]. CeD is a chronic autoimmune disorder that is triggered by gluten in the diet of individuals who have a genetic predisposition, leading to a systemic immune response. Many clinical presentations, such as specific autoantibody response presence and varying damage to the small intestinal mucosa. CeD is associated with several genes coded for Human Leukocyte Antigen (HLA) DQ2 and DQ8. CeD can manifest at any age and may lead to various complications, such as the chronic inflammation of the small intestine mucosa and submucosa and is characterized by the presence of several systemic manifestations triggered by the presence of specific-immunogenic peptides of gluten protein found in wheat, rye, and barley [14]. Furthermore, multiple genetic variations have been identified in IL-9 gene, including coding single nucleotide polymorphisms (SNPs) that occurred in the coding region of IL-9 gene leading to changes in the sequence of the amino acids that composed IL-9 protein, non-coding SNPs founded outside the coding regions (exons) that included introns, untranslated region (UTR), promoter-enhancer region, and intragenic region that positioned outside the gene [15, 16].

The previous information, along with the unclear understanding of IL-9 function in inducing the immunity induction, motivated us to enhance our knowledge regarding the association between IL-9 genotyping and CeD development risk. IL-9 is a multifunctional cytokine involved in various immune response, including type 2 immunity, autoimmunity, and anti-tumor immunity [17]. IL-9 gene is regulated by several non-coding regulatory elements that modulate its transcription through complex chromatin interactions [18]. The SNPs rs17317275 in IL-9 gene has attracted attention due to its potential impact on gene expression and its association with various diseases [13, 19, 20]. The SNP represents a single nucleotide polymorphism where adenine (A) is replaced by guanine (G), leading to three possible genotypes: AA, AG, and GG. Understanding the distribution and functional implications of these genotypes can provide insights into the role of IL-9 in disease pathogenesis and therapeutic strategies. Therefore, this study aimed to determine the frequencies of genetic single nucleotide polymorphisms (SNPs) of IL-9 rs17317275 A>G genotypes and alleles in Iraqi CeD patients, then compare the present findings of this SNP with other previous findings over the world if found.

2. Subjects

2.1 Samples collection

Fifty celiac disease blood samples were collected from patients suffering from CeD who the physician diagnosed at the specialized hospitals. The patients had a mean age of 23.50 ± 11.88 years, and Biomass Index (BMI) means ± standard deviation (SD) was 25.63 ± 6.55 kg/m^2. A control group of one hundred eighty-three healthy individuals had a mean age of 26.71 ± 10.35 years and a mean BMI of 25.86 ± 8.89 kg/m^2. A written agreement was obtained from all the participants in the present investigation through the questionnaire, including the age, gender, and other questions associated with the disease. Samples were collected between November 2021 and February 2022 and stored in Ethylenediaminetetraacetic Acid (EDTA) tubes for genetic analysis of IL-9 rs17317275 variants. The collected blood samples were kept in EDTA tubes for the genetic variant determinants of *IL-9* rs17317275.

Deoxyribonucleic Acid (DNA) was extracted using the DNA Extraction Kit (Cat procedure No.: FABGK 001-2, FAVORGEN Biotech Corporation, Vienna). The DNA yield was evaluated using a Nanodrop spectrophotometer, which revealed a purity ratio of 1.7-2.0 and a concentration range of 60-120 µg/mL. The studied groups were scanned for genotyping and alleles' frequencies for *IL-9* SNPs rs17317275 via allele-specific primers – polymerase chain reaction technique (ASP-PCR) [13, 20]. In this study, all primers underwent validation using NCBI Primer Blast, Ensemble, and UCSC In-Silico PCR websites. Three specific primers were designed based on a 218 bp sequence fragment got from the SNP database of the gene of interest. These primers included two forward primers differing by a single nucleotide (A>G at the first site of the forward primer) and a common reverse primer. Each sample was genotyped using Allele-Specific PCR (ASP-PCR), which involved two separate PCR reactions. In each reaction, one of the two forward primers (5'-AAACTTCAGCTCTGTCCCTG-3' for the *A* allele and 5'-GAACTTCAGCTCTGTCCCTG-3' for the *G* allele) was paired with the common reverse primer (5'-GCCGGTTGTGGTGTCAAT-3').

2.2. Molecular finding of IL-9 SNPs (rs17317275) by using polymerase chain reaction

All PCR reactions were managed in a total volume of 25 µl. Each reaction mixture comprised of 5µl Master mix (Intron Biotechnology, Inc., Korea), 1 µl of the one of forward primers (10 pmol/µl, Integrated DNA Technologies Inc., USA), 1µl of the common reverse primer with concentration of 10 pmol/µl (Integrated DNA Technologies Inc., USA), 3µl of the extracted DNA, and 15 µl of free nucleases water (Intron Biotechnology, Inc., Korea). Table 1

outlines the primer sequences, PCR conditions, and product lengths used for genotyping the current SNP.

Table 1: The primer sequence and PCR conditions

IL-9 SNPs rs17317275 A>G	primers sequences	PCR conditions			PCR product length
		PCR steps	Temperature (°C)	Time (sec.)	
	A forward primer AAACTTCAGCTCTGTCCCTG	**1.** Initial denaturation	94	**5**	**218 bp**
		2. Thirty-five cycles, each cycle consisted of:			
	G forward primer GAACTTCAGCTCTGTCCCTG	**A.** Denaturation	94	30	
		B. Annealing	54	30	
	Common Reverse GCCGGTTGTGGTGTCAAT	**C.** Extension	72	30	
		3. Final extension	72	10	

In this study, single nucleotide polymorphisms for *IL-9* rs17317275 were genotyped. The allele-specific primers technique (ASPs) was employed in order to genotype *IL-9* rs17317275. After PCR, the amplicons were electrophoresed on 1.5 % of agarose gel pre-stained with the Red Safe Stain® (Intron Biotechnology, Inc., Korea) [21]. The genotyping results indicated a heterozygous genotype (AG) when two bands appeared on the gel electrophoresis, while the amplicon presented with one band when electrophoresed on the gel guided to be as a homozygous genotype (AA or GG).

1.3. Statistical Analysis

Linearity, normality, and homogeneity of age and BMI data were assessed using IBM SPSS Statistics version V28.0 [22], Age and BMI were presented as mean ± SD, with statistical significance defined as $p \leq 0.05$. Numerical data were reported as frequencies and percentages. Person's chi-square and Fisher's exact tests were performed using epidemiological statistical software (WIN PEPI version 11.65) [23] to determine probabilities.
Furthermore, an online Hardy-Weinberg calculator was utilized to compute genotype and allele frequencies, odds ratios (OR), and assess Hardy-Weinberg equilibrium probability [24]. Finally, effect size and sample power were calculated with G Power (version 3.1.9.4) [25].

3. Results

3.1. Physical Characterization of the studied samples

The current results were derived from the analyzing data obtained from 233 blood samples. Linearity, normality, and homogeneity were assessed to validate the sample size for patients (n = 50) and controls (n = 138). The power of sample size was estimated using G*Power software with the following inputs: a two-tailed α error of 0.05 and an effect size (d) of 0.5. The resulting power was calculated to be 0.88, which exceeds the statistically acceptable threshold of 0.8 [25].

The mean age was 23.50 ± 11.88 and 26.71 ± 10.35 years for the both studied groups (CeD patients' group and the control group, respectively) as shown in Table 2. In addition, the BMI means were 25.63 ± 6.55 and 25.86 ± 8.89 Kg/m^2 for the CeD and control groups, respectively. The percentage of females who contributed in the current study was 56.0% for the patients' group and 57.9% for the healthy control group, while the male percentage was 44.0% for the patients' group and 42.1% for the healthy control group. The effect size was 0.8, and the sample power (2-tailed probability) was 0.99 (Table 2).

Table 2: The age and BMI mean, gender frequency, effect size, and power of samples appearances data of the studied group.

Sociodemographic	CeD patient group (n=50)	Control group (n=183)
Age (Years)	23.5 ± 11.88	26.71 ± 10.35
BMI (Kg/m^2)	25.63 ± 6.55	25.86 ± 8.89
Males' percentage (frequency)	44.0 (22)	42.1 (77)
Females' percentage (frequency)	56.0 (28)	57.9 (106)
Effect size	0.8	
Degree of Freedom (DF)	231	
Non-centrality parameter δ	5.01	
α-error probability (2 tailed)	0.009	
Power of samples (1 – β error probability) (2 tailed)	0.99	

3.2. *IL-9* SNPs rs17317275

The genetic variations of IL-9 SNPs rs17317275 were studied; (*A* allele changed to *G* allele at the site of the forward DNA strand). The genotyping was detected by ASP-PCR technique [26, 27]. The band length of *IL-9* SNP rs17317275 was 218 base pairs on 1.5% agarose gel (Figure 1).

Two alleles were determined at the position of IL-9 SNP rs17317275 (*A* and *G*), resulting in three possible genotypes (AA, AG, and GG). The AA, and AG genotypes and *A* allele were more frequent than the GG genotype in the CeD group (Table 3). The genotype frequencies observed in the two groups were found to be in Hardy-Weinberg equilibrium, as shown in Table 3. In the CeD group, the frequency of the AA genotype and *A* allele was more recurrent than AG and AA genotypes and *G* allele (72.0% and 84% for AA genotype and *A* allele versus 24.0%, 4.0% and 16.0% for AG, GG genotypes and *G* allele) (Table 3). In case-control comparison, the results of IL-9 SNPs rs17317275 presented that the AA genotype and *A* allele were significantly elevated percentage in the CeD group than the controls (72.0% and 84.0% *vs.* 10.9% and 34.0% for CeD and control, respectively) (Table 3). The frequency of AG and GG genotypes, as well as the G allele, was significantly lower proportion in the CeD group than the control group (24.0%, 4.0%, and 16.0% *vs*. 45.9%, 43.2%, and 66.0% for CeD and controls, respectively) (Table 3). The increased OR value of AA genotype and *A* allele (20.96 and 10.25, respectively) indicate their significant role in the disease development, which was 68.6% and 75.8% for AA genotype and *A* allele, while the OR value in AG, GG genotypes and *G* allele referred to the preventive role of these genotypes and allele which was 28.8%, 40.8%, and 59.7% for AG and GG genotypes and *G* allele (Table 3).

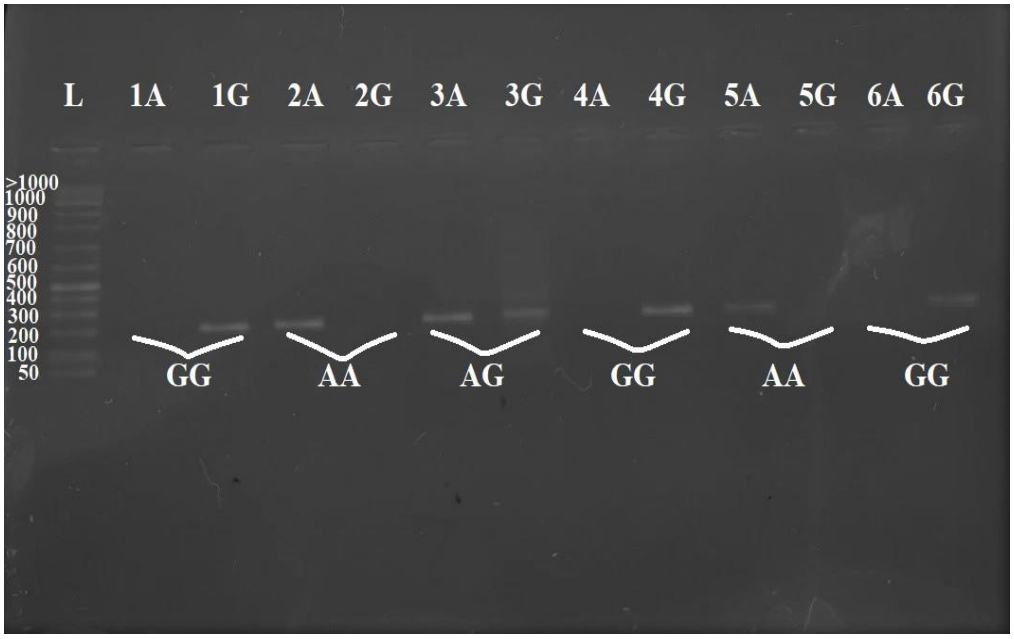

Figure 1: Gel electrophoresis of *IL-9* SNPs rs17317275 using 1.5 % of agarose gel stained with the Red Safe dye® (Intron Biotechnology, Inc., Korea).
The resulting amplicons had a band length of 218 base pairs. L: Universal DNA ladder, 1, 2, 3, 4, 5 and 6 (A or G): the examined samples. A single band in the 2nd and 5th wells referred to the AA homozygous genotype, while the 1st, 4th and 6th wells referred to the GG homozygous genotype. In contrast, the two bands in the 3rd well were referred to the AG heterozygous genotype.

Table 3: A logistic analysis comparing the alleles and genotypes frequencies of *IL-9* SNPs rs17317275 in the CeD group in comparison to the healthy control group.

Genotyping of *IL-9* SNPs rs17317275	CeD group frequency (%)		Control group frequency (%)		OR (95% CI)	Effective fraction or preventive fraction %	*P*	*Pc*
	Observed	Expected	Observed	Expected				
AA	**36 (72.0)**	35.3 (70.6)	20 (10.9)	21.0 (11.5)	20.96 (9.73 – 45.12)	68.6	6.3×10^{-17}	1.98×10^{-16}
AG	12 (24.0)	13.4 (26.9)	**84 (45.9)**	82.0 (44.8)	0.37 (0.18 – 0.75)	28.8	0.006	0.018
GG	2 (4.0)	1.3 (2.6)	**79 (43.2)**	80 (43.7)	0.05 (0.01 – 0.23)	40.8	2.2×10^{-8}	6.6×10^{-8}
Total	50 (100)	50 (100.0)	183 (100.0)	183 (100.0)				
P-HWE	0.4487		0.7400					
Alleles frequency								
A	**84 (84.0)**		124 (34.0)		10.25 (5.77 – 18.19)	75.8	7.9×10^{-9}	2.4×10^{-8}
G	16 (16.0)		**242 (66.0)**		0.10 (0.05 – 0.17)	59.7		

OR: odds ratio, 95% CI: 95% confidence intervals, *P*: Fisher's exact probability, *Pc*: Bonferroni correction probability, *P*-HWE: the probability pf Hardy – Weinberg equilibrium

4. Discussion

IL-9 SNPs rs17317275 is a novel genetic variation that has shown significant associations with several immune-related conditions. The studies indicated that the *A* allele may be linked to increased IL-9 expression, which could enhance immune responses in certain contexts [13, 17, 20]. Conversely, the *G* allele might be associated with reduced IL-9 levels, potentially impacting the severity and progression of diseases such as asthma, autoimmune disorders, and certain cancers [18]. A notable finding in the recent study was the discovery that the expression of IL-9 varied significantly depending on the genotype at the rs17317275 locus. For instance, individuals with the AA genotype tend to exhibit higher IL-9 levels compared to those with the GG genotype [17, 19]. This variation in cytokine levels can influence the immune system's ability to respond to infections, tumors, and inflammatory conditions. Moreover, the AG genotype, representing a heterogenous state, often shows intermediate levels of IL-9, suggesting a dose-dependent effect of the alleles [17, 19]. The novelty of the IL-9 SNP rs17317275 lies in its potential as a biomarker for disease susceptibility and prognosis. By identifying individuals with specific genotypes, clinicians can tailor therapeutic approaches to modulate IL-9 levels, thereby improving patient outcomes [18]. Additionally, this SNP could serve as a target for gene editing technologies aimed at correcting dysregulated IL-9 expression in various diseases [17].

The current study is the first study about the association between IL-9 serum level and its genetic polymorphism rs17317275 among CeD patients. The present results agreed with the results of Mohammed and Salloom (2021) who reported that the AA genotype was more recurrent in autoimmune disease [20]. While other studies about IL-9 genetic polymorphisms rs17317275 SNPs in rheumatoid arthritis reported that the AG genotype frequency percentage was more frequent (61.84%) than the other genotypes, while the AA and GG genotypes were less frequent (11.84% and 26.32%, respectively), and they included that the AG genotyping and *A* allele might be related to CeD development [13]. However, the exact mechanisms to understand the association between IL-9 SNPs with the autoimmune diseases still being investigated, several researchers are exploring how IL-9 influences immune responses and contributes to disease pathogenesis [1, 28, 29]. Regrettably, a thorough review of the literature revealed a lack of published studies examining the genetic and allelic variations associated with this change. However, we believe that the change from *G* to *A* allele resulting in changes in the genetic code that lead to change in the transcription and translation of the resulted amino acids and affect at the peptides series, and finally at the configuration of the protein. In addition, different studies deal with the role of IL-9 levels and their genetic polymorphisms in the immune response and autoimmune disease development [30]. Through the regulation of various immunological functions, IL-9 provides binding site to numerous transcription factors (STAT1, STAT3, STAT5, STAT6, Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκβ)) and promotes the activation and recruitment of inflammatory cells [31]. Furthermore, the increase production of IL-9 serum level especially in the intestine from the gut T cells will increase the susceptibility to food allergy, and stimulate the mast cell migration to the intestine leading to intestinal mastocytosis and increasing the permeability of the intestine and induction the inflammation in the gut [32]. The present results of elevated IL-9 level in the CeD patients' group were compatible with the investigations of Dantas *et al*. (2015) who reported the elevated IL-9 level in CeD and other autoimmune diseases [33].

Conclusions

In conclusion, the IL-9 SNPs rs17317275 represents a significant genetic variation with important implications for immune regulation and disease management. This investigation concluded that the AA genotype of the IL-9 rs17317275 SNP was more frequently observed in

patients with celiac disease, while the (AG and GG) genotypes were less common. This suggests a potential association of the AA genotype and *A* allele with autoimmune conditions like celiac disease.

Recommendations

Based on conclusions and after reviewing the literature and the present findings investigated only one SNP of *IL-9* genes, and it has been found such SNP's region encompasses further SNPs; therefore, we recommended investigating these SNPs in the forthcoming studies, The results can be better presented in terms of haplotypes of these alleles. Further research is needed to fully elucidate its role and develop targeted therapies that leverage this genetic insight.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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