

## Investigating the association between Toll-Like Receptor 9 (TLR9) rs352139 genetic variants and Systemic Lupus Erythematosus (SLE) in an Iranian population

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DOI: 10.22034/HBB.2023.25

Received: September 18, 2023; Accepted: October 14, 2023

### ABSTRACT

Numerous studies have examined the effect of genetic variations in the Toll-Like Receptor 9 (TLR9) on the development of Systemic Lupus Erythematosus (SLE). An allele-specific PCR technique was employed to analyze the genetic variation in the study participants in a cohort of SLE and healthy controls from the Golestan region of northern Iran. Our study revealed no statistically significant variations in genotype and allele distribution between individuals with SLE and the control group. When analyzing the genotype frequency for rs352139, no significant association was observed between the GA genotype and AA genotype in relation to the risk of developing SLE, using the GG genotype as the reference. These findings indicated that the TLR9 rs352139 SNP could not play a significant role in determining susceptibility to SLE.

**Keywords:** Toll-like receptor 9 (TLR9); genetic variations; polymorphism

### INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a prototypical disease associated with systemic autoimmune dysfunction. The

disease chronicity and progressive nature arise due to the immune system production of antibodies targeting self-antigens [1]. SLE symptoms can range from mild to severe, affect one or more organ systems,

and change over time, making diagnosis challenging [2]. The most reported symptoms in this category are skin manifestations, including the well-known malar butterfly rash, arthralgias, pleurisy, serositis, baldness, and lupus nephritis [3]. The etiology of SLE is still unclear. However, current research suggests that the development of SLE involves a multifaceted interplay between various genetic and environmental determinants [4,5]. Many genes have been linked to SLE through Genome-Wide Association (GWAS) studies, such as HLA-DRB1, PTPN22, IRF5, TNFAIP3 [6].

The influence of Toll-Like Receptor 9 (TLR9) on the risk of SLE among various ethnicities is currently being studied. This gene is located in regions that are more susceptible to SLE [7-9]. Essential for recognition and innate immunity activation, TLR9 is a receptor that recognizes unmethylated dinucleotide motifs of Cytosine-Phosphate-Guanine (CpG) present in bacterial, viral, and fungal DNA [10]. TLR9 facilitates type I interferon biosynthesis and plasmacytoid dendritic cell activation, which are essential in the pathophysiology of SLE when it is activated by host DNA in endosomes [11]. It is primarily found in macrophages and

dendritic cells within endosomes. TLR9 activation can also stimulate the formation of IgG2a and IgG2b autoantibodies that target host DNA, adding to autoimmune disease [12,13]. The TLR9 gene is associated with developing malaria infection, cancer, coronary artery disease, and autoimmune diseases like SLE [14-17]. Deletion of TLR9 in SLE murine models leads to more severe disease and lower anti-dsDNA antibodies [18]. TLR9 expression is increased in B cells of SLE patients, but its role as a causative or protective factor remains uncertain. Polymorphisms in TLR9 can impact key factors such as promoter activity, mRNA structure and localization, and protein function. These variations may predispose individuals to bacterial and viral infections, autoimmune disorders, and even cancer. Thus, further research is needed to fully comprehend the implications of TLR9 and its polymorphisms in these conditions [19-23]. Numerous studies have examined the relationship between TLR9 polymorphisms and susceptibility to SLE in various ethnic groups [24-26]. The findings regarding the influence of TLR9 on the risk of SLE among various ethnicities have been inconsistent, resulting in conflicting results despite numerous studies.

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The purpose of our study was to investigate the potential role of the TLR9 variant (rs352139) as a genetic susceptibility factor for SLE in the Iranian population (Golestan Province). Given the multifaceted nature of SLE, we sought to examine the impact of this SNP on various clinical phenotypes of the disease. While the TLR9 gene contains multiple polymorphisms that are widely distributed, we focused specifically on the +1174A>G polymorphism located in the intronic region of the gene. We conducted a case-control study to examine the role of this polymorphism in the susceptibility to SLE within the Iranian population. Our objective was to determine whether this SNP is linked to an increased risk of SLE in this particular population.

## **MATERIALS AND METHODS**

### ***Patients and controls***

In this research, a case-control study was conducted on a cohort of 204 participants, comprising 103 individuals diagnosed with SLE and 101 healthy controls. The study population was recruited from Golestan Province, located in the northern region of Iran, and consisted of individuals from Turkman and Fars ethnicities. In our study, we determined an effect size of approximately OR based on prior research

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by Mohammadi *et al* [27]. Setting the statistical power of 80 % ( $\beta=0.2$ ) and a significance level of 0.05. Calculating the proportion of cases as 0.5073, we used the formula  $n = [(Z\alpha/2 + Z\beta)^2 * (Pc * (1 - Pc) + P0 * (1 - P0))] / (ES^2)$  to find our sample size, with  $Z\alpha/2=1.96$ ,  $Z\beta=0.84$ , and  $ES=2$ . The SLE patient group consisted of 4 males (3.88 %) and 99 females (96.12 %), with an average age of 33.16 years ( $SD=4.28$ ). Regarding ethnicity, the majority of SLE patients were Turkmen (84; 81.55 %), followed by Fars (18; 17.48 %), and a small percentage belonged to other ethnicities (1; 0.97 %). In the normal subject group, there were 3 males (2.97 %) and 98 females (97.03 %), with an average age of 32.77 years ( $SD=3.91$ ). The majority of normal subjects were Turkmen (83; 82.18 %), followed by Fars (14; 13.86 %), and a small percentage belonged to other ethnicities (4; 3.96 %). The SLE patients were selected from the Department of Rheumatology at Sayyad-e-Shirazi Hospital, Golestan University of Medical Sciences (GoUMS). The inclusion criteria for the patient group included a confirmed diagnosis of SLE based on the American College of Rheumatology (ACR) classification criteria, which includes the presence of at least four out of eleven clinical and laboratory manifestations of SLE. The

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clinical and demographic characteristics of the SLE patients, including age, gender, disease duration, and treatment history, were recorded for further analysis. A control group comprising of 101 unrelated healthy women was also recruited during a routine physical examination conducted at Sayyad-e-Shirazi Hospital. The control group was selected based on the absence of any clinical or laboratory evidence of autoimmune or inflammatory diseases, and matched with the patient group in terms of age, gender, and ethnicity. In our study, we performed case-by-case matching without utilizing the propensity score method. This approach involved carefully selecting and pairing cases and controls based on their similar characteristics, thereby ensuring a balanced comparison between the groups and minimizing potential biases.

The study procedures, including the recruitment of participants, data collection, and analysis, were approved by the Local Ethics Committee of GoUMS (Code of Ethics: IR.GOUMS.REC.1396.177). The study was conducted in compliance with the Declaration of Helsinki and all relevant guidelines and regulations for research involving human subjects. Prior to their participation in the study, all the participants were provided with written informed consent forms and were informed

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about the purpose and procedures of the study, their rights as research participants, and the confidentiality of their personal information. Any potential risks or discomfort associated with the study procedures were minimized, and the participants were assured that their participation was voluntary and that they could withdraw from the study at any time without any consequences.

### ***DNA extraction***

Peripheral blood samples (2 ml) were obtained from each participant using EDTA anticoagulant before being stored at -20 °C until further processing. Genomic DNA was extracted from peripheral leukocytes using the standard salting-out method for the patient group. For the control group, blood samples were collected during their visit to the Sayyad-e-Shirazi Hospital, and genomic DNA was extracted from 200 µL of peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The extracted DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the purity and integrity of the DNA samples were assessed by agarose gel

electrophoresis before being stored at -80 °C for further analysis.

### **Genotyping**

In this study, we employed the highly reliable and cost-effective allele-specific polymerase chain reaction (AS-PCR) technique to determine the presence of intron +1174A>G (rs352139) polymorphism. One of the major advantages of this technique is its ability to rapidly detect the amplification products on an agarose gel. To achieve this, we designed two sets of allele-specific forward primers, each specific for one of the Single Nucleotide Polymorphisms (SNP) alleles, without any mismatch. The primer sequences used were as follows: 5'-GTGGAGTGGGTGGAGGTG-3' for A allele and 5'-AAGTGGAGTGGGTGGAGGTA-3' for G allele. Additionally, a standard reverse primer (5'-CAAGGAAAGGCTGGTGACAT-3') was prepared for both forward primers. To ensure the accuracy and reliability of the ARMS-PCR results, GAPDH was utilized as an internal control. The following primers were used for the amplification of the GAPDH gene: 5'-AACATAACCACCTCGCACCG-3' for forward, and 5'-

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AGGTATTGTGCAACGGGCTA-3' for reverse. To perform the PCR amplification of TLR9 and GAPDH genes, we used the Super PCR Master Mix 2X (Yekta Tajhiz, Iran) following the manufacturer's protocol. The reaction volume was 20 µL, containing 100 ng of genomic DNA, 10 µL of 2X master mix, and 0.5 µM of each primer. The amplification was performed under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec for TLR9 and 60°C for GAPDH, extension at 72 °C for 30 sec, and a final extension at 72 °C for 5 min. The amplicon size for TLR9 and GAPDH genes was 284 bp and 633 bp, respectively, which were confirmed on 2 % agarose gel electrophoresis. To ensure the accuracy of the genotyping results, we randomly selected three samples from each genotype in both patient and control groups and sent them for sequencing. The sequencing results confirmed the genotyping accuracy, and no discrepancies were observed between the genotyping and sequencing results.

### **Statistical analysis**

In our genetic analysis, we employed various statistical techniques to examine the relationships between genotypes,

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alleles, and disease status. We conducted direct counting to calculate genotype and allele frequencies and assessed deviations from the Hardy-Weinberg equilibrium. To compare genotype and allele distributions between patient and control groups, we employed the  $\chi^2$  test, with significance set at  $p < 0.05$ . Conditional logistic regression was considered to account for potential confounding variables in exploring genetic influences on disease status, as suggested. The selection of these statistical methods was based on the nature of the data and research objectives, allowing us to comprehensively evaluate genetic associations and deviations from expected genetic distributions.

## RESULTS

### *The genotype and allele frequency distribution for the SNP TLR9 rs352139*

Table 1 presents a comprehensive overview of the genotype and allele frequency distribution for the SNP TLR9 rs352139

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(+1174A>G) within the cohort of individuals with SLE and healthy controls. Our analysis revealed that the genotype frequencies for this SNP in both the case and control groups were consistent with the expected distribution under the HWE ( $p$ -value  $> 0.05$ ), indicating that the study population was in genetic equilibrium.

Setting the G allele with the highest frequency in healthy subjects as the reference allele, the allelic frequency analysis of rs352139 revealed no significant association between the A allele (OR: 1.10, 95 % CI: 0.75-1.61) and SLE risk ( $p$ -value=0.63). Using the GG genotype as the reference, the analysis of genotype frequency for rs352139 showed no significant association between the GA genotype (OR: 1.39, 95 % CI: 0.71-2.69) and the AA genotype (OR: 1.20, 95 % CI: 0.56-2.57) with SLE risk ( $p$ -value=0.62) under the co-dominant model.

**Table1.** Genotype and allele frequencies of TLR9 (+1174A>G) SNP in SLE patients and healthy subjects under different inheritance models

Genotypes and alleles		SLE patients (n=103)	Normal subjects (n=101)	OR (95 % CI)	p-value
		Number (%)	Number (%)		
TLR9 +1174A>G (rs352139)	G	102 (50 %)	105 (52 %)		
	A	104 (50 %)	97 (48 %)	1.10 (0.75-1.61)	0.63
	<i>Co-dominant model</i>				
	G/G	25 (24.3 %)	30(29.7)	Reference 1.00	
	G/A	52 (50.5 %)	45 (44.5 %)	1.39 (0.71-2.69)	0.62
	A/A	26 (25.2 %)	26 (25.7 %)	1.20 (0.56-2.57)	
	<i>Dominant model</i>				
	G/G	25 (24.3 %)	30 (29.7 %)	Reference 1.00	
	G/A-A/A	78 (75.7 %)	71 (70.3 %)	1.32 (0.71-2.45)	0.38
	<i>Recessive model</i>				
	G/G-G/A	77 (74.8 %)	75 (74.3 %)	Reference	
	A/A	26 (25.2 %)	26 (25.7 %)	0.97 (0.52-1.83)	0.93
	<i>Over-dominant model</i>				
	G/G-A/A	51 (49.5 %)	56 (55.5 %)	Reference	
	G/A	52 (50.5 %)	45 (44.5 %)	1.27 (0.73-2.20)	0.4
$\chi^2$ HWE*	p-value = 0.12	p-value = 0.58			

**The relationship between genotypes and clinical manifestations of SLE patients**

To explore the possible relationship between TLR9 rs352139 (+1174A>G) genotypes and clinical features of SLE, we

conducted a comparison of clinical and laboratory characteristics in SLE patients with different genotypes. Investigation into the clinical characteristics of SLE

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patients with different TLR9 genotypes (GG, GA, and AA) showed no noteworthy variations in the occurrence of malar rash, alopecia, arthritis, vasculitis, oral ulcers, lupus nephritis, or SLEDAI scores.

## DISCUSSION

SLE is a chronic autoimmune condition with an unknown underlying cause that can lead to clinical symptoms, from mild skin involvement to life-threatening harm to vital organs. As with other autoimmune disorders, SLE pathogenesis is believed to involve genetic factors, including SLE susceptibility genes [28]. This study assessed the connection between the genotype frequencies of the TLR9 +1174A>G (rs352139) polymorphism and susceptibility to SLE and disease activity in SLE patients from the Golestan province, northern Iran.

After conducting a comprehensive analysis of the TLR9 +1174A>G polymorphism (rs352139) in our study population, we found no significant association between this SNP and the risk of developing SLE, under any of the tested

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inheritance models. Our results suggest that this polymorphism is not a major contributor to SLE susceptibility in our population. However, further studies with larger sample sizes and diverse populations are required to validate our findings. Similar to our study, Azab *et al.* did not observe a significant association between the Egyptian population's TLR9 rs187084 SNP and SLE development [25]. Allelic frequencies at position +1174 in patients and healthy controls remain consistent between Korean and Chinese populations [29,30]. In contrast, an increased risk of SLE ( $p = 0.029$ ) is closely associated with the G allele at position +1174, located in intron 1 of TLR9 in the Tao *et al.* study [31]. Also, Huang *et al.* (2011) conducted studies indicating that the TLR9 -1486 T/C (rs187084) polymorphism, situated within the LD block alongside rs352140, correlates with SLE in Taiwanese patients [32].



**Table 2.** Relation of TLR-9 genotypes to demographic and clinical and laboratory data of SLE patients

Variable	Groups	G/G(n=25)	A/G(n=52)	A/A(n=26)	p-value*
Gender	Male	0(0%)	2(1.94%)	2(1.94%)	0.758
	Female	25(24.2%)	50(48.5%)	24(23.3%)	
Ethnicity	Turkman	21(20.3%)	45(43.7%)	18(17.5%)	0.333
	Fars	3(2.9%)	7(6.8%)	8(7.7%)	
	others	1(0.97%)	0	0	
Malar rash	yes	17 (16.5%)	22 (21.3%)	6 (5.8%)	0.053
	no	8 (7.7%)	30 (29.1%)	20 (19.4%)	
Hair loss	yes	16 (15.5%)	23 (22.3%)	8 (7.7%)	0.237
	no	9 (8.7%)	29 (28.1%)	18 (17.4%)	
Nephritis	yes	9 (8.7%)	9 (8.7%)	6 (5.8%)	0.427
	no	20 (19.4%)	39 (37.8%)	20 (19.4%)	
ESR range	normal	2 (2.5%)	9 (11.2%)	0	0.069
	high	19 (23.7%)	19 (23.7%)	31 (38.7%)	
WBC range	low	6 (5.8%)	7 (6.8%)	5 (4.8%)	0.275
	normal	23 (22.3%)	39 (37.8%)	21 (20.4%)	
	high	0	2 (1.9%)	0	
RF range	normal	1 (0.97%)	13 (12.6%)	2 (1.9%)	0.088
	high	13 (12.6%)	19 (18.4%)	9 (8.7%)	
Anti-dsDNA range	negative	5 (4.8%)	4 (3.5%)	7 (6.8%)	0.540
	intermediate	10 (9.7%)	19 (18.4%)	10 (9.7%)	
	positive	10 (9.7%)	29 (28.1%)	9 (8.7%)	

\* The chi-square test was used to determine whether there was a significant association between two categorical variables.

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The pathogenesis of autoimmune diseases involves immune system dysfunction, leading to impaired T-cell responses and dysregulated B-cell activation. This can lead to the production of autoantibodies, which attack healthy cells and tissues [33]. TLR9 can be activated by hypomethylated CpG DNA and potentially play a role in autoimmunity through the induction of inflammatory cells and the production of cytokines or antibodies. Moreover, several studies have shown increased TLR9 expression in Blood samples from SLE patients [34,35]. Additionally, it has been demonstrated that individuals with SLE with higher levels of anti-dsDNA antibodies also exhibit more significant expression of TLR9 mRNA [35]. Furthermore, the increased synthesis of autoantibodies can be attributed to signaling cascades of both TLR9 and IgM in B cells. This finding was established in previous studies [36].

In our study, we did not find any evidence of an association between the G/G, A/G, and A/A genotypes of TRL9 and an elevated risk of developing malar rash, arthritis, nephritis, and hair loss in patients with SLE. Furthermore, no significant association was observed between the TRL9 G/G, A/G, and A/A genotypes and the presence of anti-dsDNA antibodies in

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SLE patients. On the contrary, in the Chinese Han population, the polymorphism of the rs352140 gene was found to show a marked correlation with lupus nephritis [37]. Similar to our study, the risk of SLE-related clinical manifestations in Korean patients was not significantly associated with TLR9 polymorphisms [29]. Current research indicates that changes in TLR9 expression could potentially influence the development of renal disease in SLE, as demonstrated in recent studies [38,39]. Nonetheless, other research conducted in southern Brazil found no significant association of the SNP with SLE [40].

Shahin *et al.* recently conducted a study on the +1174A>G polymorphism (rs352139) in the TLR9 gene intron region to investigate its association with SLE in patients compared to control subjects. They discovered a strong correlation ( $p < 0.001$ ) between the GA genotype and the susceptibility to SLE. However, there were no significant differences in clinical features such as malar rash, alopecia, arthritis, vasculitis, oral ulcers, lupus nephritis, and SLEDAI between the wild genotype (GG) and polymorphic genotypes (GA and AA). Despite this, the study found a higher frequency of myositis in the group with the AG genotype ( $p = 0.032$ ) and a higher frequency of psychosis in the group

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with the AA genotype ( $p=0.014$ ). Furthermore, the wild Genotype Group (GG) had a higher frequency of photosensitivity and pleurisy ( $p=0.002$ ,  $<0.001$ , respectively). The study also found no significant differences in laboratory data between the wild genotype (GG) and the polymorphic genotypes (GA and AA) regarding anti-dsDNA, Erythrocyte Sedimentation Rate (ESR), complement consumption (C3:  $p = 0.226$ , C4:  $p = 0.371$ ), Hemoglobin (HB), Total Leukocyte Count (TLC), platelets, urea, and serum creatinine. However, patients with AA and GA genotypes at the +1174 position had a higher frequency of Antinuclear Antibody (ANA) positivity than the GG genotype ( $p=0.038$ ) [41].

The impact of TLR9 polymorphisms on SLE development and clinical features may vary depending on factors such as race, sample size, or environmental differences between populations. These observations highlight the complex interplay between genetic and environmental elements in the pathogenesis of SLE and underscore the need for ongoing research to understand this multifaceted disease. However, it is important to acknowledge the limitations of this study, including the relatively small sample size, which could potentially lead to

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less reliable findings. Therefore, further research incorporating larger sample sizes is necessary to validate and strengthen our results.

## CONCLUSION

In our study conducted on the population of Golestan in northern Iran, we did not observe any significant association between the rs352139 SNP and Systemic Lupus Erythematosus (SLE). Furthermore, the TLR9 genotypes G/G, A/G, and A/A were not found to be linked to an increased risk of malar rash, arthritis, nephritis, or hair loss in individuals with SLE. However, it is important to note that larger-scale studies, analyzing multiple loci and genes, are required to validate these findings. Additionally, functional studies are necessary to gain a better understanding of the underlying mechanisms involved in the development of SLE.

## ACKNOWLEDGMENT

We would like to express our sincere appreciation to the laboratory staff at the Department of Immunology, who contributed to the completion of this research project. We also acknowledge the financial and scientific support provided by AJA University of Medical Sciences, and Golestan University of Medical Sciences.

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