Association of rs5743899 Polymorphism of *TOLLIP* Gene With Susceptibility to Cutaneous Leishmaniasis in Southwest Iran

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Abstract

Cutaneous leishmaniasis (CL) represents a vector-transmitted infection resulting from various species of *Leishmania*. Host genetic factors, such as polymorphisms in immune-related genes, influence susceptibility to CL. The *TOLLIP* gene, a regulator of innate immunity, has been linked to various infectious diseases. This study investigates the association of the rs5743899 polymorphism in the *TOLLIP* gene with predisposition to CL in a cohort from Khuzestan province, Iran, where *Leishmania major* is endemic. The study included 67 clinically confirmed patients with cutaneous leishmaniasis who presented with active lesions, along with 101 healthy controls. Whole Blood was obtained from the subjects, and genomic DNA was extracted, and genotyping of the rs5743899 polymorphism was performed using the ARMS-PCR method. Data were analyzed using SPSS software to determine genotype frequencies and associations with CL. The genotypic frequencies were consistent with the Hardy-Weinberg equilibrium in both the case and control groups. The P-values obtained for the rs5743899 polymorphism were greater than 0.05, suggesting no association with susceptibility to CL ($p = 0.189$ for allele frequency and $p = 0.132$ for genotype frequency). Furthermore, the Odds Ratio analysis demonstrated that the presence of TT, CT, and CC genotypes did not increase the risk of developing CL. Although this study provides preliminary evidence regarding the lack of association between rs5743899 and cutaneous leishmaniasis in the studied population, the relatively small sample size may have limited the detection of modest genetic effects. Therefore, larger multicenter studies are recommended to validate these results. While these findings clarify aspects of the disease's genetic background, they also point to the need for further research into other genetic and environmental contributors to its epidemiology.

Keywords: ARMS-PCR, Genetic susceptibility, Leishmaniasis, polymorphisms, *TOLLIP*

1. Introduction

Leishmaniasis is considered a tropical zoonotic disease caused by infection with protozoan parasites of the Leishmania genus, transmitted through insect vectors. It manifests in visceral, cutaneous, and mucocutaneous forms, with symptoms ranging from mild self-limiting skin ulcers to severe, life-threatening conditions (1). Cutaneous Leishmaniasis (CL) is particularly prevalent in tropical and subtropical regions, affecting approximately 12 million people across 100 countries, with around 350 million individuals at risk (1) . The majority of cutaneous leishmaniasis cases (more than 90%) are reported from nations such as Afghanistan, Saudi Arabia, Algeria, Syria, Iran, Bolivia, Brazil, Colombia, Peru, and Nicaragua (2). The etiological agents of CL vary by region: *L. major*, *L. tropica*, and *L. aethiopica* predominate in the Old World, while *L. braziliensis, L. amazonensis, and L. mexicana* are more frequent in the New World (1).

Immune system-related factors play a significant role in susceptibility to Leishmaniasis. Additionally, various studies have indicated the involvement of genetic factors in the predisposition to different forms of Leishmaniasis. Several investigations have demonstrated the role of Toll-like receptors (TLRs) in immune response against protozoan parasites (3). Toll-like receptors function as key regulators in host immunity against Leishmania infection (4). Recent studies underscore the important role of TLR4 in inhibiting *Leishmania* proliferation within both innate and adaptive immunity (5). Genes in the TLR pathway may significantly influence predisposition to Leishmaniasis. Toll-interacting protein (TOLLIP) serves as a negative regulator in the TLR signaling, especially in suppressing TLR4 and TLR2 pathways (6). Additionally, Toll-interacting protein is proposed to modulate human TLR signaling by inhibiting pro-inflammatory cytokines such as IL-6 and TNFα, whereas it promotes IL-10 as an anti-inflammatory cytokine (7).

A number of single-nucleotide polymorphisms (SNPs) have been reported in genes encoding cytokines that play pivotal roles in the immune system regulation. Most SNPs are found in the UTRs of cytokine genes; some can enhance cytokine expression, whereas others may have minimal or no impact (8). Considering that the Th1 (T helper 1) response leads to parasite killing and that the TLR pathway is important for inhibiting parasite proliferation in animal models, we note a potential role of these pathways in host defense. Since TOLLIP acts as a key negative regulator of TLR-mediated signaling and can modulate cytokine production, genetic variations in this gene may influence the magnitude of host immune responses. Given the possible association between the *TOLLIP* gene and susceptibility to cutaneous leishmaniasis, and considering the limited studies on this disease, we focused on the rs5743899 polymorphism located in an intronic region of the *TOLLIP* gene (NM_019009). Studies on this SNP are scarce, particularly in Iranian populations, and previous research has suggested its potential involvement in susceptibility to infectious diseases, such as tuberculosis and HIV (7, 9), highlighting its possible functional relevance in immune response pathways. These findings suggest that TOLLIP

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variants, including rs5743899, might contribute to differences in susceptibility to Leishmania infection through modulation of TLR and cytokine signaling pathways. Therefore, in this study, we investigated its potential association with susceptibility to CL caused by *L.major* and *L. tropica*, with *L. major* being the predominant species responsible for cutaneous leishmaniasis in southwestern Iran, particularly in Khuzestan Province.

2. Materials and methods

2-1. Subjects

The present study was conducted in Khuzestan province, southwest of Iran, where cutaneous leishmaniasis caused by *L. major* is endemic. 67 patients with active CL lesions, confirmed by clinical and parasitological diagnosis [45 males (67%), 22 females (33%)] and 101 unrelated healthy controls [63 males (62.4%), 38 females (37.6%)] without active lesions, and without a history of CL were enrolled in the present study. The patients consisted of CL individuals referred to the leishmaniasis reference laboratory. The diagnosis was confirmed through microscopic identification of amastigotes in lesion exudates, which were smeared, methanol-fixed, and Giemsa-stained for examination under light microscopy. Parasitic density was assessed based on WHO criteria, ranging from 4+ (1–10 parasites per field) to 1+ (1–10 parasites per 1000 fields).

Equally important, the control group comprised healthy volunteers, providing a crucial basis for comparison. In this study, the age distribution was determined for sampling the age group of 15-50. Non-Iranian and non-native people were excluded from the study. All participants filled out an informed consent form before blood sample collection, after the Ethical Committee of the Dezful University of Medical Sciences approved the protocol.

2-2. DNA extraction and genotyping

Patients and controls underwent venipuncture to obtain 2mL of whole blood samples in EDTA tubes. Genomic DNA was isolated from leukocytes using a standard salting-out protocol, and the DNA quality and quantity were evaluated using a UV spectrophotometer at wavelengths of 260 and 280 nm. Finally, DNA samples were preserved at −20°C for later use.

The *TOLLIP* gene rs5743899 Polymorphism was genotyped by the ARMS-PCR method. Primers for DNA amplification and fragment analysis were designed and validated using the SNPs database and the BLAST website. Table 1 presents the primers designed for genotyping of the rs5743899 polymorphism.

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Table 1: Primers used for genotyping of rs5743899

Primer	Position	Sequence
FORW IC	Inner-forward (C allele)	CAGCTGACTGACCCCTCAGGGC
FORW IT	(T allele) Inner-forward	CAGCTGACTGACCCCTCAGGGT
REV OR	Outer-reverse	TGCTGTGAAGGGTGGTGGGTG
FORW OF	Outer-forward	TGCAAGGGGCCTGCTCCAG

The ARMS-PCR for each sample was performed in two separate PCR reactions using different primer sets of inner and outer primers. FORW IC and FORW IT primers, along with REV OR, generate products that detect the C and T alleles, respectively. The product of REV OR and FORW OF primers, meticulously designed to serve as an internal control, ensures the reliability of the results.

PCR amplification was set up in 15 microliter reaction mixtures comprising 7.5 µl of 2X PCR mastermix (Ampliqon-Denmark), 0.25 pmol/µl of each primer (metabion-Germany), and 1 µl of template DNA. PCRs were carried out using a Veriti™ Thermal Cycler (Applied Biosystems-US). The PCR steps were as follows: initial denaturation step at 95°C for 5 minutes, 35 cycles of amplification (denaturation: 95°C-30 seconds, annealing: 61°C-45 seconds), and extension step at 72°C for 45 seconds, followed by a final extension step at 72°C for 5 minutes. The PCR products were visualized by 1.5% agarose gel electrophoresis.

2-3. Statistical Analysis

The data were analyzed using the SPSS V.16 software. The mean age and the gender were compared between the case and control groups using an independent t-test and a chi-square test. Genotypic frequencies were evaluated using the χ^2 test, considering P-values < 0.05 as indicative of significance. Hardy–Weinberg equilibrium (HWE) was analyzed by the Chi-square test. The sample size was determined to achieve approximately 80% statistical power at $\alpha = 0.05$ to detect an odds ratio (OR) of 2.0. Multiple genotype comparisons were statistically adjusted between groups to identify alleles or genotypes associated with an elevated risk of developing cutaneous leishmaniasis. Odds ratios (ORs) were estimated with 95% confidence intervals.

3. Results

The analysis showed no significant difference in the mean age between the case (40.93 ± 10 years) and control (38.59 ± 12 years) groups (P = 0.20). Additionally, no significant difference in gender (P-value = 0.623) between the CL patients and controls was noted, suggesting that the matching based on these two variables was adequate.

The rs5743899 Polymorphism was genotyped by the ARMS-PCR. Figure 1 illustrates PCR products separated on a 1.5% agarose gel. The length of PCR products detecting the C and T alleles was 245 bp, while the length of the fragment used as an internal control was 481 bp.

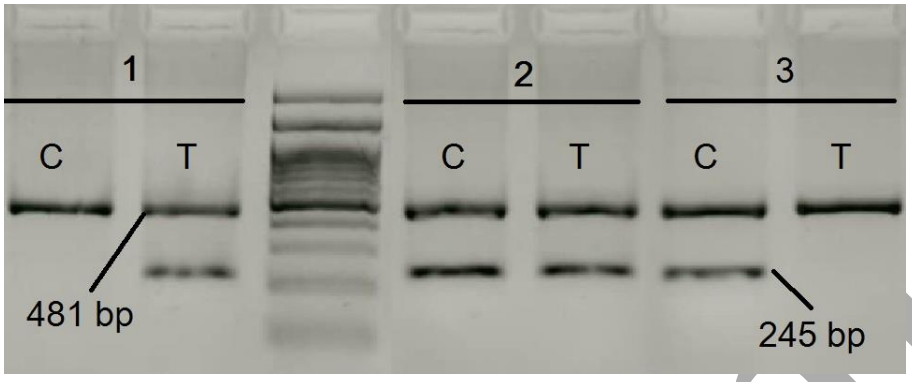

Figure 1: ARMS-PCR products separated and visualized by agarose gel electrophoresis. The 481 bp band is an internal control and must be present in all samples. The 245 bp band detects C and T alleles. 1: sample with a homozygote TT genotype. 2: sample with a heterozygote TC genotype. 3: sample with a homozygote CC genotype

The genotypic frequencies in the control and case groups were examined for HWE using the chi-square test. Both the control and case groups were consistent with HWE (p=0.919 for controls and p=0.06 for patients). Allelic and genotypic frequencies showed no significant difference. Table 2 presents the *TOLLIP* gene rs5743899 polymorphism genotype and allele frequencies in the case and control groups. According to the Pearson Chi-square test and the allele and genotype frequency table (Table 2), the P-value obtained for the polymorphism rs5743899 was greater than 0.05 ($p = 0.189$ for allele frequency and $p = 0.132$ for genotype frequency). Therefore, no significant association was observed between this polymorphism and susceptibility to cutaneous leishmaniasis. Additionally, logistic regression analysis of the rs5743899 polymorphism further confirmed that none of the genotypes (TT, CT, or CC) were significantly associated with an elevated risk of developing cutaneous leishmaniasis. Various genetic models, including allelic, codominant, dominant, recessive, and over-dominant, were evaluated. The calculated ORs showed no statistical significance in all comparisons, as evidenced by P-values >0.05 (Table 3).

Table 2: The rs5743899 genotype and allele frequencies in the case and control groups

Polymorphism	Cases (n= 67) %	Controls (n= 101) %	P-value
rs5743899T/C genotype			
TT	42 (62.7%)	57 (56.4%)	
CC	0 (0)	6 (5.9%)	0.132
CT	25 (37.3%)	38 (37.7%)	
Total	67 (100%)	101 (100%)	
rs5743899 T/C allele			
T	109 (81.3%)	152 (75/2%)	
C	25 (18.7%)	50 (24.8%)	0.189
Total	134 (100%)	202 (100%)	

The P-value for the chi-square test ($P \leq 0.05$ is considered significant)

Table 3: Risk estimates for the rs5743899 polymorphism in CL patients and controls based on logistic regression analysis

Polymorphism	Genetic model	OR	CI (95%)	P-Value
rs5743899				
T vs C	Allelic	1.434	0.836-2.460	0.190
CT vs (TT + CC)	Over-dominant	0.987	0.521-1.868	0.968
CT vs TT	Codominant	1.120	0.589-2.131	0.730
TT vs (CC+ CT)	Recessive	1.297	0.689-2.41	0.420
CC vs (TT + CT)	dominant	ND	ND	ND

Abbreviations: CI, confidence interval; ND, not determined; OR, odds ratio.

4. Discussion

Leishmaniasis, particularly Cutaneous Leishmaniasis, is an important public health challenge, especially in endemic regions like tropical and subtropical countries, including developing areas like Khuzestan province in Iran. Depending on the pathogenicity of the parasite and the efficiency of the host's immune system, it can lead to a range of clinical symptoms, from mild skin ulcers to life-threatening forms (2, 10). The contribution of genetic factors in susceptibility to infectious diseases, including Leishmaniasis, has been increasingly recognized. The *TOLLIP* gene, as a negative regulator of TLR signaling, plays an essential role in modulating the immune response to various pathogens, including *Leishmania* (4). TLR4 inhibits the growth of *L. major* by inducing nitric oxide synthase in both innate and adaptive immunity (11). Additionally, glycosylphosphatidylinositol-anchored lipophosphoglycan (LPG) acts as a virulence determinant and is one of the principal molecules of the parasite. LPG induces macrophages to secrete TNFα and IL-12 via MyD88 and relies on TLR2 to initiate NF-κB activation (12). Furthermore, it has been demonstrated that the downregulation of TLR3,

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TLR2, and MyD88 through RNA interference (RNAi) results in decreased secretion of nitric oxide and TNFα triggered by *Leishmania donovani* promastigotes (13).

Several previous studies have indicated that the *TOLLIP* gene is linked to infectious diseases, including tuberculosis (14), HIV(9), leprosy (15), and both cutaneous and Visceral Leishmaniasis (VL) (16, 17). Given these studies, the genetic background of individuals regarding susceptibility to infectious diseases is evident, although study results may vary based on the population and the specific polymorphism examined. Therefore, this study aimed to investigate the link between the rs5743899 polymorphism in the *TOLLIP* gene and susceptibility to CL in a population from Iran. In the current study, logistic regression analysis using various genetic models—including allelic, codominant, dominant, recessive, and over-dominant—revealed no statistically significant association between the rs5743899 polymorphism and predisposition to CL. Specifically, the allelic comparison (T vs C) yielded an OR of 1.43, with a 95% CI of 0.83–2.46 and a p-value of 0.19, indicating no significant increased risk. The codominant model (CT vs TT) and the over-dominant model (CT vs TT + CC) also showed non-significant associations (ORs of 1.12 and 0.98, respectively). For the recessive model (TT vs CC + CT), the OR was 1.29 (95% CI: 0.68–2.41, p = 0.42). Analysis under the dominant model (CC vs TT + CT) was not determined (ND) due to the extremely low frequency of the CC genotype in our study population, which precluded meaningful statistical comparison. Our findings suggest that rs5743899 does not play a substantial role in cutaneous leishmaniasis susceptibility in the studied population.

Interestingly, while our study did not find a significant association, other research has reported varying results regarding the role of *TOLLIP* polymorphisms in Leishmaniasis susceptibility. For instance, a study conducted in Brazil has suggested that different polymorphisms within the *TOLLIP* gene may affect the risk of developing CL, highlighting the potential for population-specific genetic factors to affect disease susceptibility (16). Other research has reported similar results regarding these genetic factors. For instance, a 2020 case-control study in India examined polymorphisms in the *TOLLIP* gene, specifically rs3550920 and rs5743899, and found no association with the rs3550920 polymorphism; however, rs5743899 was suggested as a potential risk factor for VL (17). These discrepancies may stem from differences in environmental factors, parasite species, or genetic backgrounds between populations.

Additionally, the lack of significant findings in our study might reflect the complex interplay of multiple genetic and environmental factors contributing to Leishmaniasis susceptibility. For instance, other polymorphisms in cytokine genes or TLRs may have a more pronounced influence on the immune response to *Leishmania* infection. For example, a 2021 study in India reported that the TLR9 T-1237C polymorphism increased susceptibility to visceral leishmaniasis (18), while another study in Iran found higher TLR2 and TLR4 expression in macrophages from patients with healed *L. major* lesions

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compared with non-healing cases, suggesting that both TLR2 and TLR4 may be important in the outcomes of CL caused by *L. major* (19). In another study conducted in Turkey in 2021, researchers examined the association between cytokine gene polymorphisms and predisposition to CL. They focused on the polymorphisms IL-10-1082 G/A, TNF-α-308 G/A, IL-4 -590 C/T, IFN-γ+874 T/A and IL-12B+1188 A/C. The results revealed that the IL-4-590 C/T and TNF-α-308 G/A were correlated with an increased risk of developing Cutaneous Leishmaniasis (8). The absence of association in our data may also be due to methodological constraints, including the low frequency of the CC genotype and a relatively limited sample size, both of which reduced statistical power to detect modest genetic effects. Furthermore, given that *L. major* is the predominant species in Khuzestan, regional differences in host–parasite interaction and environmental exposure might also contribute to the observed variability. Therefore, the findings of the present study should be interpreted with caution and considered preliminary. Further studies with larger cohorts and functional analyses are warranted to clarify the potential role of the *TOLLIP* rs5743899 polymorphism in cutaneous leishmaniasis susceptibility. Exploring interactions with environmental influences is also essential to gain a deeper understanding of the multifactorial nature of leishmaniasis susceptibility.

In conclusion, our study did not find significant associations between the rs5743899 polymorphism of the *TOLLIP* gene and cutaneous leishmaniasis in the Khuzestan population. Nevertheless, these findings highlight the importance of investigating genetic factors in Leishmaniasis and should be interpreted as preliminary due to the study's limited sample size. Further research with larger cohorts and additional genetic markers is warranted to better understand the immune mechanisms underlying susceptibility to Leishmaniasis and to inform targeted prevention and treatment strategies in endemic regions.

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Authors' contributions

AM and EG designed and supervised the study. FZ collected the data. AM and EG analyzed and interpreted the data. AM wrote the initial draft and finalized the manuscript. All authors wrote and reviewed the final manuscript.

Ethics approval

The ethics committee of Dezful University of Medical Sciences (IR.DUMS.REC.1401.062) approved the study proposal and protocol.

Conflict of Interests

The authors declare that there is no conflict of interest to report in this article.

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Data availability

The data generated and analyzed during the current study are not publicly available but are available from the corresponding author on request.

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Language Editing and AI Disclosure

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