RESEARCH ARTICLE

The role of Tumour Necrosis Factor Alpha (TNF-α) serum level and genetic polymorphisms with cutaneous leishmania infections

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Abstract

Objective: To assess the role of tumour necrosis factor alpha level and genotyping in susceptibility to leishmaniasis.

Method: The case-control study was conducted from March to July 2021 at Baqubah Teaching Hospital, Diyala, Iraq, and comprised patients of cutaneous leishmaniasis in group A and healthy controls in group B. The serum level and single nucleotide polymorphisms of tumour necrosis factor-alpha rs41297589 and rs1800629 were compared between the groups. Data was analysed using SPSS 28.

Results: Of the 150 subjects, there were 75(50%) in group A; 39(52%) males and 36(48%) females with mean age 23.91±13.14 years. The remaining 75(50%) subjects were in group B; 38(50.7%) males and 37(49.3%) females with mean age 22.84±4.35 years. Tumour necrosis factor-alpha level in group A was 55.81±39.64 compared to 7.51±3.61 in group B ($p<0.05$). Single nucleotide polymorphism rs41297589 showed that TT genotype and T allele were significantly increased in group A compared to group B ($p<0.05$), while rs1800629 showed that GA genotype and A allele were significantly increased in group A compared to group B ($p<0.05$). The serum level of tumour necrosis factor-alpha in group A was increased in TT genotype compared to other genotypes at rs41297589, and in GA genotype compared to other genotypes at rs1800629 ($p<0.05$).

Conclusions: There was a significant association between tumour necrosis factor-alpha serum level and genetic polymorphisms rs41297589 and rs1800629 among cutaneous leishmaniasis patients.

Keywords: Polymorphism, Nucleotide, Alleles, Psychodidae, Leishmania, Parasites, Cutaneous, Nucleotides.

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Introduction

Leishmaniasis is a parasitic ailment that impacts both humans and animals and is spread mostly by the bite of an infected phlebotomine sandfly vector.[1] Leishmaniasis has more than 20 different variants, including cutaneous leishmania (CL), mucocutaneous leishmania (ML) and visceral leishmania (VL).[2]

In the sandfly vector, leishmania species live as promastigotes, which are extracellular, flagellated and spindle-shaped organisms. The promastigotes form near the midgut endothelial cells of the sandfly.[3]

Sandflies are haematophagous, which means they inject their saliva containing the parasite into the host during a meal of blood.[4] Dendritic cells (DCs) or epidermal macrophages engulf the injected promastigotes and act as antigen-presenting cells (APCs). Promastigotes are transformed into amastigotes within the phagolysosome, which are round, immotile and non-flagellated.[5]

Amastigotes can persist inside macrophages, multiply via binary fission, and finally tear the infected cells. The released parasites by infected macrophages are then picked up by other macrophages, allowing infection to spread throughout the host.[6]

The sandfly ingests the released amastigotes after a blood meal on an infected host, and this changes back into flagellated spindle-shaped promastigotes within the fly midgut, completing the parasite's lifecycle.[7]

Approximately 300 million people live in places where leishmaniasis is endemic, in addition to the millions of new cases and thousands of fatalities related to the disease each year.[8] Furthermore, the disease, especially leishmaniasis L major, L. Mexicana and L. tropical, produces CL, which is the most frequent type of illness. The development of skin lesions, papules and ulcers, at the location of the sandfly bite characterises this version of the disease, which is normally self-resolving.[9]

The insect vector deposits meta-cyclic promastigotes in the skin of its host during the blood meal. The infection is started by these promastigotes, which are the most aggressive form of leishmania.[10]

After a variable incubation period, a little erythema develops at the location of the sandfly bite, which is the first evidence of infection. Then erythema transforms into

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a papule, then a nodule, which gradually ulcerates over a period of 2-6 months, eventually forming the distinctive lesion of localised CL.[6]

Once inside the skin, the parasites are exposed to novel microenvironments, such as connective tissue's extracellular matrix (ECM), and must interact with various barriers, including basement membrane proteins, before infecting macrophage phagolysosomes.[11-12] The many clinical symptoms of leishmaniasis are currently known to be depending on the parasite type and the state of the host's immune system.[12]

These cells are stimulated during infection, and tumour necrosis factor-alpha (TNF-α) and interferon (IFN) are generated in high amounts by both peripheral blood mononuclear cells and macrophages. This response, however, might result in tissue damage and the development of an ulcer.[13]

TNF-α is an inflammatory cytokine that plays a role in immune modulation and microbe resistance. In CL patients, high quantities have been reported[14-15]. TNF-α contributes to the inflammatory response by causing nitric oxide production, necrosis, cytotoxicity, and the development of matrix metalloproteinases (MMPs)[16-17].

The current study was planned to assess the role of TNF-α and genotyping in susceptibility to leishmaniasis.

Patients and Methods

The case-control study was conducted from March to July 2021 at Baqubah Teaching Hospital, Diyala, Iraq, and comprised CL patients in group A and healthy controls in group B. The samples were collected by random sampling technique after taking informed consent from all the participants. The study was approved, according to the Helsinki declaration,[18] by the ethics review committees of the University of Baghdad and the University of Diyala, Iraq.

The sample size was determined using G*Power calculator with two-tailed 1-β error probability 0.91, effect size 0.5 and two-tailed alpha probability 0.089.[19]

All the participants were from urban and rural regions of Diyala who visited the Baqubah Teaching Hospital for diagnosis and treatment. They were asked about the presence of chronic and inflammatory diseases, and those with such diseases were excluded.

Demographic and clinical data, including age, gender, area of residence, lesion type and shape, was gathered. Venous blood 5ml was collected from each participant; 2ml was kept in an ethylenediaminetetraacetic acid (EDTA) tube for genetic variation of TNF-α single nucleotide polymorphisms (SNPs) rs41297589 and rs1800629, while 3ml was put in a silicone gel tube and allowed to coagulate for getting the serum to assess TNF-α level in the sera.

The whole genomic deoxyribonucleic acid (DNA) was extracted using an extraction kit (Intron Company, South Korea) according to the manufacturer's guidelines. The purity of the extracted DNA reached 1.7-2.0, while the concentration was recorded to be 50-100ng/ml calculated using nanodrop.

The primers were designed using the National Center for Biotechnology Information (NCBI)[20] primer blast online resource and checked through the University of California, Santa Cruz (UCSC)[21] in-silico PCR and primer-blast online resources. The primers (Scientific Researcher Co. Ltd., Ad Diwaniya, Iraq) were prepared as per the manufacturer's instructions (Table 1).

TNF-α SNPs rs41297589 and rs1800629 were subsequently detected. There were 2 Eppendorf tubes for each sample of rs41297589 or rs1800629; 1 tube for the forward primer 1 and common reverse primer, and the second tube for the forward primer 2 and the common reverse primer. To these tubes was added 12.5µl of the master mix (Intron Company, South Korea), 2µl of the extracted DNA, and 2µl of primers (Alpha Integrated Technologies, Canada)(1µl from the forward 1 primer and 1µl from the common reverse primer for the first Eppendorf tube, and 1µl from the froward 2 primer and 1µl from the common reverse primer for the second Eppendorf tube). The final volume was 25µl, with the rest being free nuclease water.

The thermocycler protocol for both TNF-α gene SNPs rs41297589 and rs1800629 included three steps. The first step was primary initiation and included 1 cycle at 95°C for 10m. The second step involved initiation at 95°C for 35s, extension at 55°C for 35s, and elongation at 72°C for 35s. These steps were repeated 40 times each. The final step was 1 cycle at 72°C for 10m. The PCR amplicons' products were visualised through electrophoresis on 1.5% agarose gel stained with the Red Safe stain (Intron Company, South Korea). The bands were then visualised using an ultraviolet (UV) transilluminator.

Data was analysed using SPSS 28[22] and WinPepi 11.65.[23] Data homogeneity and normality was screened. For parametric data, mean and standard deviation values were calculated. For non-parametric data, frequencies and percentages were calculated. In addition, independent t-test, Tukey test and Pearson's chi-square test were used for inter-group cpmparisons, with level of significance being $p<0.05$. The odds ratios (ORs) along with 95% confidence intervals (Cis) and Fisher's exact probability were calculated using WinPepi for genotyping and allele frequencies. Online Hardy-Weinberg calculator was used

for the purpose.[24] The genotyping was done using allele-specific primer-polymerase chain reaction (ASP-PCR) technique.[25-26]

Results

Of the 150 subjects, there were 75(50%) in group A; 39(52%) males and 36(48%) females with mean age 23.91±13.14 years. The remaining 75(50%) subjects were in group B; 38(50.7%) males and 37(49.3%) females with mean age 22.84±4.35 years. TNF-α level in group A was 55.81±39.64 compared to 7.51±3.61 in group B (Table 2).

Genetic variation of TNF-α SNP rs41297589 investigated A and T alleles that corresponded to three AA, AT and TT genotypes (Figure 1).

Genotyping and allele frequencies of TNF-α SNP rs41297589 were compatible with Hardy-Weinberg equilibrium for both groups (Table 3). The TT genotype and T allele was significantly increased in group A compared to group B, while the AT genotype and A allele showed a significantly decreased frequency in group A compared to group B, and the AA genotype was not detected in group A (Table 4).

TNF-α level was significantly increased in AT and TT genotypes in group A compared to group B (Table 5).

For TNF-α SNP rs1800629, genotypes detected were GG, GA and AA (Figure 2).

Genotyping and allele frequencies of TNF-α SNP rs1800629 were compatible with the Hardy-Weinberg equilibrium for both the groups Table 6, while GA and GG genotypes as well A and G alleles were significantly increased in group A compared to group B, while the AA genotype was not detected in group B (Table 7).

TNF-α levels for SNP rs1800629 was significantly increased for GG genotype in group A compared to group B, and the difference was non-significant for the GA genotype (Table 8).

Table-1: Primers and condition of TNF-α genetic polymorphisms rs41297589 and rs1800629.

rs41297589	Sequence (5'->3')	Product length
Forward primer 1	GGAAGTTTTCCGCTGGTTGA	102 bp
Forward primer 2	GGATGTTTTCCGCTGGTTGA	
Reverse primer	TGAGGGAGCGTCTGCTG	
rs1800629		
Forward primer1	CAATAGGTTTTGAGGGGCATGGG	131 bp
Forward primer2	CAATAGGTTTTGAGGGGCATGAG	
Reverse primer	CATCAAGGATACCCCTCACACT	

TNF-α: Tumour necrosis factor-alpha, bp: Base pair.

Table-2: Demographic data.

		Patients group	Control group	Probability
Total samples size		75	75	
Gender	Males (%)	39 (52.0)	38 (50.7)	0.870
	Females (%)	36 (48.0)	37 (49.3)	
Family history	Yes	27 (36.0)	0 (0.0)	9.6 x 10-9
	No	48 (64.0)	75 (100.0)	
Chronic diseases	Yes	12 (16.0)	0 (0.0)	0.00003
	No	63 (84.0)	75 (100.0)	
Other diseases	Yes	1 (1.3)	0 (0.0)	0.316
	No	74 (98.7)	75 (100.0)	
Lesions number (mean ± SD)		2.96 ± 2.09	-	-
Power of samples (1 – β error probability (Two-tailed)				0.91
Effect size				0.5
α error probability (Two-tailed)		0.089		
Age (mean ± SD)		23.91 ± 13.14	22.84 ± 4.35	0.506
TNF-α (pg/ml)		55.81 ± 39.64	7.51 ± 3.61	1.27 x 10-16

The data of gender, family history, chronic diseases, other diseases percentage, the mean of age, lesions numbers, and the power of samples, effect size, α error probability, in addition to the total number of the studied groups mentioned in our previous published study (21)

SD: Standard deviation, TNF-α: Tumour necrosis factor-alpha.

Table-3: Genotyping frequencies of TNF-α rs41297589 between the studied groups.

Genotypes	Patients n (%)		Control n (%)	
	Observed	Expected	Observed	Expected
AA	0 (0.0)	2.08 (2.78)	10 (13.33)	12 (16.0)
AT	25 (33.33)	20.83 (27.78)	40 (53.33)	36 (48.0)
TT	50 (66.67)	52.08 (69.44)	25 (33.33)	27 (36.0)
Total	75 (100.0)	75 (100.0)	75 (100.0)	75 (100.0)
P-HWE	0.0833		0.3359	

TNF-α: Tumour necrosis factor-alpha, P-HWE: Probability of Hardy-Weinberg equilibrium.

Table-4: Genotyping and allele frequencies of TNF-α rs41297589 between the groups.

Genotypes	Patients group n (%)	Control group n (%)	OR (95% CI)	Fisher's exact probability
AA	0 (0.0)	10 (13.33)	0.04 (0.0 – 0.71)	1.4 x 10-3
AT	25 (33.33)	40 (53.33)	0.44 (0.23 – 0.84)	0.021
TT	50 (66.67)	25 (33.33)	4.0 (2.04 – 7.85)	7.8 x 10-5
Total	75 (100.0)	75 (100.0)		
		Allele's frequency		
A	25 (17.0)	60 (40.0)	0.30 (0.18 – 0.51)	1.0 x 10-5
T	125 (83.0)	90 (60.0)	3.33 (1.95 – 5.71)	

TNF-α: Tumour necrosis factor-alpha, OR: Odds ratio, 95% CI: 95% confidence interval.

Table-5: TNF-α levels among rs41297589 single nucleotide polymorphisms (SNPs) between the groups.

Genotypes	TNF- α level mean ± SD (pg/ml) Patients	Control	*p*-value
AA	-	5.71 ± 2.10A	-
AT	40.44 ± 26.63B	7.82 ± 3.70A	0.000038
TT	67.29 ± 41.36A	9.59 ± 5.35A	0.002
Probability	0.022		

Duncan test: the similar letters referred to non-significant differences ($p > 0.05$) among the same groups' genotypes, while the different letters referred to significant differences ($p < 0.05$) among the same groups' genotypes

TNF-α: Tumour necrosis factor-alpha, SD; Standard deviation.

Table-6: TNF-α genotyping frequencies of rs1800629 between the studied groups.

Genotypes	Patients n (%)		Control n (%)	
	Observed	Expected	Observed	Expected
GG	26 (34.67)	23.52 (31.36)	56 (74.67)	57.20 (76.27)
GA	32 (42.67)	36.96 (49.28)	19 (25.33)	19.59 (22.12)
AA	17 (22.67)	14.52 (19.36)	0 (0.0)	1.20 (1.60)
Total	75 (100.0)	75 (100.0)	75 (100.0)	75 (100.0)
p-HWE	0.2452		0.2091	

TNF-α: Tumour necrosis factor-alpha, *p*-HWE: *p*-value of Hardy-Weinberg Equilibrium.

Table-7: TNF-α genotyping and allele frequencies of rs1800629 between the groups.

Genotypes	Patients group n (%)	Control group n (%)	OR (95% CI)	Fisher's exact probability
GG	26 (34.67)	56 (74.67)	0.18 (0.09 – 0.36)	1.4 x 10-6
GA	32 (42.67)	19 (25.33)	2.19 (1.10 – 4.37)	0.038
AA	17 (22.67)	0 (0.0)	45.17 (2.71 – 752.61)	5.5 x 10-6
Total	75 (100.0)	75 (100.0)		
		Allele's frequency		
G	84 (56.0)	131 (87.0)	0.18 (0.10 – 0.33)	1.8 x 10-9
A	66 (44.0)	19 (13.0)	5.42 (3.04 – 9.65)	

TNF-α: Tumour necrosis factor-alpha, OR: Odds ratio, CI: Confidence interval.

Table-8: TNF-α level among rs1800629 single nucleotide polymorphisms (SNPs) between the groups.

Genotypes	TNF- α level mean ± SD (pg/ml) Patients	Control	*p*-value
GG	57.67 ± 16.21A	7.91 ± 4.24A	1.10 x 10-16
GA	58.51 ± 44.92A	5.81 ± 4.18A	0.284
AA	52.45 ± 22.09A	-	-

Duncan test: the similar letters referred to non-significant differences ($p > 0.05$) among the same groups' genotypes, while the different letters referred to significant differences ($p < 0.05$) among the same groups' genotypes

TNF-α: Tumour necrosis factor-alpha.

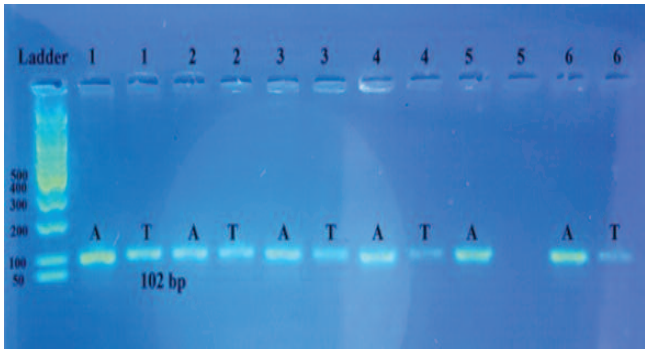

Figure 1: Gel electrophoresis of tumour necrosis factor-alpha (TNF-α) single nucleotide polymorphism (SNP) rs41297589 polymerase chain reaction (PCR) product on 1.5% agarose gel stained with Red Safe stain (Intron company, South Korea) for 45min at 100v. Ladder: Universal deoxyribonucleic acid DNA Ladder (Intron company, South Korea), 1–6: electrophoresed samples' numbers, the single band for the AA and TT genotypes (wild and mutant homozygote, respectively), the double bands for the AT genotype (mutant heterozygote).

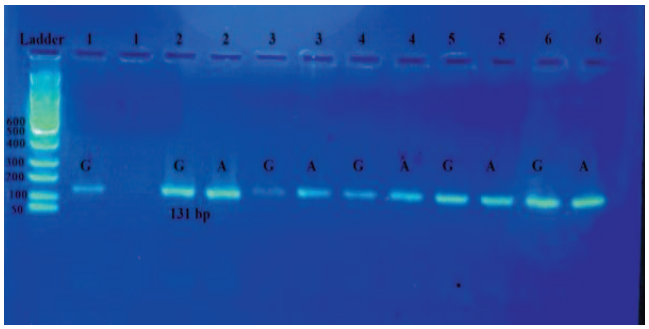

Figure 2: Gel electrophoresis of tumour necrosis factor-alpha (TNF-α) single nucleotide polymorphism (SNP) rs1800629 polymerase chain reaction (PCR) product on 1.5% agarose gel stained with Red Safe stain (Intron company, South Korea) for 45min at 100 voltages. Ladder: Universal deoxyribonucleic acid (DNA) Ladder (Intron company, South Korea), 1–6: electrophoresed samples' numbers, the single band for the GG and AA genotypes (wild and mutant homozygote, respectively), the double bands for the GA genotype (mutant heterozygote).

Discussion

TNF-α is a pro-inflammatory cytokine that plays a key role in initiating and regulating the cascade events that lead to an inflammatory response.[27] The present results showed significantly increased levels of TNF-α in patient group A compared to control group B. This was due to immune response and the role of many cytokines, including TNF-α, related to inflammation resulting from the lesions caused by CL.

The current study focussed on SNPs rs41297589 and rs1800629. The investigation of TNF-α SNP rs41297589 was done for the first time, and it appeared that TT genotyping and T allele frequency was significantly increased in group A compared to group B. In addition, the high OR value of TT genotype and T allele indicated that they might be a relative risk for CL, while the OR values related to AA, AT and A allele were a protective factor against CL. In addition, increasing TNF-α SNP rs41297589 level suggested a relationship with CL in AT and TT genotypes.

In contrast, TNF-α SNP rs1800629 showed that the frequency of GA genotype and A allele were significantly increased in group A compared to group B, while the AA genotype did not appear in group B, and the frequency of AA genotype in CL patients was 22.67%. In addition, the high OR value of GA, AA and A allele indicated that these genotypes and allele were a relative risk for CL, while the OR values of GG genotype and G allele indicated a protective factor against CL.

Furthermore, increased TNF-α SNP rs1800629 levels showed a significant association with GG genotype in CL patients, but the difference was non-significant in GA genotype between the groups.

These results agreed with earlier findings[28-29] related to TNF-

α SNP rs1800629. In a previous study conducted in Iran, the no relationship was established between TNF-α SNP rs1800629 and CL caused by L. major.[30-31] The variation in the genotypes and alleles frequencies of TNF-α SNP rs1800629 in leishmaniasis could be different owing to pathogenicity mechanisms and host responses resulting from infections with different species.[32]

Conclusion

There was a significant association between TNF-α SNP rs41297589 and rs1800629 levels and CL. The association of the relative risk of TT genotype and T allele for rs41297589 and GA, AA genotypes and A allele for rs1800629 was found with CL.

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