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. Leishmaniasis has more than 20 different variants, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL).

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.

Sandflies are haematophagous, which means they inject their saliva containing the parasite into the host during a meal of blood. 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.

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.

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.

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. 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.

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.

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

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

Eman Salman Khamaes, Ali Hafez Abbas, Nagham Yaseen Al-Bayati

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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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)\textsuperscript{20} primer blast online resource and checked through the University of California, Santa Cruz (UCSC)\textsuperscript{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 forward 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\textsuperscript{22} and WinPepi 11.65.\textsuperscript{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 comparisons, 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 genotype and allele frequencies. Online Hardy-Weinberg calculator was used

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,\textsuperscript{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.\textsuperscript{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
for the purpose. The genotyping was done using allele-specific primer-polymerase chain reaction (ASP-PCR) technique.

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.

<table>
<thead>
<tr>
<th>rs41297589</th>
<th>Sequence (5’&gt;3’)</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer 1</td>
<td>GGAAGTTTTCCGCTGGTTGA</td>
<td>102 bp</td>
</tr>
<tr>
<td>Forward primer 2</td>
<td>GGATGTTTTCCGCTGGTTGA</td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TGAGGAGGCCTCCTGCTG</td>
<td></td>
</tr>
<tr>
<td>rs1800629</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer 1</td>
<td>CAATAGGGTTTGGGGGCTATGG</td>
<td>131 bp</td>
</tr>
<tr>
<td>Forward primer 2</td>
<td>CAATAGGGTTTGGGGGCTAGAG</td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CATCAAGATACCCCTCCACT</td>
<td></td>
</tr>
</tbody>
</table>

TNF-α: Tumour necrosis factor-alpha, bp: Base pair.
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.\textsuperscript{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\textsuperscript{28,29} related to TNF-α.
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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References


