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Research Article

The Cytotoxic T Lymphocyte Antigen-4 +49A/G Single Nucleotide Polymorphism Association With Visceral Leishmaniasis

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Background: Several lines of evidence approve that innate and adaptive immunity play key roles in the defense against visceral leishmaniasis (VL). The polymorphism within the cytotoxic T lymphocyte antigen 4 (CTLA-4) gene alters its expression.

Objectives: The main aim of this study was to evaluate the polymorphism within the +49 position of the CTLA-4 gene of Iranian patients with VL in comparison with healthy controls.

Materials and Methods: In this cross-sectional study, 88 patients with clinical presentations of VL, who were seropositive for *Leishmania* (group 1), 86 patients without clinical presentations but seropositive (group 2), and 115 healthy controls (group 3) were assessed with respect to the CTLA-4 +49A/G polymorphism, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The anti-*Leishmania* antibody titration was evaluated using an immunofluorescence method.

Results: Our results indicated that both CTLA-4 +49A/G polymorphisms were significantly associated with VL.

Conclusions: According to the results, the polymorphisms within the +49 position of CTLA-4 can be associated with VL and may be considered as risk factors for the disease.

Keywords: Visceral Leishmaniasis; CTLA-4; Polymorphism

1. Background

Several *Leishmania* species are responsible for leishmaniasis, a vector-borne parasitic disease (1). Previous investigations demonstrated that although the sand fly vector is the main cause of *Leishmania* transmission into humans (1), the parasite can also be transmitted via blood transfusions (2), needlestick injury (3), or the mother/child route during pregnancy (4). Various clinical manifestations, including cutaneous, mucocutaneous, and visceral forms of the disease (5) are plausible after *Leishmania* infection, among which the visceral form (also known as Kala Azar) is the most severe form that can be lethal if left untreated (6). The quality and quantity of host immune system against the infection determines the final clinical outcome and severity of the disease (7).

T lymphocytes play key roles in combating against leishmaniasis via cytokine production (T-helper cells) and killing the parasite (8). Cytotoxic T lymphocyte antigen 4 (CTLA-4) is expressed on activated T cells and interacts with CD80 and CD86, which also are ligands for CD28, resulting in inhibition of T lymphocytes functions

(9). Based on its important roles in T lymphocyte suppression, it appears that CTLA-4 plays key roles in inducing peripheral tolerance against self and foreign antigens, which are seen in self-tolerance and chronic infections, respectively. Therefore, genetic variations that lead to up-regulation of this molecule can be associated with immune suppression and chronic infections, including visceral leishmaniasis (VL). Previous studies showed that the CTLA-4 gene contained several polymorphisms including CTLA-4 A/G and T/C at the +49 position (reference single nucleotide polymorphisms (SNP): (rs) 231775) (10). Additionally, CTLA-4 +49 A allele resulted in increase of CTLA-4 expression (11, 12). Hence, it appears that this allele increases the inhibitory function of CTLA-4 on T cell proliferation and leads to attenuated immune responses against infections.

2. Objectives

Due to the fact that the quality and quantity of immune responses identify the outcome of leishmaniasis, the

main aim of this study was to evaluate the CTLA-4 +49A/G polymorphism in seropositive patients with VL with and without clinical presentation, in comparison with healthy controls.

3. Materials and Methods

3.1. Subjects

In this cross-sectional study, 88 patients with VL with antibody against *Leishmania* (seropositive) (group 1), 86 seropositive healthy controls (group 2), and 115 seronegative healthy controls (group 3) were recruited for sample donation. VL was diagnosed by an expert specialist according to laboratory findings, medical histories, and clinical presentations. The patients with VL as well as both groups 2 and 3 were selected from Meshkin city (north-west of Iran), where *Leishmania infantum* is endemic (13-15), while other species have not been reported. The Ethical Committee of the Ardabil University of Medical Sciences approved the study protocol and the participants filled out informed consent forms prior to entrance in the study.

3.2. DNA Extraction

Genomic DNA was purified from peripheral blood samples, which were obtained from the participants in ethylene diamine tetra acetic acid (EDTA) pretreated tubes, using a commercial kit from Bioneer Company (South Korea) according to the manufacturer's instructions.

3.3. Polymorphism Detection

The CTLA-4 +49A/G gene polymorphism was examined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. PCR was performed in a final volume of 20 μ L, containing 0.3 μ L Taq DNA polymerase (5 U), 2 μ L Taq DNA polymerase buffer (10x), 2 μ L of each primer (25 ng/ μ L), 1 μ L of each dNTP [(dATP, dCTP, dGTP, dTTP) at a concentration of 10 mM], 2 μ L MgCl₂ (stock concentration: 1.5 mM), 1 μ L of prepared DNA and sterile double distilled DNase free water. All the materials were purchased from Cinna Clone company (Iran). Sequences of forward and reverse primers (Cinna Clone, Iran) for amplification of the CTLA-4 +49A/G-containing region (162 bp) were 5'- GCTCTACTTCTGAAGACCT-3' and 5'- AGTCTCACTCACCTTTGCGAG-3', respectively. The following program was used for PCR amplifications: 95°C for five minutes (denaturation), followed by 35 cycles of 30 seconds at 95°C, 53°C for 30 seconds, and 72°C for 40 seconds, using a thermal cycler (Bioneer, South Korea).

BbvI restriction enzyme (Cinna Clone, Iran) was obtained from Fermentas Company (Finland) and was used to recognize the polymorphism at the +49 position. BbvI digested the PCR products of CTLA-4 +49A/G-containing position (162 bp), in the case of G allele, into 91 bp and 71 bp sub-fragments. RFLP was performed according to the

manufacturer's guidelines. Briefly, 2 U of BbvI restriction enzyme and 5 μ L of its associated buffer were added to 10 μ L of the PCR products and incubated for 16 hours in room temperature. A 2.5% pretreated ethidium bromide agarose gel (CinnaClone, Iran) was used for electrophoresis of the PCR products and RFLP digestion products.

3.4. Immunofluorescence Assay

The titration of the anti-*Leishmania* antibody was analyzed using a commercial kit from QIAGEN Company (USA), according to the manufacturer's guidelines. Briefly, a serial dilution of each participant's serum was prepared and the latest reacted concentration was determined as the anti-*Leishmania* titration.

3.5. Statistical Analysis

Hardy-Weinberg equilibrium analysis was used for evaluating the validity of the data. χ^2 test was used from the SPSS software version 13 to identify the differences between the groups and P value less than 0.05 was considered significant.

4. Results

Our results indicated that CTLA-4 +49A/G polymorphism was significantly associated with VL ($P < 0.001$). The results from the PCR-RFLP analysis demonstrated that the CTLA-4 +49A/G genotype significantly increased in group 1 compared to both groups 2 and 3. The results in detailed are presented in Table 1 and Table 2. The statistical analysis demonstrated that the differences between the groups regarding the genotypes and alleles were significant (Tables 1 and 2). The current results also demonstrated that the patients with VL (group 1) carrying the CTLA-4 +49A/G genotype had higher mean anti-*Leishmania* antibody titers; the statistical analysis revealed that the difference was significant (Table 3) ($P < 0.001$). The results also demonstrated that the titration of anti-*Leishmania* antibody was significantly higher in group 3 participants carrying the CTLA-4 +49A/G genotype than CTLA-4 +49A/A and G/G genotypes (Table 3) ($P < 0.001$).

Table 1. The Prevalence of CTLA-4 +49A/G Polymorphism Among Patients With Clinical Presentation of VL who Were Seropositive for *Leishmania* (Group 1), Without Clinical Presentation but Seropositive (Group 2), and healthy controls (Group 3)^{a,b}

	Group 1	Group 2	Group 3
Genotypes			
A/A	33 (37.5)	52 (60.5)	48 (41.7)
A/G	48 (54.5)	22 (25.6)	22 (19.1)
G/G	7 (8)	12 (14)	45 (39.1)
Alleles			
A	114 (64.8)	126 (73.3)	118 (51.3)
G	62 (35.2)	46 (26.7)	112 (48.7)

^a Abbreviations: A, adenine; G, guanine.

^b Data are presented as No. %

Table 2. Comparisons of Group 1 versus Group 2 and Group 1 Versus Group 3 ^a

Genotypes	Group 1 vs Group 2		Group 1 vs Group 3	
	OR (95% CI)	P Value	OR (95% CI)	P Value
A/G-G/G	3.74 (1.29-10.79)	0.013	14.02 (5.46-36),	P = 0.0001
A/A-G/G	1.09 (0.38-3.04)	0.87	4.42 (1.77-10.99)	P = 0.001
A/A-A/G	0.29 (0.15-0.56)	0.0001	0.31 (0.16-0.61),	P = 0.001

^a Abbreviations: A, adenine; G, guanine; vs, versus.

Table 3. Anti-*Leishmania* Antibody Titration in Groups 1 and 2 Carrying Various CTLA-4 +49 Genotypes ^{a,b,c,d}

	Anti- <i>Leishmania</i> Antibody Titration			P Value
	CTLA-4 +49-A/A	CTLA-4 +49-A/G	CTLA-4 +49-G/G	
Group 1	2.3 ± 0.273 ^e	4.98 ± 0.216	1.86 ± 0.508	P < 0.001
Group 2	1.88 ± 0.14 ^f	3.32 ± 0.24	0.75 ± 0.15	P < 0.001

^a Since groups 1 and 2 had anti-*Leishmania* antibody, the relationship between the anti-*Leishmania* antibody titration and CTLA-4 +49 genotypes were performed within groups 1 and 2.

^b Data are presented as labeled mean ± standard error.

^c The titrations were labeled with the following numbers: 0: no antibody, 1: 1/10, 2: 1/20, 3: 1/40, 4: 1/80, 5: 1/160, and 6: 1/320.

^d Group 1, seropositive patients with VL; group 2, seropositive healthy controls.

^e The differences between CTLA-4 +49-A/G and A/A genotypes were significant.

^f The differences among CTLA-4 +49-A/A, A/G and G/G genotypes were significant.

5. Discussion

CTLA-4 is expressed on activated T lymphocytes and binds to CD80 and CD86, which results in suppression of the T lymphocyte function (16). Ectopic up-regulation of CTLA-4 during infectious diseases results in impaired immune responses and consequently induces chronic forms of infectious diseases (17). Previous studies demonstrated that expression of CTLA-4 was regulated by the polymorphisms within the exon 1 (+49 position) region of the CTLA-4 gene (11, 12). Our results showed that either genotypes or alleles of CTLA-4 +49A/G polymorphism were significantly associated with VL in the Iranian population. Additionally, the current results revealed that the CTLA-4 +49-A/G genotype was significantly increased in group 1 compared to groups 2 and 3. As mentioned in the introduction section, the CTLA-4 +49 A allele was associated with higher expression of CTLA-4; hence, it seems that higher prevalence of CTLA-4 +49-A/G genotype in Iranian patients with VL may be responsible for impaired immune responses against *Leishmania* in patients with VL.

Previous studies demonstrated that CTLA-4 plays a crucial role in suppression of immune responses against *Leishmania* during VL (18). Elevation levels of CTLA-4 in patients with VL were also reported by Katara and colleagues (19). Studies on animal models also revealed that CTLA-4 suppresses the immune responses against *Leishmania* via inducing the transforming growth factor- β (TGF- β), an anti-inflammatory cytokine (20). Murphy et al. showed that using anti-CTLA-4 antibody for blocking CTLA-4 led to enhanced host resistance to *L. donovani* (21). Murray and colleagues also identified that using anti-CTLA-4 antibody in *L. donovani* visceraally infected animals

led to parasite killing by the immune cells (22). Another study by Saha et al. demonstrated that CTLA-4 may play key roles in regulation of T-helper 1/T-helper 2 balance, which is important to determine the leishmaniasis outcome (23). Another study by Zubairi and colleagues revealed that immunotherapy with anti-CTLA-4 increased the immune responses, which resulted in killing *L. donovani* (24). Therefore, based on the aforementioned studies, it seems that overexpression of CTLA-4 can be considered as a main reason for impaired immune responses, in which, different CTLA-4 +49 genotypes may be responsible for up-regulation of this molecule in VL.

To the best of our knowledge this was the first study evaluating the CTLA-4 +49A/G polymorphism in leishmaniasis. However, there have been several studies on other chronic infections. For example, Danilovic et al. demonstrated that CTLA-4 +49A/G polymorphism was significantly associated with chronic hepatitis C infection (25). Duan and colleagues reported that patients with chronic HBV infection had higher frequencies of CTLA-4 +49-A/A genotype and also A allele (26). Raitala et al. evaluated the activity of *Helicobacter pylori* (HP), inducing indoleamine 2,3-dioxygenase, in patients with chronic HP infections and found that the activity significantly increased in patients carrying CTLA-4 +49-A/A genotype (17). Therefore, based on the aforementioned study, CTLA-4 +49-A/G polymorphism can be significantly associated with chronic infections. These results confirmed the fact that the CTLA-4 +49A/G polymorphism plays a key role in VL pathogenesis.

Our results also showed that the CTLA-4 +49A/G polymorphism was significantly associated with anti-*Leish-*

mania antibody titrations in groups 1 and 2 participants; hence, this polymorphism can be associated with antibody production against *Leishmania* in Iranian patients. The results demonstrated that anti-*Leishmania* antibody titration significantly increased in either group 1 or 2 with A/G genotype. Therefore, it appears that A/G genotype not only can be considered as a risk factor for VL, but can be associated with higher antibody production against *Leishmania*, which may be related to shift immune responses of T-helper 2 immunity and consequently impaired cellular immunity. Finally, based on our results, it seems that the CTLA-4 +49A/G polymorphism is significantly associated with VL as well as antibody production against *Leishmania* in Iranian patients with VL.

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Authors' Contributions

All the authors were involved in all the steps of manuscript preparation.

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