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Short Communication

TGF-β1 polymorphism in American tegumentary leishmaniasis in a Southern Brazilian population

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Abstract

Introduction: Genetic polymorphisms define the cytokine production leading to susceptibility or resistance to diseases. We studied the cytokine polymorphism in the development of tegumentary leishmaniasis (TL). **Methods:** Genotyping of TNF-α, TGF-β1, IFN-γ, IL-6, and IL-10 were performed by polymerase chain reaction assay. **Results:** G and C alleles of TGF- β1 (codon 25) were the most common in controls and patients, respectively. G/G was the most frequent genotype in controls, and G/C and C/C in patients. **Conclusions:** G/G genotype of codon 25 in TGF-β1 appeared to confer resistance, and G/C and C/C genotypes, susceptibility to TL in this population.

Keywords: Cutaneous leishmaniasis. Genotyping. Gene polymorphisms. Cytokines.

American tegumentary leishmaniasis (ATL) is an endemic disease prevalent in all Brazilian states. In recent years, the number of cases has been decreasing according to the Brazilian Ministry of Health[1]. During the latest survey conducted in 2016, about 12,690 cases of ATL were recorded[1].

ATL is a serious public health problem, not only due to high incidence and wide geographical distribution, but also due to the possibility of development of the lesions into destructive, disfiguring and disabling manifestations with significant psychosocial repercussions. Additionally, the treatment is complicated and expensive[2].

The etiologic agent of ATL is a protozoan of the genus *Leishmania*. In Brazil, the most frequently found parasitic species are *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis*, among which *L. (V.) braziliensis* is responsible for causing mucocutaneous leishmaniasis[2].

Upon infection, a complex interaction occurs between the parasite and the host immune system that determines the clinical manifestation and progression of ATL. Clinical and experimental pieces of evidence have suggested that *Leishmania*-specific immune responses are fundamentally mediated by T cells[3]. Cytokines associated with a Th1 profile may be present at concentrations sufficient to regulate the immune response, activate macrophages and locally control the infection[3]. According to Oliveira et al.[4], the inflammatory mediators IFN-γ and TNF-α, help to monitor the replication of intracellular pathogens and to eliminate parasites in the host. On the other hand, as an escape mechanism, parasites can induce the production of TGF-β1, blocking IFN-γ, inhibiting lymphocyte proliferation and cytokine production, and affect immune response to cutaneous leishmaniasis[4], as Th2-type cytokines, such as IL-10, that may decrease the production of inflammatory cytokines in cutaneous and mucosal leishmaniasis[4].

Some studies have emphasized that host genetic factors play a vital role in the immune-regulation of infectious diseases.

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Several genetic polymorphisms may explain the variations in production of individual cytokines that lead to different immune responses, and therefore, determine the susceptibility or resistance to diseases[5]. Abnormalities in the regulatory region of the *IL-6* gene, were shown to be important risk factors for mucosal leishmaniasis, and polymorphisms in the TNF-α and IL-10 genes were suggested to interfere with the secretion of IFN-γ in ATL patients[5,6].

Accordingly, the objective of the study was to analyze single nucleotide polymorphisms (SNPs) for genes that encode cytokines: TGF-β1 (10T/C, 25G/C) codons, IFN-? (+874T/A), IL-10 (-1082A/G, -819T/C, -592A/C) promoters, IL-6 (-174G/C) promoter, and TNF-α (-308G/A) promoter, in individuals with ATL from Southern Brazil.

In this case-control and retrospective study, 2 groups were established based on a review of medical records, by specifically focusing on the epidemiological data from diagnosed ATL patients who underwent treatment 2 years ago, and who live in endemic areas of the North and Northwest regions of the Southern state of Paraná, Brazil.

The groups were as follows: 1) ATL group composed of 102 patients (83 men and 19 women, aged between 20 and 70 years). They had a confirmed diagnosis of ATL according to the criteria of the Ministry of Health (Brazil), in addition to clinical, positive parasitological and/or immunological parameters (direct research on *Leishmania* spp., indirect immunofluorescence antibody test, and Montenegro skin test) compatible with ATL. Patients (84/102) had cutaneous lesions, 12/102 mucocutaneous lesions and 6/102 mucosal lesions, received therapy and live in Southern Brazil. 2) The control group composed of 150 healthy volunteers (79 men and 71 women, aged between 20 and 50 years). They did not show clinical manifestations of ATL, presented negative indirect immunofluorescence antibody test and lived in the same geographical area at risk of infection (areas of arboreal vegetation and presence of streams) from which the patients were selected. Ethnicity for both groups was also analyzed.

Peripheral blood samples (10 mL) were collected by venipuncture in vacuum tubes containing EDTA as an anticoagulant. Samples were centrifuged at 960 × *g* for 10 minutes to obtain leukocyte sedimentation (buffy coating) and were frozen at -80°C until use.

Genomic DNA was extracted from leukocytes using the spin-column method of the BIOPUR® kit (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were evaluated using a NanoDrop™ device (Thermo Fisher Scientific, Wilmington, DE, USA).

Genotyping for cytokines TNF-α, TGF-β1, IFN-γ, IL-6, and IL-10 was performed using the polymerase chain reaction with sequence-specific primers and Cytokine Genotyping Primer Pack® (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer's instructions. After electrophoresis, the gel was photographed and interpreted, and data were recorded using the worksheet provided by the manufacturer in the cytokine genotyping kit.

The results were entered into an Excel 2010® worksheet. Statistical analyses were performed using STATISTICA software version 7.0 (TIBCO Software Inc., Palo Alto, CA, USA) via Student's *t*-test, chi-square test and Fisher's exact test. The chi-square test and Fisher's exact test were used to test the association between variables, phenotypes, genotypes, and allele frequencies in patients and controls. $P \leq 0.05$ was considered significant for all statistical analyses.

Among the 252 subjects involved in this study, 102 patients (83 men, 81.4%) and 150 controls (79 men, 52.7%), presented a mean age of 46.44 and 32.9 years ($p < 0.01$), respectively. The proportion of mixed ethnic individuals did not present a significant difference in both groups ($p = 0.20$). The proportion of Caucasians was 123 (82.0%) for the controls and 75 (73.5%) for the patients; of mestizos was 23 (15.3%) for the controls and 25 (24.5%) for the patients; and of blacks was 4 (2.7%) for the controls and 2 (2.0%) for the patients.

The Brazilian population is one of the most heterogeneous in the world, consisting of various ethnic groups[7] and the frequencies of HLA alleles may vary according to the predominant ethnic group in a region[8, 9]. The samples tested in this study were from the state of Paraná in southern Brazil. Southern Brazil was settled mainly by Europeans, but also has significant numbers of people of African descent and Native Americans in the population[8].

The frequencies of alleles, genotypes, and phenotypes in patients and controls are shown in **Tables 1, 2, and 3**, respectively. Statistically significant associations were not found between the IL-10 gene SNP and susceptibility in either patients or controls. This finding is supported by previous studies that describe cytokine genotyping and its association with post-kala-azar dermal leishmaniasis, a skin condition that usually develops after the treatment of visceral leishmaniasis[10].

Salhi et al.[11] demonstrated that the -819C / C polymorphism located in the IL-10 promoter region might be related to the increased expression and production of this cytokine and, consequently leads, to a higher risk of lesions in ATL patients. This polymorphic IL-10 may act in synergy with TGF-β1, to decrease the antimicrobial activity of the macrophages at the wound site[11].

Although IFN-γ and TNF-α appear to play a significant role in the control of intracellular parasite replication[3], the results of the present analysis of these genes revealed that SNPs have no influence on the infection.

In fact, our results corroborate those reported by Samaranayake et al.[12] according to which SNPs of the TNF-α gene may not influence susceptibility to cutaneous leishmaniasis in the population of Sri Lanka. Another study evaluating SNPs of the TNF-α gene in tuberculosis patients, has also failed to establish this polymorphism as a risk factor for infection[13]. However, there is evidence of an association between SNPs of the TNF-α -308G / A promoter region and mucocutaneous leishmaniasis[14]. Besides, studies that involved dengue patients, showed the association between the TNF-α -308G / G genotype and resistance against the development of hemorrhagic dengue[15].

TABLE 1: Distribution of allele frequencies of cytokine genes in patients with ATL and controls.

Gene (SNP map position)	Allele	Patients (n=102)	Controls (n=150)	p*
TNF-α (-308)	G	180 (88.2%)	260 (86.7%)	0.60
	A	24 (11.8%)	40 (13.3%)	
IFN-γ (+874)	T	77 (37.7%)	130 (43.3%)	0.21
	A	127 (62.2%)	170 (56.7%)	
IL-6 (-174)	G	132 (64.7%)	212 (70.7%)	0.16
	C	72 (35.3%)	88 (29.3%)	
TGF-β1 (codon 10)	T	126 (61.8%)	173 (57.7%)	0.36
	C	78 (38.2%)	127 (42.3%)	
TGF-β1 (codon 25)	G	174 (85.3%)	278 (92.7%)	**0.01**
	C	30 (14.7%)	22 (7.3%)	
IL-10 (-1082)	G	74 (36.3%)	109 (36.3%)	0.99
	A	130 (63.7%)	191 (63.7%)	
IL-10 (-819)	C	142 (69.6%)	206 (68.7%)	0.82
	T	62 (30.4%)	94 (31.3%)	
IL-10 (-592)	A	62 (30.4%)w	94 (31.3%)	0.82
	C	142 (69.6%)	206 (68.7%)	

*Chi-square test of homogeneity or Fisher's exact test.

TABLE 2: Distribution of genotype frequencies of cytokine genes in patients with ATL and controls.

Gene (SNP map position)	Genotype	Patients (n=102)	Controls (n=150)	p*
TNF-α (-308)	G/G	79 (77.4%)	112 (74.7%)	0.90
	G/A	22 (21.6%)	36 (24.0%)	
	A/A	1 (1.00%)	2 (1.3%)	
IFN-γ (+874)	T/T	15 (14.7%)	33 (22.0%)	0.35
	T/A	47 (46.1%)	64 (42.7%)	
	A/A	40 (39.2%)	53 (35.3%)	
IL-6 (-174)	G/G	42 (41.2%)	77 (51.3%)	0.285
	G/C	48 (47.1%)	58 (38.7%)	
	C/C	12 (11.8%)	15 (10.0%)	
TGF-β1 (codon 10)	T/T	41 (40.2%)	48 (32.0%)	0.37
	T/C	44 (43.1%)	77 (51.3%)	
	C/C	17 (16.7%)	25 (16.7%)	
TGF-β1 (codon 25)	G/G	74 (72.5%)	128 (85.3%)	**0.01**
	G/C	26 (25.5%)	22 (14.7%)	
	C/C	2 (2.0%)	0 (0.0%)	
IL-10 (-1082)	G/G	18 (17.6%)	21 (14.0%)	0.47
	G/A	38 (37.2%)	67 (44.7%)	
	A/A	46 (45.1%)	62 (41.3%)	
IL-10 (-819)	C/C	50 (49.0%)	73 (48.7%)	0.92
	C/T	42 (41.2%)	60 (40.0%)	
	T/T	10 (9.8%)	17 (11.3%)	
IL-10 (-592)	A/A	10 (9.8%)	17 (11.3%)	0.92
	A/C	42 (41.2%)	60 (40.0%)	
	C/C	50 (49.0%)	73 (48.7%)	

* Chi-square test of homogeneity or Fisher's exact test.

TABLE 3: Distribution of phenotype frequencies of cytokine genes in patients with ATL and controls.

Gene	Phenotypes	Patients (n=102)	Controls (n=150)	p*
TNF-α	High	23 (22.5%)	38 (25.3%)	0.61
	Low	79 (77.4%)	112 (74.7%)	
IFN-γ	High	15 (14.7%)	33 (22.0%)	0.35
	Intermediate	47 (46.1%)	64 (42.7%)	
	Low	40 (39.2%)	53 (35.3%)	
IL-6	High	90 (88.2%)	135 (90.0%)	0.66
	Low	12 (11.8%)	15 (10.0%)	
TGF-β1	High	64 (62.7%)	113 (75.3%)	0.08
	Intermediate	30 (29.4%)	27 (18.0%)	
	Low	8 (7.8%)	10 (6.7%)	
IL-10	High	18 (17.6%)	21 (14.0%)	0.47
	Intermediate	38 (37.2%)	67 (44.7%)	
	Low	46 (45.1%)	62 (41.3%)	

* Chi-square test of homogeneity or Fisher's exact test

During the analysis of association between IFN-γ +874T/A gene polymorphism (**Table 1**), susceptibility or resistance against ATL development was not observed (statistically significant differences were not found in the frequencies of IFN-γ alleles between patients and controls). Similar results were reported previously, which suggest that the IFN-γ +874T/A SNP is not associated with the susceptibility or severity of cutaneous leishmaniasis, in spite of the influence of this cytokine on the development of the disease[5]. Matos et al.[5] reported that IFN-γ +874 T/A SNP would be involved in the pathogenesis by influencing the high cytokine levels in cutaneous leishmaniasis. In dengue hemorrhagic fever, significant differences were not found in the frequencies of phenotype, genotype and allele of the IFN-γ between patients and controls[15], as was also observed in our study.

Induction of a Th2-type immune response may increase the production of cytokines, such as IL-6, inhibiting the activation of macrophages and their antimicrobial activity and thus promoting the clinical manifestation of leishmaniasis[6]. These studies have identified an association of G/G, G/C and C/C genotypes of IL-6 with decreased or increased levels of this cytokine[6]. According to Castellucci et al.[6], the IL-6 -174C allele may be a risk factor for mucocutaneous leishmaniasis, but does not show influence on susceptibility to cutaneous leishmaniasis; these findings were similar to our results regarding the differences between patients with ATL and controls.

The frequencies for the cytokine alleles of TNF-α, IFN-γ, IL-10, and IL-6 were not statistically different between patients and controls (p>0.05). However, even though the TGF-β1 codon 10 polymorphism was not significantly different between patients and controls, the TGF-β1 codon 25 polymorphism was the most frequent among the sample population that was evaluated in our study. For the TGF-β1 codon 25G was the most common allele in the controls which was statistically different from the observation in the patient group, where the most common allele was C (p=0.01; **Table 1**).

The genotype frequencies (**Table 2**), showed significant variation (p=0.05) for TGF-β1, but not for other cytokines. In our TGF-β1 gene polymorphism analysis, the controls had a high frequency of the G allele and G/G genotype, while the patients had a high frequency of the C allele and G/C and C/C genotypes at codon 25. Statistically significant variations were found at codon 25 between C and G alleles (p = 0.01) as well as among G/G, G/C, and C/C genotypes (p = 0.01), as described in **Tables 1 and 2**, respectively. Therefore, the high frequency of C allele at codon 25 of the TGF-β1 gene seems to suggest susceptibility, whereas the high level of the G allele appears to indicate resistance to ATL.

The TGF-β1 polymorphism has been associated with various other clinical conditions such as chronic periodontitis. In a study on conditions other than ATL, the C allele at codon 25 in the TGF-β1 gene appeared to act as a preventive factor against hepatitis C viral infection[16]. Babel et al.[17] demonstrated that patients with periodontitis showed higher frequency of TGF-β1 25G/G genotype compared to controls, which suggests a possible association of the susceptibility to chronic periodontitis with this genotype. However, in a study performed in Cuba, the TGF-β1 25G/G polymorphic codon was associated with resistance against dengue hemorrhagic fever[15].

Frade et al.[18] identified a relationship between the polymorphism of the -509 T allele of the TGF-β1 gene and the risk of developing visceral leishmaniasis in an endemic

area in Brazil. However, no reports have been found for this polymorphism of TGF-β1 cytokine in ATL.

Phenotypic frequencies did not present a significant difference for the genes encoding TNF-α, IFN-γ, IL-10, IL-6, and TGF-β1 among individuals with or without ATL (p> 0.05) (**Table 3**).

Individual variations in cytokine expression are genetically determined, and the polymorphisms that involve gene regulatory regions are important to establish a low, medium or high production of these molecules. Multiples studies suggested an association between the cytokine gene polymorphisms and the immune-regulatory profile as well as patient susceptibility for various infectious diseases[5].

The limitations of this study consist particularly in the use of small sample sizes, and in the difficulties in obtaining other relevant information. Despite these limitations, this study provided important data on SNP analysis that may become a useful tool in the identification of high or low risk for the development of ATL in patients.

In conclusion, according to our data, genotype G/G and genotypes G/C and C/C at codon 25 of TGF-β1 seemed to confer resistance and susceptibility to the development of ATL, respectively, in the sample population assessed in this study. SNP analysis may become a useful tool in the identification of high or low risk for developing ATL in patients.

Ethical considerations

The present study was approved by the Ethics Committee on Human Research of the Universidade Estadual de Maringá (protocol nº 153/2009) and agrees with the Declaration of Helsinki of 1964. All volunteers have signed up a written consent form prior to the data collection and inclusion as subjects in the study.

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