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Polymorphisms in tumor necrosis factor genes and susceptibility to visceral leishmaniasis in Moroccan children

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors evaluated VL is the most severe form of leishmaniasis. Also they studied *TNF* production with susceptibility to intracellular infectious diseases. This study aimed to examine whether polymorphic alleles at these two loci are involved in the susceptibility to VL in Moroccan children.
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ABSTRACT

Objective: To examine whether polymorphic alleles at these two loci are involved in the susceptibility to visceral leishmaniasis (VL) in Moroccan children.
Methods: We have genotyped polymorphisms by PCR-restricted fragment length polymorphisms in 102 patients with VL, 92 asymptomatic carriers [positive skin test delayed-type hypersensitivity (DTH+)] and 40 healthy controls (negative skin test delayed-type hypersensitivity), with no history of *Leishmania* infection.
Results: Regression analysis showed no significant association between polymorphisms of tumor necrosis factors-α when comparing VL and DTH + group ($P > 0.05$). The associations were detected between VL and negative skin test delayed-type hypersensitivity for the heterozygote genotype ($P = 0.021$), the recessive model: 1/2 + 2/2 ($P = 0.044$) and the minor allele 2 ($P = 0.019$). The resistance to VL was found to be under the recessive model 1/2 + 2/2 of tumor necrosis factors-β, when comparing VL and DTH + group (odds ratios: 0.558, 95%; confidence interval: 0.316-0.987; $P = 0.044$).
Conclusions: These results must be regarded to preliminary but suggestive that further study with larger populations is worthwhile.

KEYWORDS

Visceral leishmaniasis, Susceptibility, *TNF*-α, *TNF*-β, Morocco

1. Introduction

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis caused by *Leishmania donovani* and *Leishmania infantum* (*L. infantum*)(*chagasi*). Annual incidence of VL is approximately 500000 cases and the mortality rate in most endemic countries is almost 10%, even when treatment is available[1,2]. Importantly, 80%–90% of human infections are subclinical or asymptomatic, usually associated with strong cell-mediated immunity [positive skin test delayed-type hypersensitivity (DTH+)][3]. Leishmaniasis due to *L. infantum* infection is a zoonotic disease presented mainly in Mediterranean basin, central Asia and Brazil. Signs and symptoms of VL include prolonged fever, fatigue, weakness, anemia, enlarged lymph nodes, splenomegaly, and hepatomegaly; if left untreated, it is almost always fatal[4,5].

In Morocco, VL is exclusively caused by *L. infantum*[6,7]. The

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endemic area extends throughout the Rif Mountains, the pre-Rif plateau, and other parts of the country. The disease continues to occur sporadically and scatter. The current active foci are located mainly in the following provinces: Fes, Taounate, Zouagha Moulay Yacoub, Al Houceima, Chefchaouen, Sefrou, Taza and Meknes. Expansion of arid areas and an increase in temperatures are thought to be synergistic risk factors for increasing the incidence of leishmaniasis in these regions[7]. Each year, 150 cases are recorded, 93% mostly in children under 10 years old[7]. Asymptomatic infection is prevalent in the endemic areas and was evaluated to 11.4%[8]. It seems obvious that the asymptomatic infection is the rule, however, this fact still remains unclear. One hypothesis is that differences in genetic background could elicit different immune response resulting in either resistance or susceptibility to the disease[3]. Tumor necrosis factor-α (*TNF-α*) (cachetin) is an inflammatory cytokine primarily produced by activated macrophages and lymphocytes T and B. It is involved in the innate phase of the immune response with a central role in the defense against intracellular pathogens[9]. This cytokine exerts a wide range of biological activities including proliferation and differentiation, apoptosis, cytotoxicity, inflammation and immunomodulation[10]. In fact, serum *TNF-α* level has been detected in patients with VL and its presence has been related to disease gravity[11]. The tumor necrosis factor-β (*TNF*-β) is a Th1 cytokine, primarily produced by activated lymphocytes T and B. *TNF*-β (lymphotoxin α) is also a key mediator in the initiation of a local vascular inflammatory response.

The *TNF-α* and the *TNF*-β genes are both located on the short arm of chromosome 6 between the class I and class II regions of the human leukocyte antigen complex. A striking feature of the entire human leukocyte antigen complex is a high degree of genetic variation. Indeed, genetic polymorphisms in *TNF-α* and *TNF*-β locus affect expression level of their genes[12]. A guanine to adenosine transition at base pair 308 in the promoter region of *TNF-α* gene has been identified (termed the A allele)[13]. A polymorphism in first intron of the *TNF*-β gene at position +252 (A > G) leads to two allelic forms; the common allele is *TNF*-β A and *TNF*-β G is variant allele[14]. Variant alleles of *TNF*2 and *TNF*-β G seem to have a strange transcriptional activation, which leads to their higher serum levels[15].

The aim of this study is to examine the implication of the polymorphisms in the -308 position of the promoter region of gene *TNF-α* and in intron 1 of +252 *TNF*-β genes in the susceptibility versus resistance to VL in children living in Moroccan leishmaniasis endemic area.

2. Materials and methods

2.1. Patients and control groups

This study involved 102 children with active VL, admitted to the Pediatric Department of Hassan II Hospital Center (Fez, Morocco). They were diagnosed clinically by serological and parasitological examinations. Two groups of controls comprised 132 unrelated children from the same endemic region: 92 asymptomatic healthy volunteers, with no history of leishmaniasis and positive leishmanin skin test (DTH+); 40 healthy volunteers, with no history of leishmaniasis and negative leishmanin skin test [negative skin test delayed-type hypersensitivity (DTH-)]. Leishmanin skin test was performed by intradermal injection of 0.1 mL *Leishmania* antigen (Pasteur Institute of Iran, Tehran, Iran)[16]. The induration was measured along two diameters by the ball-point pen technique. The induration of $\geqslant$ 5 mm in diameter was considered positive after 48-72 h. Approval for the study was provided by the Ethical Committee of the Institute Pasteur of Morocco and with children's parents consent.

2.2. Single nucleotide polymorphisms (SNPs) genotyping

Genomic DNA was extracted from peripheral blood leukocytes by phenol-chloroform procedure, as previously described[17]. The blood samples were submitted to digestion in sodium dodecyl sulfate/proteinase K buffer at 37 °C for 6 to 12 h, followed by phenol and chloroform extractions. DNA was then ethanol-precipitated and resuspended in TE buffer. Genotyping of the biallelic polymorphisms in the promoter region at position –308 (G to A) of *TNF-α* gene and in intron 1 at position +252A/G of *TNF*-β gene was performed by PCR-restricted fragment length polymorphisms[18]. Briefly, 0.5 μg of DNA was added to 20 μL of reaction mixture containing 20 pmol of each primer (*TNF-α* : 3' AGGCAATAGGTTTTGAGGGCCAT 5'; 3' TCC TCCCTGCTCCGATTCCG 5'; TFN-β: 3' CCGTGCTTCGTGCTT TGGACTA 5'; 3' AGAGCTGGTGGGACATGTCTG 5'), with an annealing temperature of 60 °C for *TNF-α* and 65 °C for *TNF*-β. In a final volume of 15 μL, 10 μL of the PCR products was digested by 5 IU of NcoI (BioLabs, New England), for 3 h at 37 °C.

Restriction fragments were separated in 3% agarose gel electrophoresis. For *TNF-α* gene, an amplified product of 107 bp, containing the G to A transition at position -308, was obtained and restriction digests generated products of 87 and 20 bp for *TNF*1 allele and 107 bp for *TNF*2 allele. For *TNF*-β gene, amplification followed by NcoI digestion generated a fragment of 740 bp for allele 1 (*TNF*-β 2) and 555 plus 185 bp for allele 2 (*TNF*-β 1).

2.3. Statistical analysis

The Hardy-Weinberg equilibrium was tested for each group, using the *Chi*-square test. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). The odds ratio (OR) and *P* values were calculated for each allele and genotype among case and control groups. Allelic, genotypic frequencies and the recessive model were compared by fisher's exact test, ORs and 95% confidence interval (CI) were estimated.

3. Results

There were no significant differences in the distribution of the mean age and sex between cases and controls ($P > 0.05$), suggesting that the matching based on these two variables was adequate. Molecular identification of the causative agent of VL was done by ITS1 PCR-restricted fragment length polymorphisms for 55 patients; all were found to be infected by *L. infantum*, the only species responsible for visceral form of leishmaniasis in Morocco (data not shown).

The observed genotypes and alleles frequencies for *TNF-α* and *TNF*-β genes in the present survey are shown in Tables 1, 2 and 3. All SNPs were in Hardy-Weinberg equilibrium. Table 1 shows the distribution of genotype and allele frequencies in *TNF* loci among cases and asymptomatic carriers DTH+. No significant difference was noted in genotype or allele frequencies of *TNF-α* and *TNF*-β gene polymorphisms between patients and DTH+ control group. Regarding

the association between *TNF-α* promoter variants with the outcome of *L. infantum* infection, associations were detected between group with active VL and DTH- control group for the heterozygote genotype (P = 0.021), the recessive model: 1/2 + 2/2 (P = 0.006) and the minor allele 2 (P = 0.019) (Table 2). Another significant association was found between DTH+ and DTH- for the heterozygote genotype 1/2 (P = 0.021) (Table 3). The minor allele 2 frequencies in *TNF-α* promoter region are 21.6% in VL, 15.8% in DTH+ and 10.3% in DTH- groups.

Table 1

Distribution of allelic and genotypic frequency of the -308 *TNF*-α and +252 *TNF*-β polymorphisms in the VL versus DTH+ groups.

TNF Polymorphism		VL	DTH+	OR (*CI*) 95%	*P*
TNF-α	1/1	66 (64.71%)	67 (72.83%)	1	
	1/2	28 (27. 45%)	21 (22.83%)	0.726 (0.377–0.140)	0.336
	2/2	8 (7.84%)	4 (4.34%)	0. 445 (0.128–0.448)	0.190
Recessive model	1/1	66 (64.71%)	67 (72.83%)	1	
	1/2 + 2/2	36 (35.29%)	25 (27.17%)	0.687 (0.376–1.256)	0.221
Allelic frequency	1	0.784 ± 0.056	0.842 ± 0.053	1	
	2	0.216 ± 0.056	0.158 ± 0.053	0.675 (0.331–1.379)	0.279
TNF-β	1/1	53 (51.96%)	61 (66.30%)	1	
	1/2	38 (37.25%)	25 (27.17%)	0.575 (0.311–1.063)	0.076
	2/2	11 (10.78%)	6 (6.52%)	0.501 (0.182–1.383)	0.177
Recessive model	1/1	88 (51.96%)	61 (66.30%)	1	
	1/2 + 2/2	16 (48.03%)	31 (33.69%)	0.558 (0.316–0.987)	0.044
Allelic frequency	1	0.706 ± 0.063	0.799 ± 0.058	1	
	2	0.294 ± 0.063	0.201 ± 0.058	0.612 (0.319–1.176)	0.138

Table 2

Distribution of allelic and genotypic frequency of the -308 *TNF*-α and +252 *TNF*-β polymorphisms in the VL versus DTH- groups.

TNF Polymorphism		VL	DTH-	OR (*CI*) 95%	*P*
TNF-α	1/1	66 (64.71%)	32 (82.05%)	1	
	1/2	28 (27.45%)	6 (15.38%)	0.440 (0.216–0.896)	0.021
	2/2	8 (7.84%)	1 (2.56%)	0.297 (0.076–1.165)	0.065
Recessive model	1/1	66 (64.71%)	32 (82.05%)	1	
	1/2 + 2/2	36 (35.29%)	7 (17.94%)	0.408 (0.212–0.785)	0.006
Allelic frequency	1	0.784 ± 0.056	0.897 ± 0.067	1	
	2	0.216 ± 0.056	0.103 ± 0.067	0.394 (0.176–0.883)	0.019
TNF-β	1/1	53 (51.96%)	23 (57.5%)	1	
	1/2	38 (37.25%)	16 (40%)	0.969 (0.541–1.736)	0.916
	2/2	11 (10.78%)	1 (2.5%)	0. 245 (0.065–0.925)	0.023
Recessive model	1/1	88 (51.96%)	23 (57.5%)	1	
	1/2 + 2/2	16 (48.03%)	17 (42.5%)	0.803 (0.460–1.401)	0.440
Allelic frequency	1	0.706 ± 0.063	0.775 ± 0.092	1	
	2	0.294 ± 0.063	0.225 ± 0.092	0.722 (0.383–1.362)	0.313

Table 3

Distribution of allelic and genotypic frequency of the -308 *TNF*-α and +252 *TNF*-β polymorphisms in the DTH+ versus DTH- groups.

TNF Polymorphism		DTH+	DTH-	OR (*CI*) 95%	*P*
TNF-α	1/1	67 (72.83%)	32 (82.05%)	1	
	1/2	21 (22.83%)	6 (15.38%)	0.607 (0.293–1.257)	0.021
	2/2	4 (4.34%)	1 (2.56%)	0.668 (0.145–3.083)	0.065
Recessive model	1/1	67 (72.83%)	32 (82.05%)	1	
	1/2 + 2/2	25 (27.17%)	7 (17.94%)	0.593 (0.302–1.165)	0.126
Allelic frequency	1	0.842 ± 0.053	0.897 ± 0.067	1	
	2	0.158 ± 0.053	0.103 ± 0.067	0.583 (0.251–1.357)	0.205
TNF-β	1/1	61 (66.30%)	23 (57.5%)	1	
	1/2	25 (27.17%)	16 (40%)	1.686 (0.923–3.079)	0.087
	2/2	6 (6.52%)	1 (2.5%)	0. 488 (0.923–1.973)	0.297
Recessive model	1/1	61 (66.30%)	23 (57.5%)	1	
	1/2 + 2/2	31 (33.69%)	17 (42.5%)	1.439 (0.813–2.549)	0.211
Allelic frequency	1	0.799 ± 0.058	0.775 ± 0.092	1	
	2	0.201 ± 0.058	0.225 ± 0.092	1.179 (0.600–2.318)	0.632

Regarding the polymorphism of *TNF*-β, the frequencies of the minor allele 2 are 29.4% in VL, 20.1% in DTH+ and 22.5% in DTH- groups. According to our statistical results, the allelic frequencies distribution in this SNP did not significantly differ between all groups. No association was found in genotypic frequencies between patients and asymptomatic group (DTH+). By using 1/1 genotype as the reference group, we showed a significant association under a recessive model, when comparing VL patients with DTH+ group (ORs: 0.558, 95% *CI*: 0.316-0.987; P = 0.044). The recessive genotype 2/2 was associated with VL patients compared to DTH- group (ORs: 0.245, 95% *CI*: 0.065- 0.925; P = 0.023).

4. Discussion

Molecular studies could contribute to a better understanding of pathogenic processes that cause major infectious diseases. *TNF*, an important proinflammatory cytokine, plays a role in innate and adaptive immune responses, and is also implicated in a wide variety of infectious and autoimmune human diseases. Several studies have attempted to show links between susceptibility to leishmaniasis and *TNF* gene polymorphisms. Controversial results from these studies prompted us to search for eventual associations of *TNF* variants with susceptibility versus resistance to *L. infantum* infection. In the present work, we analysed, thus, two biallelic polymorphisms at position –308 (G: *TNF*1 allele to A: *TNF*2 allele) of *TNF-α* and in intron 1 at position +252 of *TNF*-β. No association was found in genotypic and allelic frequencies of *TNF-α* and *TNF*-β between patient group and asymptomatic infected group (DTH+). Associations were detected between VL patients and DTH- control group for the heterozygote genotype, the minor allele2 and the recessive model for *TNF-α* , and for homozygote genotype 2/2 of *TNF*-β, but due to smaller number of DTH- cases, this result must be regarded as preliminary. These associations however, point out the need for further studies with a larger sample size of control groups. A few studies with controversial results have been performed to evaluate the *TNF* polymorphisms in different clinical types of leishmaniasis. In Brazil, an association was found between the outcome of *Leishmania chagasi* infection and alleles at *TNF* locus. The strongest association was found between asymptomatic infection DTH+ and a polymorphism in the *TNF* locus and haplotypes containing *TNF*2 were associated with symptomatic VL[19]. In agreement with this finding, a case control study of 46 patients with mucocutaneous leishmaniasis caused by *Leishmania brazililensis* suggested that the frequency of allele 2 at the -308 *TNF-α* gene polymorphism is significantly higher in patient than asymptomatic group[20]. Other studies showed that *TNF* polymorphisms are not responsible for the resistance versus susceptibility to cutaneous leishmaniasis[20,21]. In Tunisia, Meddeb-Garnaoui failed to find associations between either the -308 of *TNF-α*, gene polymorphism or the first intron of *TNF*-β gene polymorphisms and susceptibility to Mediterranean VL[22].

A number of groups have set out to determine whether the polymorphism -308 *TNF-α* could affect *TNF* transcription and expression levels. Many studies showed that the rare allele 2 *TNF-α* is strongly associated with elevated serum *TNF* levels and a more severe outcome in infectious diseases, such as cerebral malaria and mucocutaneous leishmaniasis[20,23]. In addition, individuals homozygous for this allele are more likely to die or suffer severe neurological complications due to cerebral malaria. This allele

is overrepresented in diseases where increased *TNF-α* levels are associated with poor prognosis[23]. Contrariwise, other studies have failed to show any functional difference between the two allelic forms[24,25], but although controversial, the majority of the data support a direct role for the -308 *TNF*2 allele in the elevated *TNF* levels observed in *TNF*2 homozygotes[15].

Concerning the +252 *TNF*-β, Naresh *et al*. reported that the genotype-phenotype analysis of *TNF*-β revealed a higher expression levels in patients with GG and AA genotypes as compared to controls, while, the genotype *TNF*-β +252A/G was associated with vitiligo susceptibility and influence the *TNF*-β expression[26]. *TNF* seems to be especially important during infection with intracellular pathogens. In order to assess the importance of *TNF* for protection against leishmaniasis, several studies were reported both on humans and on experimental models. Indeed, in some rheumatic disease patients under anti-*TNF* therapy, the consequence of blocking *TNF* was a reoccurrence of the clinical symptoms of leishmaniasis[27-29]. Other argument supporting the central role of *TNF* in the anti-*Leishmania* immune response has been strengthened by observations in *Leishmania major* (*L. major*) (strain BNI) infected B6. *TNF*–/– mice, which were not able to develop an efficient immune response against parasites and died 6–8 weeks after infection from visceral leishmaniasis[30]. Conversely, Ritter *et al*. showed that infection of B6. *TNF*–/– mice with *L. major* (strain FRIEDLIN) resulted in an attenuated form of disease, even though the animals were not able to resolve the local lesions but developed a chronic form of cutaneous leishmaniasis. Thus, in addition to the extensively studied host factors, the biological properties of *Leishmania* strains play an important role in the outcome of leishmaniasis[31]. Oliveira, *et al*. showed that *L. major*-infected *TNF*-receptor-1 KO mice (C57BL/6) can control parasite replication, but fail to resolve lesions. Indeed, the intense inflammatory process was observed even after 20 weeks of infection, while wild-type mice completely heal[32]. On the other hand, no significant difference in the levels of *TNF* mRNA expression was found in both strains. The information resulting from these investigations supports the notion that, *in vivo*, *TNF* is not the decisive factor responsible for the resistant versus susceptible phenotype in *Leishmania* infection.

Considering numerous genetic variations, that influence the apparently divergent findings, our data show that -308 *TNF-α* and +252 *TNF*-β genotypes do not influence susceptibility versus resistance to visceral leishmaniasis. However, some associations were detected between VL patients and DTH- control group, but due to smaller number of DTH- cases, these results should be interpreted with caution. Well-designed studies with larger population are needed to understand the specific role of *TNF* that confer protection against leishmaniasis.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

VL is the most severe form of leishmaniasis caused by *L. donovani* and *L. infantum* (*chagasi*). Annual incidence of VL is approximately 500 000 cases and the mortality rate in most endemic countries is almost 10%, even when treatment is available. The signs and symptoms of VL include prolonged fever, fatigue, weakness, anemia, enlarged lymph nodes, splenomegaly, and hepatomegaly; if left untreated, it is almost always fatal. *TNF* (cachetin) is an inflammatory cytokine primarily produced by activated macrophages and lymphocytes T and B. It is involved in the innate phase of the immune response with a central role in the defense against intracellular pathogens.

Research frontiers

This study examine the implication of the polymorphisms in the -308 position of the promoter region of gene *TNF-α* and in intron 1 of +252 *TNF*-β genes in the susceptibility versus resistance to VL in children living Moroccan leishmaniasis endemic area. The study involved 102 children with active VL admitted between 2005 and 2007. Two groups of controls comprised 132 unrelated children from the same endemic region: 92 asymptomatic healthy volunteers, with no history of leishmaniasis and positive leishmanin skin test (DTH+); 40 healthy volunteers, with no history of leishmaniasis and negative leishmanin skin test (DTH-).

Related reports

No significant difference was noted in genotype or allele frequencies of *TNF-α* and *TNF*-β gene polymorphisms between patients and DTH+ control group. The polymorphism of *TNF*-β and the frequencies of the minor allele 2 are 29.4% in VL, 20.1% in DTH+ and 22.5% in DTH- groups. A few studies with controversial results were reported to evaluate the *TNF* polymorphisms in different clinical types of leishmaniasis. However, this study in agreement with Cabera *et al.*, (1995), 46 patients with mucocutaneous leishmaniasis caused by *Leishmania brazililensis* suggested that the frequency of allele 2 at the -308 *TNF-α* gene polymorphism is significantly higher in patient than asymptomatic group.

Innovations & breakthroughs

Their data show that -308 *TNF-α* and +252 *TNF*-β genotypes do not influence susceptibility versus resistance to VL. However, some associations were detected between VL patients and DTH- control group, but due to smaller number of DTH- cases, these results should be interpreted with caution. *TNF* seems to be especially important during infection with intracellular pathogens.

Applications

To examine whether polymorphic alleles at these two loci are involved in the susceptibility to VL in children. These results must be regarded as preliminary but further study with larger populations is very important and worthwhile. VL in Morocco is exclusively caused by *L. infantum*, but can be apply in other counties also.

Peer review

This is a good study in which the authors evaluated VL is the most severe form of leishmaniasis. Also they studied *TNF* production and with susceptibility to intracellular infectious diseases. This study aimed to examine whether polymorphic alleles at these two loci are involved in the susceptibility to VL in Moroccan children.

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