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Original Article

Association of polymorphisms in the *TNFA*, *TNFRSF1A* and *TNFRSF1B* genes with lepromatous leprosy in Western Mexican patients

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Abstract

Introduction: Studies in different populations have shown that single-nucleotide polymorphisms (SNPs) of tumor necrosis factor-alpha (TNFα) and TNF receptors 1 and 2 (TNFR1 and TNFR2) may be involved in the pathogenesis of lepromatous leprosy (LL). To further explore the results in a Mexican population, we compared the frequencies of the polymorphisms in - 308 G>A *TNFA* (rs1800629), - 383 A>C *TNFRS1A* (rs2234649), and + 196 T >G *TNFSR1B* (rs1061622) genes in LL patients (n = 133) and healthy subjects (n = 198).

Methodology: The genotyping was performed with the polymerase chain reaction-based restriction fragment length polymorphism (PCR–RFLP) technique. Statistical analysis was performed using the χ^2 test, within the 95% confidence interval. Odds ratios (OR) were calculated and Hardy-Weinberg equilibrium was verified for all control subjects and patients.

Results: We found an association between the *TNFSR1* -383 A>C genotype and the risk of lepromatous leprosy when leprosy patients were compared to controls (OR = 1.71, CI: 1.08-2.69, $p = 0.02$). Furthermore, it was also associated with the risk of LL in a dominant model (AC + CC vs AA, OR: 1.65, 95% CI: 1.05-2.057, $p = 0.02$). Similar genotype and allele frequencies for the SNPs *TNFA* - 308 G>A and *TNFSR2* + 196 T>G were observed between leprosy patients and healthy subjects.

Conclusions: The *TNFSR1* -383 A>C could be a potential marker for the identification of high-risk populations. However, additional studies, using larger samples of different ethnic populations, are required.

Key words: *TNFA*; *TNFRS1A*; *TNFSR1B*; lepromatous leprosy.

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Introduction

Leprosy is caused by the intracellular pathogen *Mycobacterium leprae* which infects macrophages and Schwann cells. It causes damage to the skin and peripheral nerves that lead to distinct clinical manifestations based on the host immune response against the pathogen. Leprosy is characterized by a spectrum of clinical presentations and can be categorized into two stable forms or poles known as the tuberculoid (TT) and lepromatous leprosy (LL); as well as the three borderline forms that are known as borderline-tuberculoid leprosy (BT), borderline leprosy (BB) and borderline lepromatous leprosy (BL). TT patients manifest a strong cellular immune response, mediated by macrophages and Th1 lymphocytes, which results in few, localized, and often self-healing paucibacillary lesions. In LL, the opposite pole, the immune response is mediated by antibodies and Th2 cytokines, with the presence of foamy macrophages that allow the bacilli to propagate and cause extended multibacillary lesions on the skin and nerves [1]. This variability of the host response to infection seems to be influenced by genetic and environmental factors.

The tumor necrosis factor alpha (TNFα) functions as a key immunoregulatory cytokine. It is secreted by macrophage/monocytes, lymphocytes, and endothelial cells, with important biological effects on the inflammatory response in several infectious and autoimmune diseases [2,3]. This cytokine plays an important role in the host response against intracellular bacterial infections and contributes to granuloma formation, synthesis of nitric oxide, and chemotaxis of immune cells [4,5]. The functions of TNFα are mediated by the TNFR1 and TNFR2 receptors, which induce activation of the transcriptional factors NF-κB and AP-1 [6]. TNFR1 is expressed in several cells and it is the main regulator of the TNFα functions, such as proliferation, apoptosis, and necroptosis [6,7]. TNFR2 is mainly expressed in T and B lymphocytes, endothelial cells, and myeloid cells [6,8]. Interaction of TNFα with TNFR2 activates a signaling pathway that induces cell proliferation and survival thus having a major pro-inflammatory effect [6].

The genes encoding TNFα and its receptors have polymorphic variants that have been associated with several pathologies. The *TNFA* gene is located on chromosome 6 (6p21.31). A single nucleotide polymorphism (SNP) in the promoter region of this gene at the -308 position replaces guanine with adenine (-308G>A, rs1800629), leading to enhanced transcription of the gene and increased activity of the cytokine. This polymorphism has been associated with leprosy [9–11] and pulmonary tuberculosis in different populations [12].

TNF receptor superfamily member 1A (also called CD120a, TNFR1, and TNFRp55/p60) is encoded by the *TNFRSF1A* gene which is located on chromosome 12 (12p13.31). A SNP at the -383 position results in a change of an adenine to a cytosine (-383A>C, rs1061622) in the promoter region, conferring an increase in the gene transcription. This has also been studied in different autoimmune pathologies, such as ankylosing spondylitis, type 1 diabetes, and rheumatoid arthritis in different populations [13–16].

The TNF receptor superfamily member 1B (also called CD120b and p75/p80) is encoded by the gene *TNFRSF1B* which is located on chromosome 1 (1p36.22). It contains a SNP that substitutes thymine for guanine at position +196 of the gene (rs1061622, ATG → AGG), which leads to a change of methionine (M) to arginine (R) in the extracellular domain of the receptor and affects receptor ability to activate NF-kB [16–18]. This SNP is associated with autoimmune disorders, such as systemic lupus erythematosus and rheumatoid arthritis in Asian populations [19,20].

Based on the above, the objective of this study was to determine the association of the polymorphisms -308 G>A *TNFA*, -383 A>C *TNFRS1A*, and 196 T>G *TNFSR1B* with lepromatous leprosy in the mestizo population of western Mexico.

Table 1. Primer sequences of *TNFA*, *TNFR1*, and *TNFR2* genes.

Gene	Primers	PCR conditions	Restriction enzyme	Reference
***TNFA* (-308)**	5′-AGGCAATAGGTTTTGAGGGCCAT-3′ 5′-TCCTCCCTGCTCCGATTCCG-3′	Initial denaturation: 94°C for 3 min 35 cycles of: 94°C for 30 sec 60°C for 30 sec 72°C for 30 sec Final extension: 72°C for 1 min	*NcoI* 2% agarose gel electrophoresis	**[24]**
***TNFR1* (-383)**	5′-TTATTGCCCCTTGGTGTTTGGTTG-3′ 5′-GGAGGGGAAGAGTGAGGCAGTGTT-3′	Initial denaturation: 95°C for 5 min 30 cycles of: 95°C for 1 min 60°C for 1 min 72°C for 1 min Final extension: 72°C for 5 min	*BglII* 2% agarose gel electrophoresis	**[25]**
***TNFR2* (+196)**	5′-ACTCTCCTATCCTGCCTGCT-3′ 5′-TTCTGGAGTTGGCTGCGTGT-3′	Initial denaturation: 95°C for 5 min 35 cycles of: 95° C for 1 min 57°C for 1 min 72°C for 1 min Final extension: 72°C for 5 min	*NlaIII* 4% agarose gel electrophoresis	**[26]**

PCR: polymerase chain reaction.

Table 2. Clinical and demographic characteristics of patients with lepromatous leprosy and healthy subjects.

Characteristics	LL (n = 133)	HS (n = 198)
Age, mean ± SD	53 ± 18.08	40.21 ± 15.18
Disease duration (years), mean ± SD	10 ± 7.9	-
Gender		
Female (%)	41	47
Male (%)	59	53
Family history (%)	44	-
Bacilloscopy (%, ++/+++)	100	-
Treatment (PCT) (%)	53	-
New cases (%)	47	-

Quantitative variables are expressed as means ± standard deviations (SD) and qualitative variables as frequencies and percentages (%) as noted. Family history refers to at least one first-grade family who has been infected with leprosy bacilli. Bacilloscopy samples were taken from ear smears. LL, lepromatous leprosy; HS, healthy subjects. PCT, polychemotherapy (rifampicin, clofazimine, dapsone).

Methodology

Patients and healthy subjects

We took samples of peripheral blood from 133 patients diagnosed with LL according to the international criteria defined by Ridley and Jopling [21] from the Instituto Dermatologico de Jalisco, SSA, "Dr. Jose Barba Rubio" in Guadalajara, Mexico. All patients were treated with the multidrug therapy (MTD) as proposed by the World Health Organization (WHO). The control group consisted of 198 healthy subjects (HS) who were gender and age matched. All HS were at least 18 years old. Patients and HS were mestizos from Western Mexico. Mestizos are a population of mixed ancestry resulting from the colonial Spaniards and Amerindians [22], and are genealogically native from Western Mexico ancestors for at least three generations.

DNA sample preparation

Whole blood samples were collected in tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant via venipuncture from patients and HS. Genomic DNA was isolated by the standard methodology described previously [23].

PCR-RFLP screening of TNFA TNFR1 and TNFR2 polymorphisms

Polymorphisms analysis for *TNFA* (-308 A>G), *TNFR1* (-383 A>C), and *TNFR2* (T>G; codon 196) were performed according to modified protocols from previously reported assays [24–26]. Briefly, PCR amplification of the promoter or coding region of the genes was performed using specifically designed pairs of oligonucleotide primers, which were then identified by a restriction enzyme assay. The primer sequences, annealing temperatures for PCR, and restriction enzymes used in each assay are listed in Table 1.

Ethical considerations

The protocol was approved by the ethics, research, and biosecurity committees of the Instituto Dermatologico de Jalisco, SSA, "Dr. Jose Barba Rubio", Secretaria de Salud from Jalisco state, Mexico. All research was performed according to the Declaration of Helsinki amended in Brazil in 2013 [27] and according to Mexico's regulations for studies on human health. Informed consent was signed by all the individuals included in the study.

Statistical analysis

Hardy-Weinberg equilibrium was tested among the healthy subjects in the population under investigation. The relative association of genotype and allelic frequencies among patients and controls was assessed by the Chi-square (χ^2) test or Fisher's exact test when necessary. Odds ratio (OR) and 95% confidence interval (CI) for relative risks were calculated. The statistical significance level was $p < 0.05$. All statistical calculations were performed with Statistical Package for Social Sciences (SPSS, version 11.0, for Windows).

Results

Clinical evaluation of the study groups

The demographic and clinical features of the LL patients and the HS included in the study are presented in Table 2. The diagnosis of LL was based on clinical, histopathological, and bacilloscopic studies. The LL group consisted of 59% males and 41% females, with a mean age of 53 ± 18.8 years, and a disease duration of 10 ± 7.9 years. The reference group included 198 healthy volunteers, with a mean age of 43 ± 15.18 years, matched to the patients by age and gender.

Genotypic and allelic frequencies of TNF - 308 G>A, TNFRSF1A - 383 A>C, and TNFRSF1B + 196 T>G variants

Allelic and genotypic frequencies of *TNF* - 308 G>A, *TNFRSF1A* -383 A>C, and *TNFRSF1B* + 196

Table 3. Genotype and allele distributions of *TNFA - 308 G>A*, *TNFRSF1A - 383 A>C,* and *TNFRSF1B 196 T>G* polymorphisms of patients with lepromatous leprosy and healthy subjects.

Polymorphism	LL (n = 133); % (n)	HS (n =198); % (n)	OR (CI 95%)	*p* value
***TNFA* -308 G > A** (rs1800629)				
Genotype				0.32
GG[b]	61.65 (82)	66.16 (131)	1	
GA	38.35 (51)	32.83 (65)	1.25 (0.77-2.033)	0.33
AA	0 (0)	1.01 (2)	0.80 (0.01-15.6)	0.85
EHW $p = 0.05$				
Allele				
G[b]	82.82 (215)	82.57 (327)	1	
A	19.17 (51)	17.42 (69)	1.12 (0.74-1.71)	0.57
Do				
GG[b]	61.65 (82)	66.16 (131)	1	
GA+AA	38.34 (51)	33.83 (67)	1.21 (0.75-1.97)	0.40
***TNFRSF1A* -383 A > C** (rs2234649)				
Genotype				0.07
AA[b]	40.60 (54)	53.03 (105)	1	
AC	54.89 (73)	41.92 (83)	1.71 (1.06-2.76)	**0.02**
CC	4.51 (6)	5.05 (10)	1.17 (0.33-3.76)	0.78
HWE $p = 0.27$				
Allele				
A[b]	68.04 (181)	73.98 (293)	1	
C	31.95 (85)	26.01 (103)	1.33 (0.93-1.90)	0.1
Do				
AA[b]	40.60 (54)	53.03 (105)	1	
AC+CC	59.39 (79)	46.96 (93)	1.65 (1.03-2.64)	**0.03**
***TNFRSF1B* +196 T > G** (rs1061622)				
Genotype				0.66
TT[b]	62.41 (83)	62.63 (124)	1	
TG	30.83 (41)	32.83 (65)	0.94 (0.56-1.56)	0.80
GG	6.77 (9)	4.55 (9)	1.49 (0.50-4.44)	0.41
HWE $p = 0.83$				
Allele				
T[b]	77.81 (207)	79.04 (313)	1	
G	22.18 (59)	20.95 (83)	1.07 (0.72-1.59)	0.71
Do				
TT[b]	62.41 (83)	62.63 (124)	1	
TG + GG	37.59 (50)	37.37 (74)	1.00 (0.62-1.63)	0.97

Percentages were obtained by direct count; *p* value was calculated by χ^2 test; LL: lepromatous leprosy; OR: odds ratio; 95% CI: 95% confidence interval; HWE: Hardy-Weinberg equilibrium; Do: analysis of dominant and codominant model; [b] reference category.

T>G polymorphisms were calculated in all subjects to identify the polymorphisms involved in LL susceptibility (Table 3). All the variants were in Hardy-Weinberg equilibrium in the HS group ($p>0.05$).

No significant differences were observed in the frequencies of the *TNF* -308 G>A SNP between the groups of patients and HS. Our results showed that the *TNF* - 308 GG was the most frequent genotype among LL (61.65%,82/133) and HS (66.16%, 131/198) and the genotype distribution pattern did not differ significantly ($p = 0.31$).

The A allele of the *TNFRSF1A* - 383 polymorphism was more frequent in the HS group (73.98%, 293/396) than LL group (68.04%, 181/266) (Table 3), although no significant differences were observed. Comparing the genotypic frequencies between LL patients and HS, we found a higher frequency of the AC genotype among LL patients (54.89 %, 73/133) than HS (41.92 %, 83/198; OR = 1.71, CI: 1.08-2.69, $p = 0.02$).

Regarding the *TNFRSF1B* + 196 T>G variant analysis, no statistically significant differences were observed in the allele distribution between LL patients and HS ($p = 0.66$). The frequency of the TT genotype was 62.41% (83/133) in the LL group and 62.63% (124/198) in the HS group.

Allelic frequencies of all three polymorphisms showed similar distribution patterns between HS and

LL and none of the genetic models was associated with the risk of developing LL (p>0.05).

Dominant and codominant genetic models were applied to analyze the associations between the *TNF* - 308 G>A, *TNFRSF1A* - 383 A>C, and *TNFRSF1B* + 196 T>G polymorphisms and LL. The results showed that the *TNFRSF1A* - 383 AA genotype was significantly associated with increased risk for LL in the dominant model (AC + CC vs AA, OR: 1.65, 95% CI: 1.05-2.057, p = 0.02). However, no significant association was found for *TNF* - 308 G>A and *TNFRSF1B* + 196 T>G when we compared the LL group with the HS group in any genetic model of inheritance analyzed.

Discussion

Leprosy is a chronic infectious disease, caused by the obligate intracellular pathogen *M. leprae*. The TT spectrum of leprosy is characterized by a strong cell immune response accompanied by the expression of Th1 cytokines like TNFα that induce the activation of macrophages. These in turn produce inducible nitric oxide synthase (iNOS) and release free radicals to destroy the mycobacteria. Meanwhile, the expression of Th2 cytokines in the LL spectrum leads to a humoral immune response, which is inefficient against an intracellular pathogen such as *M. leprae* [28].

Not all individuals who are chronically exposed to this mycobacterium develop the clinical manifestations of leprosy. Several studies have tried to elucidate the genetic factors involved in the development of this complex disease. It is unlikely that a single genetic marker can provide an efficient prognosis since the immune response depends on the controlled expression of several genes, which ultimately induce an efficient immune response and thus contribute to specific clinical manifestations in patients with leprosy.

SNPs provide relevant information since they can be used as genotypic markers of specific disease phenotypes and can regulate biological phenomena that influence mRNA expression, thereby altering mRNA isoforms (unraveling cryptic splicing sites) or may be involved in the modification of the enzymatic activity of genes related to leprosy. Many SNPs of immunoregulatory genes have been studied to describe their participation in the susceptibility of the host leading to the development of leprosy *per se* or some leprosy poles [29]. A significant association between the *TNFA* promoter polymorphism at the - 308 position (G>A transition) has been extensively associated with several autoimmune and inflammatory disorders, as well as infections such as leprosy due to the increased production of TNFα.

We failed to find an association between the - 308 G>A polymorphism in the *TNFA* gene with susceptibility to LL in Mexican patients. However, Mexico is a country with high genetic heterogeneity and distinctive patterns of linkage disequilibrium according to the geographic regions. Our results regarding the distribution of this SNP are similar to a previous study carried out on the population of Mexican mestizo patients from the northwest state of Sinaloa [30]. On the other hand, our results are in contrast with a previous investigation in which a significant association was found between the *TNFA* - 308 A allele and LL patients in India [31]. Furthermore, in a study of Brazilian patients, the G/G genotype was associated with resistance against LL compared with healthy controls [32]. Nevertheless, according to Cordeiro dos Santos *et al.*, no significant associations were observed between the *TNFA* - 308 polymorphism and the susceptibility to leprosy in Brazilian Amazon patients (MB and PB forms) [33].

TNFα exerts its biological effects by binding to its receptors, TNFR1 and TNFR2 [6]. Some studies have described that the pro-inflammatory and pathogen-killing functions of TNF are regulated mainly through its binding to TNFR1 [34]. Recent evidence suggests that TNFR1 has a relevant role in the pro-inflammatory and antitumoral responses, as well as resistance to viral and bacterial infections [35–39].

The - 383 A>C *TNFR1A* (rs2234649) SNP has been identified as a susceptibility factor or predictive marker in patients with invasive pulmonary aspergillosis, ankylosing spondylitis, rheumatoid arthritis, Crohn's disease, and Sjögren syndrome [40–44]. To the best of our knowledge, its association with leprosy susceptibility has not been previously evaluated. Our data suggest that subjects who are heterozygous (AC) for this SNP have a higher risk of LL development. Another important finding of our study is the genetic risk for LL in the combined AC/CC genotype compared to the AA genotype when we applied a dominant model of inheritance analysis.

Therefore, it is necessary to perform additional studies to establish the role of *TNFR1* - 383 A>C SNP with leprosy *per se*, the spectrum of leprosy, response to treatment, and prognosis. These studies could be focused on examining the functional role of this SNP in leprosy and the regulation of the cellular events involved in the gene expression, as well as the production of either soluble or membrane-bound TNFR1. In this sense, an alternative transcription may

play a role in the regulation of the expression of *TNFRSF1A*. This regulation may be modulated by polymorphisms in the gene (rs4149570, rs767455, and rs1800692) that lead to the elimination of exons 2 and 6 during mRNA maturation, which have been described as markers of susceptibility to inflammatory diseases [45–48]. Therefore, it would be interesting to perform further functional and genetic analysis of those SNPs to establish the combined effect of genotypes (haplotypes) and their association with the host susceptibility to *M. leprae* infection or leprosy clinical manifestations.

A relevant point to consider in the regulation of the immune response in leprosy is the interaction of TNFα with its receptor TNFR2, a transmembrane protein necessary for differentiation [49] and survival of T cells [50], as well as signaling in the regulation of inflammatory responses mediated by TNFα [51]. Studies carried out in TNFR2 knock-out mice suggest that this receptor participates in the development of neurovascular lesions in experimental models of malaria [52], in the early control of experimental melioidosis [39], as well as in the regulation of the inflammatory process in pleurisy induced by mycobacteria [53]. On the other hand, changes in the regulation of this receptor could be involved in various inflammatory, infectious and autoimmune diseases [16,54–57]. The TNFR2 deleterious effects in these pathologies have been attributed to an increase of the soluble form in biological fluids due to the *TNFR2* + 196 polymorphisms.

The association between genetic polymorphisms and susceptibility to infectious diseases has been demonstrated by several authors. Ghamari *et al.* analyzed the + 196 T>G polymorphism of *TNFRSF1B* in Iranian patients with pulmonary tuberculosis but did not find any significant association [12]. Accordingly, we found no association between the + 196 T>G polymorphism and the LL patients. Therefore, it would be interesting to evaluate other genetic variants of *TNFR2* to elucidate the complex regulation of these pathways. In addition, further studies could analyze their association with changes in the soluble TNFR2 levels and TNFR2 membrane expression, which could affect the cytokine profile and contribute to the resistance or susceptibility of the host to *M. leprae*.

This study had some limitations. We were able to recruit only five patients with TT and we did not have patients with the other clinical forms of the disease. Therefore, it was not possible to perform an association analysis with a group of patients who had different clinical presentations than LL.

Our study also had some strengths. The healthy subjects group belonged to the same geographic region (Western Mexico) as the patients' group, and, therefore, shared their ethnic composition.

Based on the results of our study, we consider that it is necessary to delve into association studies of genes involved in immunological pathways of the innate and adaptive immune response that participate in the establishment of the infection, the diverse host responses that determine the development of one of the clinical spectra of leprosy, and the probable disease evolution. The information gathered from these studies may help to determine the eventual outcome more accurately.

Conclusions

Our results suggest that the *TNFRSF1A* - 383 A>C is a SNP associated with susceptibility to LL in Western Mexican patients. However, we did not observe any association between leprosy and the studied SNPs in the *TNFA* and of *TNFRS1B* genes. Therefore, it would be interesting to analyze other genetic variants in *TNFR1* and their participation in the immune responses in the different clinical forms of leprosy and other infectious diseases. In addition, much remains to be known about how these genes and their interaction with environmental factors may participate and determine the final phenotype in patients with leprosy. Accurate profiling of genetic variants may help identify risk populations and new treatment strategies.

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