Association between STR-794 CATT<sub>5-8</sub> and SNP-173 G/C polymorphisms in the MIF gene and Lepromatous Leprosy in Mestizo patients of western Mexico

M.A. Martinez-Guzman<sup>a,b</sup>, A. Alvarado-Navarro<sup>b</sup>, A.L. Pereira-Suarez<sup>b</sup>, J.F. Muñoz-Valle<sup>a</sup>, M. Fafutis-Morris<sup>b</sup>,* 

<sup>a</sup>Doctorado en Ciencias Biomédicas con Orientación en Inmunología, Departamento de Fisiología, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, México, Sierra Mojada 950, Col. Independencia, 44340 Guadalajara, Jalisco, Mexico 
<sup>b</sup>Centro de Investigación en Inmunología y Dermatología/Universidad de Guadalajara, México, Av. Federalismo Norte 3102, Col. Atemajac del Valle, 45190 Zapopan, Jalisco, Mexico

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**A B S T R A C T**

Lepromatous Leprosy (LL) is the most common presentation of leprosy in Mexico. LL patients are unable to activate an effective inflammatory response against *Mycobacterium leprae* probably due to the genetics of the host. Macrophage Migration Inhibitory Factor (MIF) is important to trigger inflammation processes. Two polymorphisms have been reported for human MIF: STR-794 CATT<sub>5-8</sub> and SNP-173 G/C. 7-8 CATT repeats at -794 and the C allele at -173 increase the expression of MIF. We aim to determine the association between the polymorphisms in MIF gene and LL. We carried a case and controls study with 100 Mexican LL patients and 100 healthy subjects (HS). PCR was used for genotyping of STR-794 CATT<sub>5-8</sub> polymorphism and PCR-RFLP for -173 G/C. We found that LL patients possess high -794 CATT repeats (47.1%) more often than HS (32.7%). In conclusion, a MIF polymorphism is associated with susceptibility to LL in Western Mexican population.

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**1. Introduction**

Leprosy is a chronic infectious disease in humans caused by the intracellular pathogen *Mycobacterium leprae*, which infects macrophages and Schwann cells, thus damaging peripheral nerves and giving rise to loss of sensitivity. The clinical presentation of the disease is diverse and depends on the immune response of the host, ranging from Tuberculoid Leprosy (TT) to Lepromatous Leprosy (LL) poles through intermediate Borderline Tuberculoid (BT), Borderline Lepromatous (BL) poles. Cellular immune response present in TT and BT is mediated by macrophages and Th1 lymphocytes; however, in BB, LL, and mainly in LL, the immune response is antibody-mediated accompanied by ineffective foamy infected macrophages, whose bactericidal activities are further hindered by the Th2 cytokine profile [1]. Both poles may be distinguished by bacterial density in histopathological smears: a maximum of 10 bacilli in 100 microscopic fields is observed in samples of patients with TT and BT, equivalent to a Bacterial Index (BI) of 1+; in BB, BL, and LL from 1 to >1000 bacilli can be observed in a single microscopic field, representing from a score of 2+ to 6+ on the BI [2].

Due to its status as a Neglected Tropical Disease (NTD), the World Health Organization (WHO) has proposed treating contagious patients with polychemotherapy to stop the transmission of leprosy. Despite the significant reduction in worldwide prevalence with this strategy, new cases continue to present constantly...
However, this approach ignores that the role of genetic factors in the risk of contracting and combating the disease has been proven. IL-12B, HLA-DRB1, TNF, LTA, IL10, PARK2, NOD2, RIPK2, and LACC1 are some of the genes previously associated with the disease. Because leprosy is a complex disease and that final outcomes largely depend on host immunological factors, it is necessary to maintain the investigation of genetic markers of susceptibility by means of molecular techniques in an ongoing manner [4,5].

The Macrophage Migration Inhibitory Factor (MIF) enhances the production of Reactive Oxygen Species (ROS) by macrophages and their migration to infection. Unlike other cytokines, MIF is usually maintained in vesicles in its mature and active form, which confers upon it the capacity to be one of the first cytokines to be released on infectious stimuli. Furthermore, MIF promotes production and establishes a network of positive feedback with Tumor Necrosis Factor alpha (TNF-α) [6].

MIF gene is located in chromosome 22 (22q11.2) and contains two clinically relevant polymorphisms within the promoter region [6] that have been associated with susceptibility to several diseases [7,8]. A Short Tandem Repeat (STR) polymorphism is located at locus -794, for which seven or eight repeats of CATT have been associated with higher MIF production and greater susceptibility to inflammatory diseases when compared with five or six CATT repeats [7]. Likewise, the C allele of a Single Nucleotide Polymorphism (SNP) at position -173 creates a binding site for Activator Protein 4 (AP-4), and its presence is associated with higher severity and inflammation in autoimmune diseases than in the Wild-Type (WT) G allele [9,10].

The prevalence of leprosy cases has diminished in Mexico due to the recommended polychemotherapy treatment [3,11]. Four regions of distinct prevalence can be localized in this country, among which the western region presents the highest number of cases, perhaps because of higher susceptibility to the disease. In this region, LL comprises the main presentation of leprosy [12]. Considering that genetic background is one of the most relevant factors for developing LL and that MIF polymorphisms have been associated with susceptibility to several diseases, the aim of this study was to study the association of STR -794 CATT_{5,8} and SNP -173 G/C polymorphisms in the MIF gene and the development of LL in the population of western Mexico.

2. Materials and methods

2.1. Ethical considerations

We designed the present study according to the Declaration of Helsinki, as last reviewed at Fortaleza, Brazil, in 2013. Patients and Healthy Subjects (HS) were informed about the objective of this research and they voluntarily signed an informed consent letter in agreement (Universidad de Guadalajara ethical committee number CI-02515).

2.2. Characteristics of patients and HS

We included 100 patients with a clinical, histopathological, and bacilloscopic diagnosis of LL who were seen at an external appointment at the Dr. José Barba Rubio Dermatological Institute of Jalisco. 100 HS were paired with patients regarding age and gender. Both HS and patients were genealogically native to western Mexico for at least three generations.

2.3. DNA extraction

We obtained 8 mL peripheral blood from both study groups using EDTA as anticoagulant, and DNA was purified by the salting-out technique. Briefly, erythrocytes were lysed using hypotonic ammonium solution, leukocytes membrane was disrupted using SDS for 48 h at 37 °C and proteinase K, proteins were precipitated by increasing salt concentration and finally DNA was purified by cold absolute ethanol precipitation followed by 2 washes with ethanol 70% [13].

2.4. Genotyping of the STR -794 CATT_{5,8} polymorphism

To analyze the STR -794 CATT_{5,8} polymorphism, we amplified polymorphic fragments by end-point Polymerase Chain Reaction (PCR), for which we used the protocol previously reported by Muñoz-Valle group [7,14]. The reaction mix contained Buffer A 1X, TaqPol 0.032 U/μl (Vivantis Technologies Sdn. Bhd., USA), MgCl2 5 mM, an equimolar mix of dioxynucleotide triphosphate (dNTP) 0.1 mM and primers 300 nM (Invitrogen, USA), and 500 ng of DNA as substrate. The amplification protocol included an initial denaturation at 95 °C for 4 min, 30 denaturation cycles at 95 °C for 30 s, alignment at 60 °C for 30 s, and an extension at 72 °C for 30 s, followed by a final extension at 72 °C for 2 min. As a result, fragments of 208, 212, 216, or 220 base pairs (bp) were obtained for alleles of 5, 6, 7, or 8 CATT repetitions, respectively. The PCR products were visualized on 10% polyacrylamide gels 29:1 (Sigma-Aldrich, USA) stained with 0.2% AgNO3 (Caledon, Canada) after electrophoresing at 60V for 25 h. Genetic dominance analysis was performed considering as dominant the seven and eight CATT repetitions high repeats (HR).

2.5. Genotyping the SNP -173 G/C polymorphism

We determined the genotypes of the SNP -173 G/C polymorphism by PCR-Restriction Fragment Length Polymorphism (RFLP). For PCR, we employed a modification to the primers as reported by Makihja et al. [15] as follows: forward primer 5’-ACT-AAG-AA A-GAC-CCG-AGG-3’ and reverse primer 5’-GGG-GCA-CTG-TGG-TG T-TTA-CG-3’. The reaction mix contained Buffer A 0.93X, TaqPol 0.112 U/μl (Vivantis Technologies), MgCl2 4.7 mM, equimolar mix of dNTP 0.09 mM, primers 84 nM (Invitrogen, USA) and betaine 1.12 M (Sigma-Aldrich, USA), and 100 ng of DNA as substrate. The amplification protocol included initial denaturation at 95 °C for 4 min, 30 denaturation cycles at 95 °C for 30 s, alignment at 60 °C for 30 s, and an extension at 72 °C for 30 s, followed by a final extension at 72 °C for 2 min, which resulted in a 366-bp-long fragment. Finally, we digested the amplified products using 0.032 U/μl of AluI enzyme (New England Biolabs, USA) for 16 h at 37 °C, and the products were observed on 6% polyacrylamide gels 29:1 (Sigma-Aldrich, USA) stained with 0.2% AgNO3 (Caledon, Canada) after electrophoresing at 150 V for 1 h. As a result, we obtained 268-bp fragments for G/G subjects, two fragments of 206 and 62 bp for C/C subjects, and all three fragments were observed for G/C subjects; additionally, a 98-bp fragment was observed in all subjects due to the presence of another restriction site in amplified products. Additionally, 10 random samples were sequenced in order to confirm the results obtained by RFLP. Genetic dominance analysis was performed considering as dominant the C allele.

2.6. Statistical analysis

Regarding the clinical and demographic characteristics of patients and HS, qualitative variables were expressed as frequencies and quantitative variables, as means and Standard Deviations (SD). Analyses were carried out with IBM SPSS Statistics ver. 20 and Microsoft Excel 2010 in Windows 7. Genotype and allele distribution in the study groups was determined by direct counting and was expressed as frequencies with Standard Errors (SE), and their association with the disease was studied using Odds Ratios (OR) and 95% Confidence Intervals (95% CI) computed by χ².
Homogeneity in subjects of HS groups was determined by Hardy–Weinberg equilibrium. Differences were considered statistically significant when \( p < 0.05 \).

3. Results

3.1. Clinical and demographic characteristics of patients and Healthy Subjects

For this study, we included 100 patients with leprosy and 100 Healthy Subjects (HS) from western Mexico. Average disease-evolution time was 10.79 years, and 47% of patients with leprosy had family members who have presented this disease. All patients were positive with at least 2+ on the BI (Table 1).

### 3.2. Genotype and allele frequencies of STR -794 CATT<sub>5-8</sub> of the MIF gene in patients and HS

The genotype distribution was in Hardy-Weinberg equilibrium in the HS group. For our study we grouped STR genotypes as low CATT repeats (LR: 5/5, 5/6, 6/6) and high repeats (HR: 7/X, 8/X; whereas X = any allele). We found that HR genotypes are significantly more prevalent in patients (47.1%) than in HS (32.7%) \( (p = 0.046) \). We also found that the allele CATT<sub>7</sub> was more frequent in patients (28.4%) than in HS (17.8%) with statistical significance \( (p = 0.0147) \) (Table 2).

### 3.3. Genotype and allele frequencies of SNP -173 C/G of the MIF gene in patients and in HS

When we analyzed the frequencies of SNP -173 C/G genotypes, we found that the C/G genotype is more frequent in HS (58.5%) than in patients (50.5%); thus, the C/G is more frequent in patients (43.2%) than in HS (34.3%), without these differences being not statistically significant \( (p = 0.407) \). Furthermore, genotypes with the dominant C allele are more frequently found in patients (49.5%) than in HS (41.2%), but this difference is not statistically significant \( (p = 0.271) \) (Table 2). The sequenced samples matched the genotypes observed by RFLP.

4. Discussion

*Mycobacterium leprae* is the causal agent of leprosy. LL, which is the most common manifestation of this disease in western Mexico, is characterized by absence of cellular immune response. Even though it is not clear why some patients develop this pole of the disease, it is known that genetic factors play an important role in defining the host immune response [1]. We studied *MIF* gene polymorphisms because the protein that it encodes possesses important inflammatory properties that include stimulation of macrophages to produce ROS, increase in both the levels and response to TNF-α and leukocytes recruitment [6]. Previous studies have associated *MIF* polymorphisms with susceptibility to several diseases [9–22]. However, this is the first study that analyzes the association between STR -794 CATT<sub>5-8</sub> and SNP -173 C/G MIF gene polymorphisms and leprosy.

We found not-statistically significant more men than women among the patients, unlike the accepted paradigm of males being twice as likely to present the disease than females [3]. Instead of assuming a biological difference in Western Mexican population, this difference may be due to the gender idiosyncrasy in our population, where women may spend more time studying their own skin and attending clinical check-ups than men.

We found that STR -794 CATT<sub>5-8</sub> genotype and allele distributions in HS was similar to that reported by previous works in western Mexico population. Morales-Zambrano et al., also found that 6 CATT repeats was the most frequent allele (55%) and that so was genotype 6/6 (34%) [14]. However, it is worth of notice that we found 2 individuals with alleles of 8 CATT repeats in each group; this is a very rare allele and some studies commonly fail to find this. We also compared the frequencies we found with those in other populations: genotypes 5/6 (33.5%) and 6/6 (43.7%) were the most common in HS from United Kingdom too [17]. In contrast, the STR -794 CATT<sub>5-8</sub> genotype distribution found in HS from Kenya greatly differs from what we found: they found the 5/6 genotype to be the most frequent (33.9%) followed by 5/5...
(25.8%), which barely reached 5% prevalence in our study [10]. It has been reported that higher CATT repeats are associated to migration and genetic mixing [18], thus explaining why the CATT5 allele is more prevalent in African populations. Since the genetics of Mexican population is the result of interbreeding of American native, European, Asian and African subjects [23], this intensive mixing would also explain the higher prevalence of CATT6 and CATT7 alleles in Mexican population.

We also made comparisons of SNP -173 G/C genotype distributions in other studies. Morales-Zambrano et al. also found that the most frequent SNP -173 G/C allele in western Mexican population was G (75%) and that the most prevalent genotypes were G/G (54%) and G/C (42%) [14]. In United Kingdom, G/G (77.2%) genotype was the most frequently found and, unlike western Mexico, C/C genotype is less than 1% frequent [17]. However, genotype distributions in Kenya were different for this polymorphism: C/C was the most prevalent genotype (46%) while G/G was the least (16%) [19].

Here we found that individuals with LL are carriers of high CATT repeats of STR -794 CATT5-8 more frequently than HS. Inverted CCAAT box-binding protein of 90 kDa (ICBP90) has been shown to induce MIF transcription through the binding to its promoter region in a site that includes the STR -794. Furthermore, high CATT repeat alleles stabilize this binding and respond more actively to ICBP90, thereby promoting the inflammatory environments that are characteristic of the above mentioned inflammatory pathologies [24].

High CATT repeats have also been associated to bad prognosis in infectious diseases e.g. malaria. To study the role of MIF in this disease, MIF knockout mice were infected with P. chabaudi and their survival time was measured and they found that MIF−/− mice had higher survival index than WT infected mice [25]. They also studied MIF genotypes in malaria patients and they observed that the genotypes of high MIF expression may confer bad prognosis because of inflammatory complications and reduction of erythrocytosis. However, other results suggest that high expression MIF alleles are associated to decreased serum levels and that MIF absence could be a risk factor in this pathology [10,26]. In agreement with this, it has been reported that MIF has microbicidal activity against Leishmania major, an intracellular parasite whose clinical presentation ranges in several manifestations similar to leprosy [27]. Furthermore, genotype 5/5 of STR -794 CATT5-8 was identified as a susceptibility factor to development of tuberculosis in a cohort of Uganda individuals. Additionally, they confirmed that mice express higher MIF serum levels when transfected with a gene containing high CATT repeats in the promoter region compared to those transfected with low CATT repeats. Finally, the former mice were more efficient at destructing M. tuberculosis, which suggests an important role of MIF against this pathogen [8]. Therefore, MIF could enhance immune response against pathogens and aid to resolve infections more quickly, as it has been observed for other protozoa [28]. In contrast, we have found that high CATT repeats of STR -794 CATT5-8 are associated to susceptibility to develop LL. Noteworthy, leprosy shares some susceptibility genes with autoimmune diseases such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis and lupus erythematosus, to an even greater extent than it does with infectious diseases like tuberculosis, HIV and HBV [29]. Indeed, associations between high CATT repeats of STR -794 CATT5-8 and rheumatoid arthritis [30], systemic erythematous lupus [7] and psoriatic arthritis [14] have also been reported.

C/G genotype of SNP -173 G/C has also been associated to severity of inflammatory diseases in studies of polyarthritis [17]; systemic erythematous lupus [7]; psoriatic arthritis [14] and hepatitis B virus infection [20]. Also, SNP –173 G/C has been found to be associated to cancer [21] and intestinal inflammatory disease [22] through meta-analyses studies. Surprisingly, we did not find this polymorphism associated to LL in our population.

In Mexico, the incidence of leprosy is around 200 new cases per year, while the prevalence rounds 500 since 2009. Furthermore, Mexico is divided in regions according to leprosy prevalence. In this study, we focused on the genetics concerns of MIF gene in 100 LL patients gathered of the Western Mexico region, which seems to be more susceptible to leprosy than other Mexican populations. However, the relative higher incidence in this region, new cases detections is further delayed due to stigma and lack of access to health Institutions [12]. Though other genotyping strategies like sequencing all samples and Taqman-based probes could prove to be more accurate, the genotyping technique we employed was validated by previous studies for the STR-794 [14] and the SNP -173 [19], as well as by sequencing random samples in our study. Allegedly, median age of the HS group is lower than the patients group, which could be a limitation of this work. Also, further studies regarding systemic and local levels of MIF in patients with LL need to be conducted to understand the role of this cytokine on the physiopathology of this disease.

In conclusion, we found that patients with LL of western Mexico present high CATT repeats of STR -794 CATT5-8 more frequently than HS of the same population. However, we did not find statistical association of the disease with SNP -173 G/C.

Authors’ contributions

MMG carried out experimental work including sampling, processing and genotyping, and also drafted the manuscript. AAN designed part of the experiments. APS designed part of the experiments. JMV designed experiments and aided to conception of the project. MFM designed the experiments and conceived the project. All authors read, reviewed and approved the final manuscript.

Declaration of interest

The authors declare that are no financial or labor relations that may constitute a conflict interest in this work. We have not received “profit in money, goods, entertainment or subsidies” from any source that has a particular interest in the results of this research.

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