

Original Research Article

Analysis of the frequency of the Toll-like 2 gene polymorphism in leprosy

ABSTRACT

Polymorphisms in genes that are responsible for encoding cytokines and receptors involved in the immune response, such as Toll-like Receptor (TLR) 2 in leprosy, are of great interest for immunogenetic studies. This work aimed to analyze the possible association of single nucleotide polymorphism (SNP), synonymous, rs3804100 of the TLR2 gene with leprosy. A case-control study was conducted in Bacteriology and Mycology section of Evandro Chagas Institute, Brazil between August 2020 and July 2021. The scope of the study consisted of 122 individuals from municipalities of Goianésia, Rondon, Curionópolis, Altamira, Parauapebas and Redenção of the State of Pará, Brazil. Genotyping was performed by conventional PCR and sequencing in the ABI 3130 Genetic Analyzer (Applied Biosystems®) using primer nucleotides designed by the Primer3Plus program from the genomic region "Homo sapiens toll like receptor 2 (TLR2) transcript variant X6, mRNA", deposited in GenBank with reference XM_011532216.2. The analyzes were performed based on Fisher's exact test. It was managed in accordance with Helsinki Declaration and the Brazilian National Health Council and with approval of the ethics committee at Evandro Chagas Institute, under opinion number: 3.950.570. No associations between gender and leprosy were possible ($P > 0.05$). However, associations were observed between age groups, which were significant between those over 46 years old ($P = 0.004$) and the 2nd dose of BCG as a more protective agent between the groups analyzed ($P = 0.004$). For the subjects with the typed genotypes, 68 contacts had T/T genotype and only 4 T/C genotypes, while in MB group only 1 T/C genotype was found and none in PB ($P > 0.05$). We conclude that there is no association between the TLR2 SNP rs3804100 and leprosy in the Pará population, which still indicates the need for new immunogenetic studies with other genes involved in the immune response and a greater number of polymorphisms.

Keywords: Epidemiology; Leprosy; Genetic polymorphism; Immunogenetics.

1. INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, a bacillus-shaped mycobacterium that acts as an obligate intracellular parasite, mainly affecting the skin and nervous tissues [1]. Moreover, *M. leprae* infects predominantly macrophages, dendritic cells, and Schwann cells, which may lead to nerve damage and disability [2].

Epidemiological data position Brazil as the second in prevalence of cases globally, surpassed only by India [3]. According to data from the Brazilian Ministry of Health, high incidence of leprosy cases occurs in the Midwest, Northeast and North regions, and with the state of Pará as the 3rd in number of detected cases in 2018 within the Brazilian territory [4].

Leprosy progression is linked to immunogenetic factors that orientate the host's immune response against the pathogen. The tuberculoid form (TT) (paucibacillary) is understood by the high host resistance to infection, which is characterized by

a T helper 1 (Th1) lymphocyte response. Oppositely, the lepromatous form (LL) (multibacillary) a reduced Th1 response is observed, causing infection spread, bacillary multiplication, and a Th2-based immune response. Finally, transitions clinical manifestations between the polar clinical (TT and LL) forms, correspond to the borderline group [5].

Toll-like receptors (TLRs) are a group of transmembrane receptors present from *Drosophila* flies to mammals. Among humans, the group of these receptors consists of 10 protein subtypes predominantly characterized by single nucleotide polymorphisms (SNPs). TLRs are specific for several components of infectious agents, including viruses, bacteria and fungi, also having major participation in the activation of the innate immune response, cytokine production, and adaptive immune response [6,7].

Host cell signaling by *M. leprae* begins through activation of pattern recognition receptors (PRRs) by antigen-presenting cells (APCs), such as the TLR1/TLR2 heterodimer, which is able to detect mycobacterial components. Hence, they enable mobilization and phenotypic differentiation of macrophages, and immune responses mediated by pro-inflammatory cytokines, which is related with the generation of free radicals, oxygen and nitrogen reactive species, leading to Th1, Th2, Th17, Treg (regulatory) responses, and also through Natural Killer (NK) cells. Therefore, polymorphic variants (SNPs) of the TLR2 gene, are possible candidates for factors of susceptibility or resistance to infection [8].

The present study aimed to verify the relationship between the SNP rs3804100 of the TLR2 gene and leprosy by comparing the genotypic and allelic frequencies among leprosy cases and their healthy contacts.

2. MATERIAL AND METHODS

2.1 Sampling and Study Location

This is a case-control study involving patients from Altamira, Goianésia, Rondon, Parauapebas, Redenção and Curionópolis cities, state of Pará, Brazil. The sample consisted of spontaneous demand of 50 patients, including 25 TT and 25 LL. Additionally, a group of 72 household contacts, who did not present clinical symptoms of leprosy, were included as controls [9]. Blood samples were collected from patients and contacts by venipuncture in 5ml tubes and stored at -20°C for laboratory procedures at the Bacteriology and Mycology section, Evandro Chagas Institute. Household contacts with a history or suspicion of leprosy were excluded.

2.2 SNP rs3804100 typing of the TLR2 gene

The SNP under analysis is rs3804100 from Thymine to Cytosine (T>C) at position 450 of the extracellular domain of TLR2 at nucleotide 1350 (1350T/C). The typing was based on two PCR primer nucleotides: Primer 1 - F: 5'ATTCAGCCTGTGAGGATGCC 3' and R: 5' TTGCTGCTCCTGAGTGAAGG 3' generating a 512 bp fragment and Primer 2 - F: 5' ACCGGAGAGACTTTGCTCAC 3' and R: 5' GCTTGCTGCTCCTGAGTGAA 3' generating fragment of 437 bp. Primers used in PCR essays were generated on the Primer3Plus program based on the genomic region "Homo sapiens toll like receptor 2 (TLR2) transcript variant X6, mRNA", deposited in GenBank with the reference XM_011532216.2.

DNA extractions were performed using the DNeasy Blood & Tissue kit (QIAGEN), following the manufacturer's guidelines. Conventional PCR essays for TLR2 gene amplification were performed using PlatinumTaq under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. Amplified products were submitted to electrophoresis on 3,6% agarose gel stained with 3,0 µL of SybrSafe for visualization of the amplified DNA fragments. Reaction products were purified and bidirectionally sequenced using Big Dye Terminator v3.1 chemistry on ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were analyzed and compared to those available at BLAST on the National Center for Biotechnology Information (NCBI) website.

2.3 Data Analysis

The results were organized in a database of the Epi info™ 7 program. The observed proportions of polymorphism presence within each studied group were analyzed using Fisher's exact test. The analysis for SNPs prospecting considering the dominant and variant alleles of the searched nucleotide base was performed by means of a bioinformatics program, Bioedit™ v7.2.5.

2.4 Ethical Considerations

The present study was conducted in accordance with Helsinki Declaration and the Brazilian National Health Council and with approval of the ethics committee at Evandro Chagas Institute, under opinion number: 3.950.570.

3. RESULTS

From a total of 122 individuals with the defined genotypes, 39 were from Altamira (31,97%), followed by Redenção (33 – 27,05%), Goianésia (19 - 15,57%), Parauapebas (12- 9,84%), Rondon (10 - 8,20%), and Curionópolis (9 - 7,38%). Regarding household contacts individuals, 32 were from Altamira (44,44%), followed by Redenção (21 - 29,17%) and Goianésia (8 - 11,11%). Most of the patients group came from Redenção with 12 (24,00%), then from Goianésia with 11 (22,00%) and Parauapebas with 10 (20,00%). Distribution of individuals according to location and clinical manifestation is presented in Table 1.

Table 1. Distribution of number and frequency of sampling of contacts, LL, TT, and patients according to municipalities.

MUNICIPALITIES	CONTACTS N (%)	LL N (%)	TT N (%)	PATIENTS N (%)	TOTAL N (%)
ALTAMIRA	32 (44,44%)	6 (24,00%)	1 (4,00%)	7 (14,00%)	39 (31,97%)
REDENÇÃO	21 (29,17%)	6 (24,00%)	6 (24,00%)	12 (24,00%)	33 (27,05%)
GOIANÉSIA	8 (11,11%)	1 (4,00%)	10 (40,00%)	11 (22,00%)	19 (15,57%)
PARAUPEBAS	2 (2,78%)	9 (36,00%)	1 (4,00%)	10 (20,00%)	12 (9,84%)
RONDON	2 (2,78%)	3 (12,00%)	5 (20,00%)	8 (16,00%)	10 (8,20%)
CURIONÓPOLIS	7 (9,72%)	0 (0,00%)	2 (8,00%)	2 (4,00%)	9 (7,38%)
TOTAL	72 (100,00%)	25 (100,00%)	25 (100,00%)	50 (100,00%)	122 (100,00%)

Socio-epidemiological data revealed that most study subjects were women (78 - 63,93%), over 46 years of age (41,80%), and having taken only 1 dose of BCG (bacillus *Calmett-Guerin*) vaccine (63 - 51,64%). Among the group of household contacts, 49 were females (68,08%), over 46 years of age (27 - 37,50%), and having taken only 1 dose of BCG vaccine (40 - 55,56%). The group of patients was also mostly female, 29 (58%), with a predominance of 24 of them older than 46 years (48%), and 25 of them had not had a BCG vaccination (50%) (Table 2).

Table 2. Demographics of the subjects as to sex, age, and BCG.

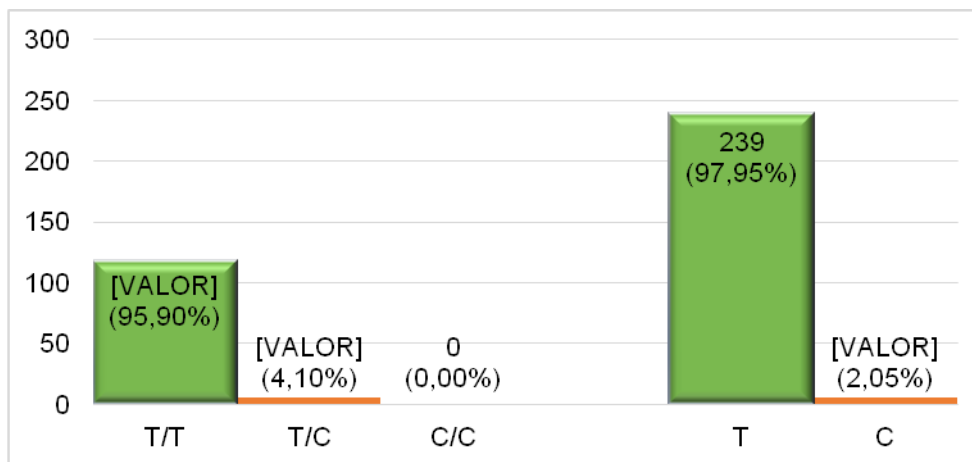
	CONTACTS N (%)	LL N (%)	TT N (%)	PATIENTS N (%)	TOTAL N (%)
Sex					
Male	23 (31,94%)	12 (48,00%)	9 (36,00%)	21 (42,00%)	44 (36,07%)
Female	49 (68,06%)	13 (52,00%)	16 (64,00%)	29 (58,00%)	78 (63,93%)
Age					
0-15	15(20,83%) ^{a,b,c,d}	1 (4,00%) ^{a,c,e}	1 (4,00%) ^{a,d,e}	2 (4,00%) ^b	17 (13,93%)
16-31	16 (22,22%) ^{a,b,c,d}	2 (8,00%) ^{a,c,e}	3 (12,00%) ^{a,d,e}	5 (10,00%) ^b	21 (17,21%)
32-46	14 (19,44%) ^{a,b,c,d}	6 (24,00%) ^{a,c,e}	13 (52,00%) ^{a,d,e}	19 (38,00%) ^b	33 (27,05%)
>46	27 (37,50%) ^{a,b,c,d}	16 (64,00%) ^{a,c,e}	8 (32,00%) ^{a,d,e}	24 (48,00%) ^b	51 (41,80%)
BCG					
0	18 (25,00%) ^{f,g,h,i}	12 (48,00%) ^{f,h,j}	13 (52,00%) ^{f,i,j}	25 (50,00%) ^g	43 (35,25%)
1	40 (55,56%) ^{f,g,h,i}	12 (48,00%) ^{f,h,j}	11 (44,00%) ^{f,i,j}	23 (46,00%) ^g	63 (51,64%)

≥2 14 (19,44%)^{f,g,h,i} 1 (4,00%)^{f,h,j} 1 (4,00%)^{f,i,j} 2 (4,00%)^g 16 (13,11%)

$P > 0.05$; ^a $P = 0.003$; ^b $P = 0.004$; ^c $P = 0.001$; ^d $P = 0.003$; ^e $P = 0.002$; ^f $P = 0.024$; ^g $P = 0.004$; ^h $P = 0.025$; ⁱ $P = 0.015$; ^j $P = 0.036$, Fisher's Exact Test

The genotype distribution for the SNP in TLR2 revealed that 117 individuals presented the T/T genotype (95,90%), followed by 5 presenting the T/C genotype. (4,10%). As for the alleles, 239 were T (97,95%) and 5 were C (2,05%) (Graph 1).

Graph 1. Distribution of the T/T, T/C, C/C genotypes and of the T and C alleles for the SNP in TLR2.



Considering the distribution of SNP rs3804100 genotypes of the TLR-2 gene among the evaluated groups, the majority of household contacts presented T/T genotype (68 – 94,4%) and only four the T/C genotype (5,56%). Among the LL group, one individual presented the T/C genotype (4,00%), while the T/T genotype was detected among all TT individuals (Table 3).

Table 3. Distribution of genotypes in the contact, LL, TT and patient groups for SNP rs3804100.

GENOTYPE	CONTACT N (%)	LL N (%)	TT N (%)	PATIENT N (%)
T/C	4 (5,56%)	1 (4,00%)	0 (0,00%)	1 (2,00%)
T/T	68 (94,44%)	24 (96,00%)	25 (100,00%)	49 (98,00%)
TOTAL	72 (100,00%)	25 (100,00%)	25 (100,00%)	50 (100,00%)

$P > 0.05$, Fisher's Exact Test

Allele distribution for SNP rs3804100 among the groups demonstrated that all TT individuals had only the T allele (100%), while in LL there was only 1 C allele, mutant (2%). In the contact group there were only 4 C alleles (2,78%). In the patient group, the only C allele found represented 1% (Table 4).

Table 4. Allele distribution for the contact, LL, TT and patient groups for SNP rs3804100.

ALLELE	CONTACT N (%)	LL N (%)	TT N (%)	PATIENT N (%)
T	140 (97,22%)	49 (98,00%)	50 (100,00%)	99 (99,00%)
C	4 (2,78%)	1 (2,00%)	0 (0,00%)	1 (1,00%)
TOTAL	144 (100,00%)	50 (100,00%)	50 (100,00%)	100 (100,00%)

$P > 0.05$, Fisher's Exact Test

4. DISCUSSION

Even though not fatal, and despite the advances in therapy, diagnosis and research, leprosy remains as a global public health problem, being one of the most frequent causes of non-traumatic peripheral neuropathy worldwide. Leprosy reactions, as a result of acute episodes of clinical inflammation during the chronic course of the disease are a challenging

issue as nerve damage increases morbidity even after treatment. In addition, its endemicity in tropical and developing countries is delimited by epidemiological, immunogenetic, socioeconomic and environmental risk factors [10].

Immunogenetic studies in relation with leprosy are essential to identify new markers (genes) in the genetic profile which may be associated with susceptibility and/or protection against the disease [11]. The innate immune response mediated by PRRs, such as TLR2, is the first line of host defense, which takes into account pathogen recognition. TLR2 is also required for the induction of IL-12, as the IL-12-dependent IFN- γ pathway is of paramount importance in cellular immunity, promoting Th1 response. The human TLR2 gene is located on chromosome 4 (4q32), consists of 3 exons, two coding and one non-coding [12]. TLR2 SNP rs3804100 has been described to affect and reduce exonic splicing sites, but still with uncertain role in TLR2 function [13].

Mycobacterium tuberculosis and *M. leprae* are mycobacteria that have common surface elements detected by the host. Krutzik et al., 2003 demonstrated that TLR1/2 heterodimers mediate monocyte activation promoted by *M. leprae*, indicating the presence of mycobacterial lipoproteins in addition to lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM), characterizing examples of TLR2 binding to mycobacterial components [14,15]. Thus, SNPs in the TLR2 gene may impair binding of the TLR1/2 heterodimer to mycobacterial lipoproteins, as in the case of SNP rs121917864, which promoted susceptibility to tuberculosis and leprosy in studies by Ben-Ali et al., 2004 and Kang and Chae, 2001 [16,17], respectively. The 19 kDa adhesion lipoprotein from *M. tuberculosis* shares 47% amino acid sequence similarity with the lipoprotein from *M. leprae* [18].

Genotyping studies have allowed associations between SNPs in the TLR gene and infectious diseases, such as those caused by mycobacteria. A study by Chen et al., 2010 in a Taiwanese population correlated the SNP rs3804100 and susceptibility to latent tuberculosis conferred by the CC genotype (in 4,3% of patients and in 4,3% of controls), which triggered elevation in the NK cell counts in the blood and, consequently, lead to disease progression [19]. Human NK cells have been reported to directly recognize mycobacteria through TLR2 and release TNF- α and IFN- γ [20]. However, these results are divergent in different populations due to distinction in the genetic makeup [21,22]. This genetic makeup corresponds to the genetic basis of the individual, which undergoes variations over time and due to environmental exposure [23].

There were no statistically significant associations between sex and leprosy, which was also observed among a Indian population in study by De Vries and Perry, 1985 [24]. However, most of the recent studies portray a discrepancy between the sexes: in Asian countries, leprosy affects men more than women, likewise in Brazilian studies, while in African countries women are more affected. Interestingly, among LL groups, African and Brazilian men were more affected [25–29]. The detection of leprosy among African women is probably explained by an aesthetics (body care) compared to men, as well as easier access to health services such as prenatal care [25]. The predominance of leprosy cases among men globally is probably due to the higher environmental exposure to *M. leprae* and/or the lack of women undergoing a complete physical examination in some cultures, leading to delayed diagnosis and the generation of physical disabilities [30].

In Brazil, studies by Rocha et al., 2020 and Romão and Mazzoni, 2013 revealed that higher detection of new cases are observed among those older than 46 years, likewise in the present study [31,32], which is probably linked to the lower exposure of younger people to the bacillus and longer incubation period for LL cases [30]. Analysis regarding age highlight the involvement of the economically active population and low transmission of the disease, in addition to contributing factors related to age-related immunological changes and higher susceptibility to infectious diseases among elderly people. Despite this concerning fact, most of cases among elderly are excluded from the Global Health Strategies for leprosy, making it difficult to conduct surveillance and control programs for the disease [33,34].

Since the 1960s, studies conducted in six different countries support the results obtained in the present study regarding the protection conferred by the second dose of BCG vaccine against leprosy among the groups, as 12,5% of the group of patients who took the 2nd dose in our study population [35,36]. In the present study, within the group of patients, about 50% had no BCG vaccination scar, revealing the lack of protective factor against the disease. The presence of a vaccination scar was the most common finding among all the groups analyzed. The calculation of BCG vaccine efficacy ranged from 0 to 50%, respectively, in Brazil and Malawi [37,38]. In India, the protection afforded by the first dose of BCG vaccine was 14% in the general population and 80% in contacts with leprosy. Several case-control studies have been conducted to evaluate the protective effect of BCG vaccine in leprosy, 12 of which included individuals with the first dose [36,39–42]. Most of these studies were conducted with people in tuberculosis control programs. The overall protection observed in these studies was between 36% and 90%, which is in line with the present study [40,42–45].

As similar in study by Bochud et al., 2008, the TLR2 SNPs rs3804099; rs3804100 were associated with leprosy reverse reaction, involving Ethiopian population, and for rs3804100 no significant associations were observed ($P= 0.73$), with CC

genotype present in 0,2% of patients and 0% among controls, with C allele being found rarely [46], corroborating the information in the present study about the lack of association between the studied SNP and leprosy. The T/C genotype was also more detected in both studies in the LL group compared to the TT group, which means similarity between contacts and TT due to lack of mutation in these groups, which could indicate that the T allele is related to protection from the disease and/or its more severe form.

Furthermore, in vivo recognition of mycobacteria seems to depend on multiple TLRs, since mice lacking a single type of receptor (such as TLR2) are only slightly more susceptible when exposed to aerosolized *M. tuberculosis* [47]. Another study by Bafica et al., 2005 demonstrated that concomitant recognition of mycobacteria through TLR2 and TLR9 produced most or all of the stimuli required to induce protective antimycobacterial immune responses that depend on TLRs [48].

The results of the present study have not shown associations between SNP rs3804100 and susceptibility or protection to leprosy, also demonstrating that this genomic region is well preserved in the Brazilian Amazon population. Thus, due to the low frequency of SNPs evaluated in the population investigated, further in-depth studies are required, in addition to the investigation of other polymorphisms, in order to elucidate the relationship between SNP rs3804100 in the TLRs gene and the immune response developed by the pathogen-host interaction.

5. CONCLUSION

In conclusion, no significant association between sex and leprosy was observed in this study. An association between age group and leprosy was observed, which was significant among those older than 46 years. Correlation of 2nd dose of BCG was found to be more protective among the groups analyzed. The allelic and genotypic frequencies of the SNPs found were undifferentiated among the three study groups. No association was observed between the SNP rs3804100 of TLR2 and leprosy. A predominance of T/T genotype and T alleles was observed in all PB group individuals. The mutant genotype, T/C was observed only in contacts and TT.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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