

Single Nucleotide Polymorphisms (SNPs) at -238 and -308 Positions in the TNF α Promoter: Clinical and Bacteriological Evaluation in Leprosy¹

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ABSTRACT

Tumor necrosis factor alpha (TNF α) plays a key role in orchestrating the complex events involved in inflammation and immune response. The presence of single nucleotide polymorphisms (SNPs) within the promoter region of the TNF α gene has been associated with a number of diseases. The aim of this study was to investigate the distribution of polymorphisms at positions -238 (G/A) and -308 (G/A) at the TNF α promoter, and its association to the outcome of different clinical forms of leprosy. Furthermore, the bacteriological index (BI) was evaluated among genotyped multibacillary (MB) patients in order to investigate the possible influence of each polymorphism on the bacterial load. This study included a total of 631 leprosy patients being 401 MB and 230 paucibacillary (PB), that was further separated according to its ethnicity (Afro- and Euro-Brazilians). The combination of SNPs in haplotypes generated three different arrangements: TNFG-G, TNFG-A and TNFA-G. In spite of the marked differences observed in the frequency of the haplotypes along the ethnic groups, no statistical differences were observed in haplotype frequencies between MB and PB patients. The BI analyses showed a lower bacteriological index among the -308 carriers, while the BI of the -238 carriers was higher. Although no significance has been achieved in this analysis regarding the influence of the polymorphisms to the development of the clinical outcome, it seems that in a different stage (among the MB patients) the polymorphisms could contribute to the degree of severity observed.

RÉSUMÉ

Le facteur alpha de nécrose tumorale (TNF α) joue un rôle important dans l'ajustement des événements complexes qui régulent l'inflammation et la réponse immunitaire. La présence de polymorphismes mono-nucléotidiques (SNPs) au sein de la région promotrice du gène codant pour TNF α a été associée à un certain nombre de maladies. Le but de cet article était d'explorer la distribution de polymorphismes aux positions -238 (G/A) et -308 (G/A) du promoteur de TNF α et son association aux résultats phénotypiques des différentes formes de lèpre. De plus, l'index bactérioscopique (IB) a été évalué parmi les patients multibacillaires (MB) génotypés dans le but d'évaluer la possible influence de chaque polymorphisme sur la charge bactérienne. Cette étude a porté sur 631 lépreux comportant 401 MB et 230 PB, qui furent encore séparés par ethnie (Afro et Euro-brésiliens). La combinaison des SNPs en haplotypes a généré 3 arrangements différents : TNF-G, TNF-A et TNFA-G. En dépit de différences marquées observées dans les fréquences haplotypiques entre les groupes ethniques, aucune différence statistiquement significative ne fut observée entre les patients MB et PB. Les analyses de IB ont montré un index bactérioscopique plus faible parmi les porteurs -308, tandis que le IB des porteurs -238 était plus élevé. Bien que cette analyse de polymorphismes n'ait pas démontré de différence significative sur l'issue clinique de la

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lepre, il semble qu'à un certain stade de la maladie des patients multibacillaires, ces polymorphismes pourraient contribuer au degré de sévérité observé.

RESUMEN

El factor de necrosis tumoral alfa (TNF α) juega un papel importante en la orquestación de los complejos eventos que ocurren en la inflamación y la respuesta inmunitaria. La presencia de polimorfismos simples en polinucleótidos (SNPs) dentro de la región promotora del gene TNF α ha sido asociada a numerosas enfermedades. El objetivo del estudio fue el investigar la distribución de los polimorfismos en las posiciones -238 (G/A) y -308 (G/A) del promotor del TNF α , y su asociación con las diferentes formas clínicas de la lepra. Se evaluó, además, el índice bacteriológico entre los pacientes MB estudiados para poder investigar la posible influencia de cada polimorfismo sobre la carga bacilar. En el estudio se incluyeron 631 pacientes con lepra (401 MB y 230 PB) los cuales fueron adicionalmente separados de acuerdo a su etnicidad como Afro- y Euro-brasileños. La combinación de SNPs en los haplotipos generó 3 diferentes arreglos: TNFG-G, TNFG-A y TNFA-G. No obstante las marcadas diferencias en la frecuencia de los haplotipos entre los grupos étnicos, no se observaron diferencias estadísticas en las frecuencias de los haplotipos entre los pacientes MB y PB. Los análisis mostraron un menor índice bacteriológico entre los portadores-308 que entre los portadores-238 donde el IB fue mayor. Aunque en este análisis sobre la relación entre los polimorfismos y el tipo clínico de la enfermedad no se llegó a observar significancia estadística, parecería ser que al menos entre los pacientes MB los polimorfismos pueden contribuir al grado de severidad observado.

Leprosy, a chronic human disease, is the result of infection by *Mycobacterium leprae*. The clinical spectrum of the disease includes two polar (lepromatous-LL and tuberculoid-TT) and three borderline forms (tuberculoid-BT, borderline-BB, and lepromatous-BL) (19). The multibacillary (MB) form, including LL, BB, and BL, is characterized by low immune responsiveness and high bacterial load. The paucibacillary (PB) form, including TT and BT, is marked by strong cell-mediated immunity against the bacillus (22). Much evidence has implicated cytokines in the immune response of leprosy (16), mainly the tumor necrosis factor-alpha (TNF α), which has a beneficial function in host defense but, if produced in high levels, contributes to tissue damage (25).

The presence of single nucleotide polymorphisms (SNPs) in the TNF α promoter region and their association with autoimmune and infectious diseases has been extensively studied (30). The polymorphism detected at position -308 (G/A) within the promoter region of the TNF α gene (29) was the first one found to be associated with disease (18). This polymorphism has been observed in association with several other infectious diseases where excessive TNF α production seems to play a role, such as in cerebral malaria (12) and mucocutaneous leishmaniasis (2). An association of the -308 polymorphism with the development of lepromatous leprosy was previously reported

in an Indian population (21). However, in Brazilian leprosy patients the association of this polymorphism with protection has been described (23, 24, 27). A plausible explanation for these divergent findings may involve the ethnicity, where the allelic frequency of TNF α -308A in leprosy patients range from 7.0 to 10.8 in Indian and Brazilian, respectively (21, 24). This allelic frequency variation is wide among populations such as Caucasian Irish (23.0), African Zulu (22.1), Arabian Omani (8.1), Singapore Chinese (12.0) and Mexican Mestizos (2.5) (13). Studies at another G/A transition polymorphism (-238) in the TNF α promoter region (4) has shown that the A allele was increased among patients with chronic hepatitis B and C, suggesting association to disease susceptibility (7, 8) while in cancer a protective effect was observed (9). In fact, the complex relationship between SNPs in the human genome and disease association indicates the need for the construction of haplotypes (specific combination of SNPs on the same chromosome) on the locus studied because they are more informative than any single SNP (1, 26).

In the recent analyses from Brazilian leprosy patients we have shown in paucibacillary forms an increased allelic frequency of the TNF-308A in comparison to the multibacillary (0.14 and 0.09, respectively) with a borderline significance ($\chi^2 = 3.47$; $p = 0.06$) (24). This data did not define if TNF

-308A was a trend marker for protection, i.e., allele frequency in control > PB > MB or whether TNF-308A discriminates between cases and controls only, being a resistance locus of leprosy *per se*. Thus, the aim of this study was to describe whether there was a difference between PB and MB using an increased number of patients. To overcome a possible cryptic stratification that would impact the SNP frequency and mask an association effect, patients were separated according to ethnicity. Besides, the -308 and -238 SNPs were combined in haplotypes that better analyze the TNF promoter region. In addition, to understand the impact of polymorphisms of TNF α promoter in relation to progression of the disease, the comparison between SNPs in -308 and -238 position and the bacteriological index (BI) was set out in MB patients.

MATERIALS AND METHODS

Patients. Six hundred and thirty one leprosy patients from the Leprosy Out-Patient Unit, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were included in this study. They were diagnosed on the basis of clinical and bacteriological criteria and classified according to the Ridley and Jopling Scale (20). Four hundred one MB and 230 PB patients were studied, including 405 males and 226 females. Brazilians were classified according to their ethnic origin after careful inspection of facial morphological features, hair type and skin color. Two groups were ascertained: Afro-Brazilians and Euro-Brazilians with N = 251 and N = 235, respectively. Asians and Amerindians are not commonly represented in the population of Rio de Janeiro and were not observed among the individuals inspected.

Bacteriological index determination. Bacteriological index (BI) was determined according to Ridley, 1964 (19) among the multibacillary patients in slit skin smears from six different anatomic sites and ranged from 0.16 to 5.33 (mean = 2.50 \pm 1.46).

DNA extraction and SNPs genotyping. Genomic DNA was prepared from frozen whole blood collected with sodium citrate buffer by a commercially available DNAzol extraction kit (Invitrogen Life Technologies, Gaithersburg, MD, USA). Genotyping of the TNF α promoter region for analysis of polymorphisms at the -238 and -308 positions was performed by polymerase chain

reaction (PCR)-restriction fragment length polymorphism (RFLP), a single PCR step and further restriction analysis (6, 29). Restricted amplified products were visualized by electrophoresis in 3% agarose gel and ethidium bromide staining.

Statistical analysis. The statistical significance of TNF α promoter polymorphism distributions was analyzed by way of the χ^2 test Odds-ratio (OR) and 95% confidence intervals (CI). Yates' correction or Fisher's exact test was used when appropriate (Epi Info 6: CDC, Atlanta, GA). The significance level adopted was p < 0.05. The haplotype frequencies were estimated using the EH program (28).

Generalized additive model. the variability of the bacteriological index (BI) in relation of the presence of the mutation in the TNF α promoter was studied through a generalized additive model (GAM). The GAM is a special regression model, in which some of regression assumptions are relaxed. So, GAM models may have advantages in many biological phenomena, and may replace traditional linear or logistic regression models. In mathematical terms, GAM models allow predictors (or covariates) to be replaced by arbitrary smooth functions. For instance, B-splines (polynomials that can be adjusted to a set of points) could be used as a smoothing function. In this context, a GAM model was used to study the association between the occurrence of the G/A substitution in the positions -308 and -238 of the promoter gene of TNF α , and the bacilloscopic index, calculated as previously described. For the adjustment of the model, one should indicate the number of nodes necessary. A node is the number of points used for the curve to be adjusted. The SPLUS 2000 program, Professional Release 3, MathSoft, Inc. was used to perform these analyses.

RESULTS

Analyses of TNF α haplotypes frequencies in leprosy patients in different Brazilian ethnic groups. Haplotypes of TNF α were estimated using a maximum-likelihood probability test from -308 and -238 SNP genotypes of unrelated patients stratified in Euro- and Afro-Brazilian (The Table). Three different haplotypes on the TNF α gene have been identified (TNFG-G, TNFA-G, TNFG-A). No statistical differ-

THE TABLE. *Haplotypes frequencies of the TNF α promoter polymorphisms among MB and PB leprosy patients within distinct ethnic groups.*

Haplotypes	Brazilian		Euro-Brazilian		Afro-Brazilian	
	MB	PB	MB	PB	MB	PB
-308/-238						
G-G	0.83	0.81	0.82	0.78	0.84	0.86
G-A	0.05	0.05	0.05	0.04	0.04	0.04
A-G	0.10	0.12	0.12	0.16	0.10	0.08

ences were observed in haplotypes from TNF α promoter between MB and PB patients irrespective to the ethnic group analyzed. The TNFG-G haplotype in both PB and MB forms presented the highest frequency among patients in both ethnic groups. Marked differences between Euro- and Afro-Brazilians were observed when other haplotypes were analyzed. Among PB patients, an increased frequency of the TNFA-G haplotype in Euro-Brazilian was detected in comparison to Afro-Brazilian (0.16 and 0.08, respectively) was detected.

Analyses of the bacteriological index (BI) from multibacillary patients genotyped for SNPs at position -308 and -238. Bacteriological index (BI) variability in relation to the presence of the -238 (N = 343) and -308 (N = 341) polymorphisms was analyzed via the GAM (Fig. 1A, B). The GAM regression model was performed to study the association between the presence of the A allele at the positions -238 and -308 of the promoter gene of TNF α , as the dependent variable, and the variability of the baciloscopic index, as a predictor variable. For the model using the substitution at position -238, a regression polynomial spline with 1,5 nodes was used. It was observed that the probability for the occurrence of the A allele at the -238 position increases with higher bacteriological index (Fig. 1A).

For the model using the substitution at position -308, a regression polynomial spline with 1,1 nodes was used. In contrast to the result obtained with -238, the probability for the occurrence of the A allele at the -308 position is greater with a low BI (Fig. 1B).

DISCUSSION

Studies using the frequency of single nucleotide polymorphisms in candidate genes are interesting approaches to the investigation of the susceptibility and severity of diseases. It is believed that SNPs are relevant

since they can be used as genotypic markers of specific disease phenotypes or can regulate biological phenomena influencing mRNA expression, thereby altering mRNA isoforms (unravelling cryptic splicing sites) or modifying enzymatic activity of genes (¹¹). The problem is that SNP frequencies vary enormously among populations (¹³), especially Brazilians who originated from a variety of ethnicities, mainly Portuguese explorers mixed with native Amerindians and Africans (³). The outcome of this admixed colonization is a dense population without a clear genetic/morphological ethnic cut off (¹⁷). However, some cryptic stratification may still be functioning as confounding factors in Brazilians in population-based studies. Indeed, the separation according to the morphological features of the patients better discriminate Afro- and Euro-Brazilians, where a difference in the -308A/-238G haplotype frequency from Afro-Brazilian (9%) to Euro-Brazilian (13%) patients was observed. Still, no statistical differences were observed when PB and MB were compared, demonstrating that if there is some cryptic stratification due to admixture, it is not being detected by conventional morphological inspection in our patient population. Thus, to scrutinize the stratification in Brazilian population-based studies it would be necessary to use genomic controls (⁵). Moreover, a recent study performed in Gambian and Malawian populations studying SNPs spanning 4.4kb of the TNF α /LT α locus demonstrated the need to Type 8 in Gambians, and 7 out 12 SNPs in Malawians, to detect the haplotypic structure and informative SNPs in this region due to the high frequency of recombination (¹). We do not have data for the Brazilians but it seems to be necessary to enlarge the focused region of the TNF locus to capture more information about severity in leprosy.

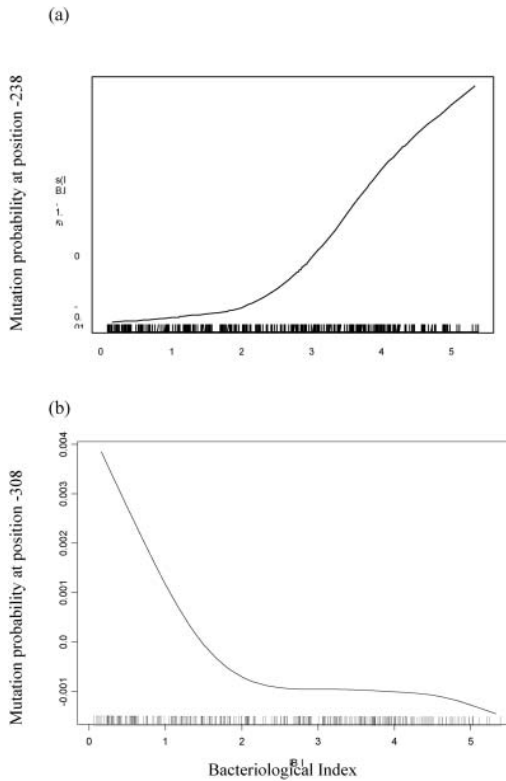


FIG. 1. Analyses of the bacteriological index (BI) variability of TNF α promoter polymorphism -238 (a) and -308 (b) by Generalized Additive Model (GAM) in 341 and 343 multibacillary genotyped patients, respectively. The “y” axis means the probability of “A” allele occurrence (homozygous or heterozygous) and BI at “x” axis, range from 0.16 to 5.33. Each individual BI is represented as a small bar.

On the other hand, the analysis of BI and its association with polymorphisms at positions -308 and -238 in TNF α suggested these polymorphisms are functionally relevant. We previously demonstrated that TNF α -308A was associated with a stronger response in Mitsuda reaction⁽¹⁵⁾. In this study, GAM analyses in -308A, revealed that such patients have lower BIs. The results of our previous findings⁽¹⁵⁾ with this new data is that TNF -308A could be upregulating the secretion of TNF α that, in turn, induces a stronger DTH skin response in paucibacillary patients and restricts *M. leprae* growth in multibacillary patients.

The opposite was verified concerning the study of -238A and BIs. In this case, the presence of the A allele was more frequent in multibacillary patients with higher BIs. This

data is in accordance with the literature, where it has been demonstrated that the -238A polymorphism is associated with lower levels of TNF α ⁽¹⁰⁾.

The possibility of using slit skin smears is one of the few alternatives for *in vivo* determination of the bacterial load. The BI is one of the clinical parameters indicating disease progression and severity, representing a clear risk factor for the development of the acute inflammatory episodes in leprosy⁽¹⁴⁾. Thus, by way of the adjusted model (GAM), the existence of a clinical significance for the variability of BI in relation to the presence of polymorphisms in the TNF α promoter suggests a functional dichotomy between the -308 and -238 SNPs in relation to TNF regulation and leprosy progression.

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