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Haplotype of the Promoter Region of *TNF* Gene May Mark Resistance to Tuberculosis in the Amazonas State, Brazil

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Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is worldwide and keeps on increasing with the appearance of HIV. Host genes involved in the immune response play key role in conveying resistance or susceptibility to TB. We investigated a possible association of the different single nucleotide polymorphisms (SNPs) present in the promoter region of the *TNF* with either susceptibility or resistance TB. Nucleotide sequencing of the promoter region of *TNF* encompassing the SNPs –238 G/A, –308 G/A, –857 C/T, –863 A/C and –1031 T/C was performed on 230 TB patients and 293 control subjects. The –863 A/C and –857 C/C genotypes show protection to TB. The –308A allele is associated with protection to TB. Haplotypes analysis revealed that the haplotype –1031T/–863C/–857C/–308A/–238G is protective to TB (p=0.024). Our data suggests that SNPs present in the promoter region of *TNF* are associated with resistance to the development of TB.

Keywords: TNF; Polymorphism; Tuberculosis; Genetic marker

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major infectious disease and is worldwide. Approximately 9 million individuals developed TB in the year 2013 [1]. One-third of the world population is considered infected by *M. tuberculosis*, but only 5 to 10% develop active TB. Pulmonary TB is the major clinical form while extrapulmonary TB is present in only 10-20% of patients with TB [2-4].

Host genes involved in the immune response play key role to susceptibility or protection to *M. tuberculosis* infection [2,5-9]. TNF-α play an important role in the cellular immune response to TB [6,10]. Macrophages infected by *M. tuberculosis* display enhanced production of TNF-α cytokine [11]. TNF-α participates directly in the macrophage activation, reactive nitrogen production and in the maintenance of granulomas to limit the spread of the mycobacterial growth [10,12-14].

The *TNF* gene, approximately four kilobases, is located on chromosome 6p21.3 within the major histocompatibility complex (MHC) class III region and consists of four exons. The promoter region presents various single-nucleotide polymorphisms (SNPs) that have been suggested to influence the gene expression [15-17]. In this study, we investigated whether the SNPs –238 G/A (rs361525), –308 G/A (rs1800629), –857 C/T (rs1799724), –863 A/C (rs1800630) and –1031 T/C (rs1799964) in the promoter region of the *TNF* gene are associated with either susceptibility or resistance to TB in patients from the Brazilian state of Amazonas.

Subjects and Methods

Study population

This is a case-control study including 230 unrelated patients with TB and 293 unrelated healthy control subjects aged between 18 and 65 years from the Brazilian Legal Amazon states. All of the patients with TB were followed at the reference center for diagnosis and treatment of TB, the Policlínica de Referência em Pneumologia Sanitária Cardoso Fontes, Manaus, Amazonas, Brazil. Unrelated healthy individuals of the same ethnicity as the patients were selected to represent a control group. Healthy subjects with a family history of mycobacterial diseases were excluded. All of the participants in the study gave a written informed consent according to the Institutional Ethical Committee. This study was approved by the institutional review board (number: 05925212.8.0000.0006).

All patients with TB were initially diagnosed by experienced professionals. Clinical evaluation is based on chest radiography (X-ray), sputum-smear microscopy tests, molecular test (geneXpert), *M. tuberculosis* culture with biochemical analysis and PCR for confirmation of MTB.

The control subjects were also evaluated clinically and individuals with any respiratory symptom was submitted for mycobacteriology assays such as the detection of MTB in sputum and culture for *M. tuberculosis* to confirm the absence of the disease.

Biological material and DNA purification

Peripheral blood samples from all the participants were collected for DNA purification with the QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The eluted DNA was stored at -80°C until use.

Polymerase chain reaction (PCR) and purification of PCR product

The promoter region of *TNF* encompassing the SNPs –238 G/A (rs361525), –308 G/A (rs1800629), –857 C/T (rs1799724), –863 A/C (rs1800630) and –1031 T/C (rs1799964) was PCR amplified by the following pair of primers: Forward 5'-GAATGGAGGGAGGGACAGAGGGCT-3' and Reverse 5'-CGAGAGGAGGGCGGGGAAAGAATC-3' generating a PCR product 1146 base pairs. The cycling conditions of the PCR, as well as the purification of the PCR product were performed as described elsewhere [18].

Nucleotide sequencing and sequence analysis

Approximately 10-40 ng of purified PCR product was applied in the sequencing reaction. Nucleotide sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing (Applied Biosystems, Foster City, California) and the above primers individually. A third internal primer, the upstream primer 5'-GCCTCAAGCCTGCCACCAAGC-3' was also used to cover the sequencing of the all 1146 bp fragment. Capillary electrophoresis was performed in the ABI 3130 DNA sequencer with the polymer POP-7™. The sequences were initially analyzed with the Sequencing Analysis software (Applied Biosystems, v5.3.1) and further analyzed with the Geneious software (Biomatters, v6.0.5) to build contigs compared with the *TNF* reference gene sequence (GenBank ID: NG_007462).

Haplotype analysis

The haplotypes frequencies were calculated using the Haploview software (v.4.2), as a measure of the Linkage Disequilibrium (LD). Haplotypes with frequencies <1% were not considered as relevant for comparisons. The degree of pairwise between nucleotides was analyzed by LD structure, considering r2 values >0.8 as strong LD, <0.8 for weak and <0.1 as negative LD.

Statistical analysis

The χ^2 and Mann-Whitney tests were used to compare the variation in gender and age between TB patients and control subjects. The genotype and allele frequencies were determined by direct counting. The Hardy-Weinberg equilibrium was calculated by comparing estimated to observed frequencies of genotypes by χ^2 test. The haplotypes frequencies were compared between patients and control subjects using the Haploview (v.4.2). Comparison of genotypes and alleles frequencies between patients with TB and healthy controls were performed by χ^2 and Fisher exact test along with the Odds Ratio and 95% confidence of interval using the site (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl.).

Results

Study population

Basic characteristics of the patients and controls included in the study are shown in Table 1. The mean age of the TB patients and controls are 33.4 ± SD 12.2 and 33.6 ± SD 12.1 respectively. Male subjects were prevalent among patients (65.2%) compared to healthy controls (43%) and was statistically significant. There was a higher prevalence of multibacillary TB (65.7%) than paucibacillary TB among patients (21.3%). Pulmonary TB was common (87.8%).

	Patients (n=230)	**Controls (n=293)**
Age (years)	*n* (%)	*n* (%)
Mean	33.4 ± 12.2	33.6 ± 12.1
Gender		
Male	150 (65.2)	126 (43)
Female	80 (34.8)	167 (57)
Bacteriologic index (BI)		
Paucibacillary	49 (21.3)	
Multibacillary	151 (65.7)	
No defined	30 (13)	
Tuberculosis form		
Pulmonary	202 (87.8)	
Extrapulmonary	28 (12.2)	
Mann Whitney Test for age between patients and control subjects p=0.84;		
χ^2 Test for gender p<0.0001.		

Table 1: Characteristics of the study population.

Genotype and allele frequencies of the polymorphisms in the *TNF* promoter region

The genotypes and alleles distribution of the SNPs *TNF* –1031 T/C, –863 A/C, –857 C/T, –308 G/A and –238 G/A are shown in Table 2. All the SNPs were in Hardy-Weinberg equilibrium in both patients with TB and healthy controls.

The *TNF* –1031 T allele was more prevalent in patients with TB and shows a trend of association with susceptibility (OR=1.34; 95% CI=1.00–1.78; p=0.055). The –308 G allele is associated with susceptibility to TB (OR=1.86; 95% CI=1.09–3.16; p=0.026) while the –308 A allele with protection (OR=0.53; 95% CI=0.31–0.90). The –308 G/G genotype confers twice a risk of developing TB in comparison to carriers of the –308 A allele (OR=1.85; 95% CI=1.07–3.20; p=0.035). The –863 A/C genotype shows protection to TB (OR=0.65; 95% CI=0.44–0.95; p=0.036). Similarly, the –857 C/C genotype was significantly higher among the control subjects (73%) than in TB patients (63.5%) (OR=0.64; 95% CI=0.44–0.93; p=0.024).

The genotype and allele frequencies for each SNP were similar among paucibacillary and multibacillary groups (data not shown).

	N (Frequency)			
Genotype/ Allele	**Patients**	**Controls**	**OR (95% CI)**	***p* value**
-1031 T/C				
T/T	143 (62.2)	157 (53.6)	1.42 (1.00 - 2.02)	0.059
T/C	76 (33)	116 (39.6)	0.75 (0.52 - 1.08)	0.146
C/C	11 (4.8)	20 (6.8)	0.68 (0.32 - 1.46)	0.426
T	362 (78.7)	430 (73.4)	1.34 (1.00 - 1.78)	
C	98 (21.3)	156 (26.6)	0.74 (0.55 - 0.99)	0.055
-863 A/C				
A/A	10 (4.4)	6 (2.1)	2.17 (0.77 - 6.07)	0.207
A/C	56 (24.3)	97 (33.1)	0.65 (0.44 - 0.95)	0.036
C/C	164 (71.3)	190 (64.8)	1.34 (0.92 - 1.95)	0.14
A	76 (16.5)	109 (18.6)	0.86 (0.62 - 1.19)	
C	384 (83.5)	477 (81.4)	1.15 (0.83 - 1.59)	0.427
-857 C/T				
C/C	146 (63.5)	214 (73)	0.64 (0.44 - 0.93)	0.024
C/T	77 (33.5)	68 (23.2)	1.66 (1.13 - 2.44)	0.012
T/T	7 (3)	11 (3.8)	0.80 (0.30 - 2.10)	0.84
C	369 (80.2)	496 (84.6)	0.73 (0.53 - 1.01)	
T	91 (19.8)	90 (15.4)	1.35 (0.98 - 1.87)	0.072
-308 G/A				
G/G	209 (90.9)	247 (84.3)	1.85 (1.07 - 3.20)	0.035
G/A	21 (9.1)	44 (15)	0.56 (0.32 - 0.98)	0.058
A/A	0 (0)	2 (0.7)	NA	NA
G	439 (95.4)	538 (91.8)	1.86 (1.09 - 3.16)	
A	21 (4.6)	48 (8.2)	0.53 (0.31 - 0.90)	0.026
-238 G/A				
G/G	210 (91.3)	254 (86.7)	1.61 (0.91 - 2.84)	0.129
G/A	19 (8.3)	37 (12.6)	0.62 (0.34 - 1.11)	0.144
A/A	1 (0.4)	2 (0.7)	0.63 (0.06 - 7.05)	0.833
G	439 (95.4)	545 (93)	1.57 (0.91 - 2.70)	
A	21 (4.6)	41 (7)	0.63 (0.37 - 1.09)	0.128
NA: not available due to zero values present				

Table 2: Genotype and allele distribution of polymorphisms in the *TNF* promoter region between tuberculosis patients and control subjects.

Haplotypes distribution

The LD among the five SNPs is shown in Figure 1 and pairwise nucleotides analysis generates six haplotypes (Table 3). Comparison of the haplotypes between patients and controls revealed an association of the haplotype –1031T/–863C/–857C/–308A/–238G with protection to TB (p=0.024). Comparison of this haplotype according to sputum

						N (frequency)			
Haplotype	**-1031**	**-863**	**-857**	**-308**	**-238**	**Patients**	**Controls**	**Chi Square**	***p* value**
1	T	C	C	G	G	125 (54.4)	146 (49.7)	2.256	0.133
2	C	A	C	G	G	37 (16.3)	53 (18.1)	0.573	0.449
3	T	C	T	G	G	45 (19.5)	45 (15.1)	3.509	0.061
4	T	C	C	A	G	11 (4.6)	24 (8.0)	5.044	0.024
5	C	C	C	G	A	11 (4.6)	20 (7.0)	2.732	0.098
6	C	C	C	G	G	1 (0.4)	5 (1.7)	3.671	0.055

Table 3: Haplotypes of the promoter region of TNF present in patients with TB and control subjects.

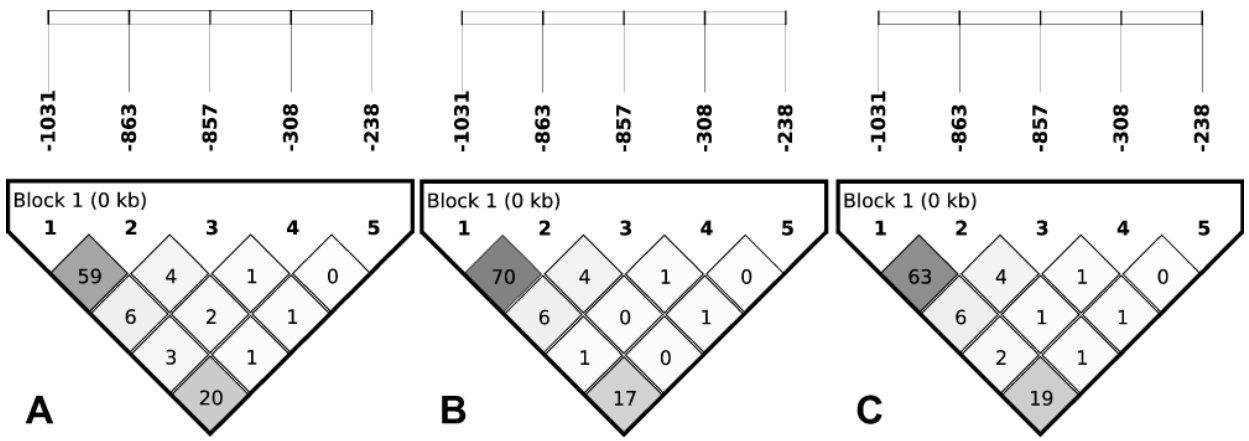

Figure 1: Linkage disequilibrium structure of the promoter region of *TNF*. **(A)** LD in control subjects, **(B)** LD in tuberculosis patients and **(C)** LD in study population. Numbers in the boxes indicate the correlation coefficient value of LD (r^2) multiplied by 100. The box trends toward to white indicate decreased r^2 value, strong LD represented by black box. Strong LD is only observed between the SNPs –1031 and –863.

smear status showed no statistical difference between AFB smear positive (16/302; 5%) and AFB smear negative (2/98; 2%).

Discussion

In the course of infection caused by *M. tuberculosis*, macrophages are activated initially to produce inflammatory cytokines liberated by cells of the innate and adaptive immune response [7]. The TNF-α cytokine plays an important role in the development of the cellular immune response against *M. tuberculosis* and participates in the granulomas formation, as well as in the cellular orchestration and activation of the molecular mechanisms of macrophages [6,10,12-14]. Several SNPs present in the promoter region of *TNF* are reported to correlate with TNF-α levels [17,19-22]. The –308 A allele correlates with higher levels of the cytokine [19] while the haplotype –1031 C/–863 A/–857 T with higher transcriptional activity [20].

The frequencies of the different SNPs in the promoter region of *TNF* observed in this study is similar to those observed in other populations from America, Asia and Europe [23-26], except for the frequencies of the –308 G/A SNP which differs in the African population. The –308 A allele is around 20% in the African population [23]. The genotypic distributions of the –1031 T/C, –863 A/C, –857 C/T SNPs found in the North Indians population of India [27] also differ from our study.

A recent study showed a possible association for the risk of TB in HIV-infected subjects carriers of the –1031 T allele [28]. Peripheral blood mononuclear cells from healthy carriers of the *TNF* –1031 C allele activated with Concanavalin-A (Con-A) secreted higher levels of the TNF-α cytokine [20]. Recently, we showed that individuals with the –1031 C/C genotype have nine times more chance to present resistance to leprosy caused by *M. leprae* than carriers of the T/T genotype [18]. In this study, the –1031 T allele is associated with susceptibility to TB and reinforces that the -1031T allele may present a risk to infectious diseases.

Several studies showed a lack of association of the *TNF* –863 A/C SNP with TB in Asian subjects [24,27,29]. A meta-analysis study also confirmed this lack of association [30]. However, in one study, carriers of the –863 A/A genotype were shown to have twice the chance of developing TB [25]. Our data shows that heterozygosity for the –863 SNP is associated with protection to TB. Of note, this allele –863 C when associated with the –308 A allele haplotype-wise confers protection to TB but not with the –308G allele. Importantly, the –308 A allele or the –863 C allele are reported to bind with better affinity to

transcriptional factors than their counterpart allele [19,22] and TNF-α has been shown necessary for protection against *M. tuberculosis* infection in mice model [10].

Various studies showed discrepancy results for the *TNF* –857 C/T SNP to TB. The –857 T/T genotype was associated with protection in the Chinese population [25] in contrast to the Iranian population [29] and our study where the –857 C/C genotype confers protection to TB. However, a meta-analysis study performed among Asian subjects with a total of 3630 patients with TB and 4055 controls, showed association of the –857 C/T genotype with higher risk to TB [30]. Our data is consistent with the meta-analysis study.

The *TNF* –308 G/A and –238 G/A SNPs have been extensively investigated in different case-controls TB studies. Controversial results have been observed. Some have shown lack of association [24,29,31–35], while others suggested association with TB [36–38]. Moreover, three meta-analyses of the –308 SNP, one with ten studies consisting of 1.167 patients and 1.720 controls [39] and the other two with a higher sample size [30,40], showed lack of association between the –308 G/A SNP and TB. Our data differs to the meta-analysis studies and is consistent with the study of Correa et al. [36] where the -308A is protective to TB.

Three studies showed different association of the *TNF* –238 G/A SNP with TB [36-38]. Two of the studies cited the –238A allele as protective to TB [37,38] in contrast to the other affording susceptibility [36]. This –238 SNP did not show any association to TB in this study despite the frequency of the –238 A allele is 7% among the healthy individuals compared to 4.6% in TB patients. The lack of association may be due to the genetic background of our population compared to the Iranian [37] or the Colombian population [36]. Interestingly, the frequency of the minor allele in the healthy Iranian and Colombian population is 19 and 11% respectively compared to 7% in our population. This may suggest that an increase in our sample size may detect an association. However, this may not be likely as we detected an association with the –308 A allele and the frequency is 4.8% among the TB patients and 8% in healthy individuals.

A number of functional studies showed differential transcriptional activity of the different SNPs in the promoter region of the *TNF* using the luciferase reporter gene assay. The –308A allele was associated with a higher transcriptional activity compared to the –308G allele [17,19,41]. A cloned fragment containing the *TNF* SNPs –1031 T/C (rs1799964), –863 A/C (rs1800630), –857 C/T (rs1799724), –308 G/A (rs1800629) and –238 G/A (rs361525) upstream of the luciferase gene in the pGL3 basic vector lacking a eukaryotic promoter showed different transcriptional activity [20,42]. The cloned fragment containing the –863A allele was related with reduced transcriptional activity of the *TNF* gene in monocytes and hepatic cells [42]. Peripheral blood mononuclear cells from healthy Japanese carriers of at least one of the alleles –1031T, –863C, –857C and –308A showed higher levels of TNF-α when stimulated with Con-A [20]. In healthy Indian subjects, the haplotype –1031T/–863C/–857C/–308G was significantly associated with a higher production of the TNF-α cytokine in comparison to the –1031C/–863A/–857C/–308G and other haplotypes [22]. Electrophoretic mobility shift assays have shown that combination –863C/–857/T or –863A/–857/C bind with nuclear binding proteins such as the Octamer Binding Transcription Factor 1 (OCT-1) [21,22,42]. However, the –863C/–857/T binds with a better affinity. Here, we showed that the haplotype TCCAG is protective. It is highly possible that individuals, carriers of the haplotype –1031T/–863C/–857C/–308A/–238G correlated with higher levels of transcription, are protected to TB development. TNF has been shown to play an important role in the protective immune response against *Mycobacterium tuberculosis* in mice [10]. It is of interest to see if TB patients with the haplotype TCCAG respond better to chemotherapy in future studies. Although our sample size is not large enough to generate higher number of individuals with this haplotype (only 11 patients and 24 controls), functional studies by other groups showing this haplotype correlates with higher levels of TNF-α do confirm this haplotype may be protective. The haplotype CCCGA did not show any statistical difference despite present in 20 control subjects compared to 11 TB patients. An increase in our sample size may probably detect an association. Notably, the –863 C allele has been correlated to higher levels of TNF-α and combining both haplotypes may show a higher protective association.

Altogether, the SNPs present in the promoter region of *TNF* may mark resistance to the development of TB in the Brazilian population of the state of Amazonas. Different haplotypes of these SNPs may potentially influence different immune responses against infection caused by *M. tuberculosis* due differential transcriptional activity of the *TNF* gene. It will be of utmost interest in reproducing the results of this study with a higher sample size and to perform functional studies with the different haplotypes to correlate with either transcriptional activity or levels of TNF-α.

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