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Influence of the Interferon–Gamma (IFN– γ) and Tumor Necrosis Factor Alpha (TNF– α) Gene Polymorphisms in TB Occurrence and Clinical Spectrum

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1. Introduction

Tuberculosis (TB) is a major public concern and is the most important single infectious cause of mortality and morbidity worldwide. According the World Health Organization (WHO) records, in 2009, there were an estimated 9.4 million new cases, 14 million prevalent cases, and approximately 1.7 million deaths by TB [1]. Additionally, approximately one third of the world's population is infected with the causative bacterium, *Mycobacterium tuberculosis* (*Mtb*), and is at risk for developing active tuberculosis. Interestingly, while approximately 9 million people develop active TB each year, the majority remain asymptotically (latently) infected with the pathogen presumably due to a protective immune response. Without intervention, approximately five to ten percent of those latently infected will develop overt disease and the potential to transmit *Mtb* to others [2].

Familial clustering data, twin studies and complex segregation analysis have all suggested a strong genetic component in the human susceptibility to the chronic mycobacterial diseases [3-7] but also a complex picture of geographic heterogeneity in genetic effects on the different

mycobacterial infections is involved [8, 9]. Several non-HLA genes have been implicated in TB susceptibility. However, the discrepant data reported may be attributed to a number of different factors, such as the types of studies, ethnicity, genetic background, and clinical status of patients with tuberculosis that may be associated with a particular genetic profile. The interaction among lung cells with pro and anti-inflammatory mediators during the infection with *Mtb* have been deeply investigated [10]. Among involved cytokines, the key role of interferon-gamma (IFN- γ) and tumor necrosis factor (TNF- α) in eliciting an inflammatory response against *Mtb* have been emphasized [11-13].

In human studies, the crucial role of TNF- α in protective host immunity against reactivation of latent TB was highlighted by the observation that the relapse and severe course of TB is over-represented in rheumatoid arthritis patients following the use of anti-TNF- α antibodies [14]. Concerning the IFN- γ , it is well established that deficiency in IFN- γ gene expression is associated with severe impairment of resistance to infections, in particular those that are normally killed by activated macrophages [15, 16]. Low synthesis of this cytokine has been associated with active tuberculosis [17]. However, on the contrary of TNF- α , the Interferon gamma coding gene (*IFNG*) is highly conserved and few single nucleotide polymorphisms (SNPs) are found in the intragenic region. Several case-control studies to evaluate association of SNPs in these genes with TB have produced mixed results, with little consensus in most cases on whether any TNF polymorphisms are actually associated with active TB disease [18, 19].

In the present study we aimed to analyse the existing promoter variability of the *IFNG* and *TNF- α* genes by partial mapping of this region in samples from Brazilians, followed by an association study of the identified SNPs and TB outcome after infection with *Mtb*.

2. Method used

2.1. Study population

In a case-control design, five hundred consanguineously unrelated individuals admitted at the University Hospital Complex: Thoracic Institute/Clementino Fraga University Hospital from Federal University of Rio de Janeiro-UFRJ were enrolled in this study after signing informed consent approved by the local Ethics Committee of HUCFF-UFRJ.

Demographic, clinical, and microbiological data as well as the HIV status of the subjects (age > 18 years old) were collected. Active TB cases (n=265) were defined as those after a positive culture confirmation in clinical specimen or with clinical, radiographic and laboratory improvement according to the American Thoracic Statements. They comprised 265 TB patients to be used for the descriptive genetic analysis. For the association study, TB-HIV comorbidity was considered as an exclusion criteria and sample size was reduced as follow: 140 TB patients, being 121 with pulmonary TB (PTB) and 19 extrapulmonary forms of TB (TBE). The mean age of TB patients was \pm 51 years (range 18-84 years) including 73 males and 67 females.

For the control group, a complete questionnaire to document TB risk factors since baseline testing was used. Individuals were eligible as controls if they had no previous TB history, consanguinity and negative HIV status. In formations concerning Tuberculin Skin Test (TST) response were available for all controls. They comprised 235 individuals, to be used for descriptive genetic analysis. For the association study, after application of the exclusion criteria, 154 individuals were included in this group, of which, 96 were TST positive (TST+) and 58 TST negative (TST-). The mean age in this group was ± 50 (range 18 - 82 years) and included 55 males and 99 females.

Sample Collection and handling

A volume of 3 mL of venous blood was collected from each volunteer and stored at -20°C . Genomic DNA was isolated from 100 μL of frozen whole blood using the FlexiGene DNA Kit (Qiagen Inc., USA), according to the manufacturer's specifications. After extraction, DNA samples were stored at -20°C .

2.2. *IFNG* and *TNF- α* genotyping

Genotyping of the proximal portion of the promoter region in *TNF- α* and *IFNG* genes was achieved by direct sequencing of PCR products. Two sets of primers for PCR amplification and sequencing of *IFNG*, DNA fragment of 863bp, (IFN-EF: 5' GGA ACTCCCCCTGGGAATATTCT 3', IFNER: 5' AGCTGATCAGGTCCAAAGGA3', IFNIF: 5' CGAAGTGGGGAGGT AAAAA 3' and IFNIR: 5' CCCAGGAAACTGCTACTCTG 3'), and *TNF- α* , DNA fragment of 855bp (TNFEF: 5' CAGGACCTCCAGGTATGGAA3', TNFER: 5' TAGCTGGTCCTCTGCTGTCC3', TNFIF: 5' CCTGCATCCTGTCTGGAAGT 3' and TNFIR: 5' TTTCAACCCCTGTGTGTTTCG 3') were designed by using the Primer3 software [20].

For PCR-mediated DNA amplification of *IFNG*, 100 ng of genomic DNA were added to a 50 μL reaction mixture containing 200ng of each primer (IFN-EF and IFN-ER), 0.2mM of each dNTPs, 2.0mM MgCl_2 and 1U *Taq* DNA polymerase (Invitrogen by Life Technologies, USA) and submitted to an initial denaturation at 94°C for 5 min., followed by 35 cycles of 1 min. at 94°C , 1 min. at 65.3°C and 1 min at 72°C . Final extension was performed for 5 min. at 72°C . Likewise, for amplification of the 855pb *TNF- α* fragment, 100ng of genomic DNA were added to a 25 μL reaction mixture containing 200ng of each primer (TNF-EF and TNF-ER), 2mM MgCl_2 , 0.2mM of each dNTPs and 0.5U of *Taq* gold DNA polymerase (PE Applied BioSystems) and submitted to initial denaturation at 94°C for 15 min, followed by 35 cycles of 1 min. at 94°C , 1 min. at 65.9°C and 1 min at 72°C with a final extension at 72°C for 5 min. Evaluation of PCR products was done by electrophoresis on 1.2% agarose gel followed by ethidium bromide staining.

For sequencing, PCR products were purified with ChargeSwitch Kit (Invitrogen Life Technologies), according to the manufacturer's recommendations. Sequencing of the amplified fragments was performed in both DNA strands using a combination of the internal and external primers using ABI PRISM Big Dye Terminator v. 3.1 Kit (PE Applied BioSystems), according to the manufacturer's recommendations, on an ABI PRISM 3730 DNA Analyser (PE

Applied BioSystems). All singletons and even new/rare mutation identified were confirmed by re-amplification and re-sequencing.

2.3. Computational analysis

The SNPs identification in each individual sample was achieved after alignment of the generated sequences with the GenBank reference sequences AF3757790 and AB088112 for *IFNG* and *TNF- α* respectively. Transcription starting site sequence definition adopted for both genes considered as starting point, the first nucleotide immediately preceding position (-1) out of mRNA. Sequence analysis was carried out through SeqScapev. 2.6 software (Applied Biosystem). Haplotype reconstruction was achieved through the use of PHASE Vs. 2.1.1 software [21, 22].

2.4. Statistical analysis

Pair-wise linkage disequilibrium was tested for the loci studies. The Hardy-Weinberg equilibrium using χ^2 test. Statistics were performed in XLSTAT 2008.7 (Addinsoft Software Inc - New York USA). The magnitude of the associations was estimated by odds ratio values and the coefficient of associations. All tests were performed at the 0.05 level of significance by Epi Info version 3.5.1 2008 (Centers for Disease Control and Prevention, USA).

3. Results

In this work, a partial mapping of the promoter regions of *IFNG* and *TNF- α* genes was performed by direct PCR sequencing approach in 265 TB patients and 235 healthy controls residents in Rio de Janeiro, Brazil. Sequencing approach allowed the identification of new SNPs and consequently new haplotypes for both genes. Expected genotype frequencies were calculated from respective single allele frequencies and were consistent with Hard Weinberg Equilibrium using χ^2 test.

3.1. Partial mapping of the *IFNG* promoter region in samples from Brazilians residents in Rio de Janeiro

Sequence analysis of the proximal portion of *IFNG* promoter region (863 bp upstream of the transcription starting site) revealed the presence of seven SNPs, of which, four were new, and located at positions (-787C>T, -599C>G, -517C>T, and -255A>G). The three remaining SNPs, already deposited in GenBank-Entrez SNP database, were located at positions (-785C>T, -200G>T, reported as (-183 and -179) and -172A>G (reported as -155). Table 1 show the allele and genotype frequencies of the identified SNPs in the whole studied population (500 samples). All SNPs were found in a very low frequency, sometimes as a singleton (-255A>G). In this case, the SNP was confirmed by new PCR amplification and re-sequencing. The two more frequent SNPs were the ones located at positions -599C>G and -200G>T, both with 1.4%. No homozygosity was identified in these positions.

Locus <i>IFNG</i>	Genotype	Subjects (n = 500)	Absolute Frequency	Allele frequency
-787*	CC	495	0.99	
	TC	5	.001	0.05
	(f) T	5	–	
-785	CC	495	0.99	
	CT	5	0.01	0.05
	(f) T	5	–	
-599*	CC	487	0.974	
	CG	12	0.024	
	GG	1	0.002	0.014
	(f) G	2	-	
-517*	CC	497	0.994	
	CT	3	0.06	0.003
	(f)T	3	-	
-255*	AA	499	0.998	
	AG	1	0.002	0.001
	(f) G	1	-	
-200	GG	486	0.972	
	GT	14	0.028	0.014
	(f) T	14	-	
	AA	498	0.996	
-172	AG	2	0.004	0.002
	(f) G	2		

Table 1. Genotype and allele frequencies of SNPs within *IFNG* promoter in Brazilians from Rio de Janeiro.

3.2. *IFNG* haplotypes characterization

Haplotype reconstruction was achieved from genotyping data by using Phase Vs. 2.1.1 software. A total of eight different haplotypes were characterized with basis on the combination of the seven SNPs identified within the *IFNG* promoter. Table 2 shows the frequencies of the identified haplotypes in the total population. The haplotype 4 was the, more frequent among the whole samples analyzed.

Haplotypes	-787	-785	-599	-517	-255	-200	-172	Frequency
1	C	C	C	C	A	G	A	0.916
2	C	T	C	C	A	G	A	0.010
3	C	C	G	C	A	G	A	0.024
4	C	C	C	C	A	T	A	0.028
5	T	C	C	C	A	G	A	0.010
6	C	C	C	C	G	G	A	0.002
7	C	C	C	C	A	G	G	0.004
8	C	C	C	T	A	G	A	0.006

Table 2. Characterization of the identified haplotypes within *IFNG* proximal promoter region in Brazilians from Rio de Janeiro (n=500).

3.3. Partial mapping of the *TNF- α* promoter region in samples from Brazilians residents in Rio de Janeiro

The partial mapping of the proximal portion (855 bp upstream of the transcription starting site) of *TNF- α* promoter was also performed by direct sequencing of PCR products. Upon analysis of the generated sequences seven SNPs, all described in the literature, and were identified in a total of 500 samples. Table 3 shows the allele and genotype frequencies. With the exception of the most studied SNPs (-238 -308, and -376) presenting frequencies higher than 3%, all others were present in less than one percent.

3.4. *TNF- α* haplotypes characterization

A total of fourteen different haplotypes were characterized. Except for the wild-type, haplotype 1, the higher frequent was the haplotype 3, presenting a mutant variation only at -308 position. As expected, the rare combination presenting polymorphisms only at positions -238 and -308 was present in the sample studied although in a low frequency (Table 4).

Locus	Genotype	Subjects (n=500)	Absolute Frequency	Allele Frequency
	GG	495	0.990	-
-646	GA	5	0.010	-
	A	5	-	0.005
	AA	495	0.990	-
-572	AC	5	0.010	-
	C	5	-	0.005
	CC	499	0.998	-
-422	CT	1	0.002	-
	T	1	-	0.001
	GG	471	0.942	-
-376	GA	28	0.056	-
	AA	1	0.002	-
	A	30	-	0.030
	GG	418	0.836	-
-308	GA	77	0.154	-
	AA	5	0.010	-
	A	87	-	0.087
	GG	489	0.978	-
-244	GA	11	0.022	-
	A	11	-	0.011
	GG	453	0.906	-
-238	GA	44	0.088	-
	AA	3	0.006	-
	A	50	-	0.050

Table 3. Genotype and allele frequencies of SNPs within *TNF- α* promoter in Brazilians from Rio de Janeiro.

Haplotype	-646	-572	-422	-376	-308	-238	-244	Frequency
1	G	A	C	G	G	G	G	0.710
2	G	A	C	A	G	G	G	0.006
3	G	A	C	G	A	G	G	0.146
4	G	C	C	G	G	G	G	0.006
5	G	A	C	G	G	G	A	0.020
6	G	A	C	G	G	A	G	0.040
7	A	A	C	G	G	G	G	0.008
8	G	A	T	G	G	G	G	0.002
9	G	A	C	A	G	A	G	0.044
10	G	A	C	A	A	A	G	0.060
11	G	A	C	A	A	G	G	0.002
12	G	A	C	G	A	G	A	0.002
13	G	A	C	G	A	A	G	0.004
14	G	C	C	G	A	G	G	0.004

Table 4. Haplotypes description and frequencies within *TNF- α* promoter in Brazilians from Rio de Janeiro.

3.5. Association of the *IFNG* SNPs and TB outcomes

Association of the identified SNPs variations within the analyzed region of *IFNG* with different TB outcomes (susceptibility *per se*, protection, severity and susceptibility to latent *M. tuberculosis* infection) was assessed based in the comparison of allele, genotype and haplotype frequencies between the stratified groups. The groups used for each evaluation were as follow: a) susceptibility *per se* to TB (TB patients versus TST+ controls), b) disease severity (PTB versus TBE) and c) susceptibility to the latent infection (healthy controls TST+ versus TST-).

As previously stated, for this analysis, the sample size was reduced in groups, (patients and controls) because of the exclusion criteria of TB-HIV co-infection and consanguinity. After exclusions, because of the very low frequency of the -255 A>G and -172 A>G these SNPs were also excluded.

Results of the association study upon comparison of genotype frequencies of the five remaining SNPs between TB patients (TBP/EPTB) versus TST+ controls are shown in Table 5. Only the SNP -200G>T presented a significantly higher frequency of the GT genotype in the control group indicating an association of this genotype with protection to the occurrence of active TB ($\chi^2 = 3.86$, $p = 0.033$, OR = 0.18 CI = 0.03 -1.00). Evaluation of the identified SNPs with the other outcomes did not show any association (data not shown).

Loci	Genotype	Patientes (N=140)	Controls TST+* (N=96)	χ^2	p-value	OR	IC
-787	CC	138	96	1.383	0.515	#	#
	CT	2	0				
-785	CC	140	94	2.942	0.16	#	0.00<2.79
	CT	0	2				
-599	CC	135	93	0.035	NS	1.15	0.23<6.23
	CG	5	3				
-517	CC	140	93	2.29	0.066	#	0.00<1.52
	CG	0	3				
-200	GG	138	89	3.86	0.033	0.18	0.03<1.00
	GT	2	7				

Table 5. Genotype distribution of the *IFNG* SNPs among TB patients and healthy controls (TST+).

Given that the SNP -200 *IFNG* was the only one that was associated with any of the studied outcomes at genotype level, allele frequency was also tested for the same outcomes. Table 6 shows the comparison of the -200T variant between the stratified groups. The results confirm the association with protection to the occurrence of active TB and, additionally to TBP. Association of the -200T variant was also seen to occurrence of latent infection ($p=0.035$).

Different outcomes	Groups		SNP IFNG -200		
			P-value*	OR	IC
Occurrence of TBactive	Pacientes 0.0071	TST+ 0.036	0.033	0.19	0.03<1.01
Occurrence pulmonary TB	TBP 0.0082	TST+ 0.036	0.043	0.22	0.033<1.17
disease severity	TBP 0.0082	TBE 0.00	1.00	#	#
latent infection	TST+ 0.036	TST- 0.000	0.035	#	#

Table 6. Distribution of allele frequencies of -200T variant mutant groups according to the different outcomes.

Finally, the more prevalent *IFNG* polymorphisms (-599C>G and -200G>T) were tested against demographic variables, such as, gender and age. No significant association was found after stratified analysis at allele, genotype or haplotype levels (data not shown).

3.6. Association of the *TNF- α* SNPs and TB outcomes

Table 7 summarizes the distribution and comparison of genotype frequencies of each individual SNP among TB patients and TST+ controls. No significant difference was observed. The evaluation of the possible association of different genotypes of *TNF- α* gene with susceptibility to the occurrence of TBP or TBE was also carried out separately, however, no association was found (data not shown).

Locus	Genotype	Patients (N=140)	Controls TST+ (N= 96)	<i>p-value</i>	OR	IC
-646	GG	139	95			
	GA	1	1	1.00	0.68	0.02<25.33
-572	AA	138	95			
	CA	2	1	1.00	1.38	0.10<38.92
-422	CC	140	96			
	CT	0	0	-	-	-
	GG	127	93			
-376	GA	11	3	0.11	3.17	0.81<14.46
	AA	2	0			
-308	GG	118	83			
	GA	19	11	0.78	1.19	0.54<2.67
	AA	3	2			
-244	GG	136	92			
	GA	4	4	0.71	0.68	0.14<3.31
-238	GG	123	88			
	GA	15	8	0.47	1.50	0.58<3.97
	AA	2	0			

Table 7. Genotype distribution of the *TNF- α* SNPs among PTB patients and healthy controls (TST+).

Comparison of the *TNF- α* SNPs frequencies between TBP and TBE is shown in (Table 8). Only the -572A>C (CA genotype) presented a significant difference between these groups, being absent among the 121 TBP subjects, (RR = 8.12, CI = 5.20 < 12.67 and *p*-value = 0.0175). The results indicate a risk for disease severity. This association was confirmed upon allele frequency evaluation (RR = 7.72, CI = 5.69 < 10.47 and *p* = 0.0179) data not shown.

Locus	Genotype	PTB N=121	TBE N=19	<i>p</i> -value	RR	IC
-646	GG	120	19	1.00	0.00	0,00<115.2
	GA	1	0			
-572	AA	121	17	0.0175	8.12	5.20<12.67
	AC	0	2			
-422	CC	121	19	-	-	-
	CT	0	0			
-376	GG	111	17	0.506	1.25	0.33<4.79
	GA	10	1			
-308	AA	0	1	0.392	0.63	0.16<2.54
	GG	101	17			
-244	GA	18	1	0.554	#	#
	AA	2	1			
-238	GG	117	19	0.585	0.85	0.22<3.37
	GA	4	0			
	GG	106	17			
	GA	14	1			
	AA	1	1			

Table 8. Genotypes distribution of the *TNF- α* SNPs among TBP and TBE.

The association between the *TNF- α* genotypes with latent infection was also evaluated. No significant difference was found (data not shown).

The final evaluation of independent SNPs with the different TB outcomes was performed based in the allele frequencies comparison for the most common *TNF- α* SNPs (-376G>A, -308G>A, -244G>A and -238G>A). The SNP -376G>A, allele variant -376A, showed a significant association with susceptibility to the occurrence of active TB ($p = 0.035$, OR = 3.57, CI = 0.95 < 15.72) and severity ($p = 0.038$ and RR = 2.68) (Table 9). All other outcomes showed no significant association with any of the variants tested, (data not shown).

Different outcomes	Study Group		SNP <i>TNF-α</i> -376A		
			<i>p</i> -valor	OR	IC
Occurrence of activeTB	Patients	TST+	0.035	3.57	0.95<15.72
	(<i>fa</i>) 0.054	0.016			
Occurrence of PTB	PTB	TST+	0.201	2.72	0,68<12.62
	(<i>fa</i>) 0.041	0.016			
Severity of disease	PTB	TBE	0.038	2.68*	1.22<5.86
	(<i>fa</i>) 0.041	0.052			
Latent infection	TST+	TST-	0.90	0.623	0.12<7.86
	(<i>fa</i>) 0.016	0.017			

Table 9. Distribution of allele frequency of the *TNF- α* -376A variant and association analysis with different outcomes studied.

Table 10 shows the distribution of the 14 identified haplotypes in the different groups used for the association study. No significant difference was observed in the haplotypes frequencies between groups (data not shown) and their distribution was quite homogeneous.

Haplotype	General TB n= 140	PTB n=121	EPTB n=19	Controls PPD+ n=96	Controls PPD- n=58
1	95(67.9%)	82(67.8%)	0	70(72.9%)	40(69%)
2	2(1.4%)	1(0.8%)	0	0	0
3	19(13.6%)	17(14.1%)	2(10.5%)	11(11.5%)	7(12.1%)
4	1(0.7%)	0	1(5.26%)	0	1(1.72%)
5	3(2.1%)	3(2.5%)	0	4(4.2%)	2(3.4%)
6	7(5%)	6(5%)	1(5.3%)	5(5.2%)	5(8.6%)
7	1(0.7%)	1(0.8%)	0	1(1.1%)	1(1.7%)
9	9(6%)	8(6.6%)	1(5.3%)	3(3.1%)	2(3.4%)
11	1(0.7%)	1(0.8%)	0	1(1.1%)	0
12	1(0.7%)	1(0.8%)	0	0	0
13	1(0.7%)	1(0.8%)	0	0	0
14	1(0.71%)	0	1(5.26%)	1(1.04%)	0

Table 10. Frequency of *TNF*- α haplotypes in the different groups studied

After the genotyping of all samples and evaluation of the possible association with the different TB outcomes, the most frequent polymorphisms (-376G>A; -308G>A; -244G>A and -238G>A) were tested in a stratified analysis against the demographic variables gender and age. No significant differences were found for gender or age (data not shown).

4. Discussion

It is well known that to *M. tuberculosis*, etiologic agent of human TB can cause a broad spectrum of effects ranging from no infection to different clinical disease phenotypes [2, 16, 23-25]. However, the reasons for individual or ethnic differences in acquiring infection, active disease, disease severity, and different clinical outcomes have not been completely clarified. It has long been realized that many human diseases arise from the complex interplay between environmental exposures and host genetics susceptibilities [26]. In addition, several genetic factors have also been associated with different outcomes: host susceptibility *per se* the occurrence of active TB, disease severity and / or protection for the occurrence of active disease [27-33].

The establishment of an efficient immune response involves many different molecules, among which, cytokines and their receptors play an extremely important role. Thus, any genetic alteration leading to changes in the regulation of gene expression may reflect this response. It is known that the interindividual variation in the production of these molecules is directly related to the genetic "background". Literature data have clearly demonstrated that genetic variability of the genes encoding these molecules can affect the regulation of gene expression positively or negatively influencing the final yield of the molecule in question. In the last decade, several single nucleotide polymorphisms (SNPs) in the regulatory region of different cytokine genes have been described and associated with susceptibility, severity or protection for a growing number of diseases of different etiologies including tuberculosis [7, 34-35].

Among the possible genetic variations associated with an increased risk of developing TB, there are several polymorphisms, mainly SNPs, in genes coding for cytokines, cytokine receptors and several other molecules such as vitamin D receptor, NRAMP1 (SLC11A1), HLA genes, etc.

The immune defense against *M. tuberculosis* is complex and involves the interaction between T CD4⁺, T CD8⁺ lymphocytes, macrophages, and monocytes along with the production of cytokines, such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) [36].

Convincing evidence indicating the importance of IFN- γ in particular, in the control of mycobacterial infections has been found in both experimental and clinical studies [37-38].

Among the mainly important cytokines involved in TB progress after infection with *M. tuberculosis*, TNF- α plays a key role. It is also a potent proinflammatory cytokine acting in protection against intracellular pathogens [39-40].

The genetic variability of *TNF- α* and *IFNG* has been described in the last decade [41-46] including association studies with tuberculosis. However, the frequencies of the polymorphisms already described varies according to the ethnicity of the population studied, hampering the better interpretation of the value of association studies. Unfortunately, most of these are performed in ethnically homogeneous populations, and therefore, many of the associations described for a particular allelic variant in a certain gene may not represent genetic risk factor in other populations. In Brazil, a country characterized by ethnically mixed population, there are few data regarding the frequency of single nucleotide polymorphisms in these genes (*IFNG*, *TNF- α*) and the few existing studies refers to one or two SNPs only. In view of the importance of the promoter region with respect to regulation of gene expression, the major goal of this work was to proceed a partial mapping of the promoter region of *IFNG* and *TNF- α* genes (approximately, 800bp upstream of the transcription starting site) through PCR-sequencing approach in samples from TB patients and healthy controls from Rio de Janeiro, Brazil. Subsequently, based on frequencies of the different SNPs found individually for each gene, we performed an association study with different TB outcomes.

4.1. Polymorphisms in the promoter region of *IFNG* and its association with TB

Characterization of the important portion within the *IFNG* was firstly identified two decades ago by deletion analysis studies [47-48]. According to authors, it comprises a highly conserved

region from positions -117 to -47 and contains two sub regions that can be complexes with proteins. The sub-proximal region (-90 to -65) shows strong homology to the IL-2 promoter [49]. Several transcription factors activate transcription of *IFNG* by binding to this region. Conversely, several others inhibit factors binds in other regions affecting transcription. Hence, the interest in investigating the polymorphic sites within *IFNG* gene promoter, particularly considering the importance of this cytokine in eliciting the immune response.

Here, analysis of the generated sequences identified seven polymorphic sites, four of which were new. The transition C → T, was identified at position-787 from the transcription starting site in five subjects, all heterozygous. The second C → T transition, previously described in the data base of SNPs at position-785 was also found in five individuals, all heterozygous, however, no reference to this SNP was found in the literature. The other three SNPs not yet described, were C to G transition at position -599; C to T at position -517 and A to G at position -255. Finally, two additional SNPs, transition from G to T at position -200 and A to G at position -172, already described and well characterized [45] were found in our population.

One of the main problem found during this mapping was the confirmation of the identified SNPs based on literature data and from different SNPs data bases available online because of the lack of standardization regarding to the reference nucleotide to define the promoter region (transcription starting site nt +1). Many authors describe the SNPs identified in relation to the site of translation or use reference sequences containing sequencing errors leading to misclassification of SNPs (eg SNP-200G>T, originally described as -183 [45], later called as -179 [50] and finally, confirmed in this study as -200). The current name, confirmed in this study is based on the correction of the reference sequence used in previous studies and now available online. These types of errors greatly hampered the beginning of the sequence analysis regarding the identification of novel SNPs.

The frequency of each polymorphism was determined in the study population. As noted in Table 1, the allele frequencies for all the identified SNPs were less than one percent, except for the variants -599G and -200T, both in a frequency of 1.4%.

Functionally, it is known that polymorphisms (-200 and -172) can affect transcription of the *IFNG*. The region from -213 to -200 induces transcription factor through (AP-1) [51]. A polymorphism at this site (position -200, for example) must change the connection of AP-1 and the promoter activity in T cells. The polymorphism -172 is near to the nuclear factors-activated T-cells site (NFAT site) (-186TAAAGGAAA-178) and should affect the stability of this region [50].

The variant *IFNG* -200T is highly inducible by TNF- α and binds constitutively to nuclear extracts obtained from T cells, whereas the allele -200G does not respond to TNF- α [50; 52]. The induction of transcriptional activity, when the T allele is present, increases protection against tuberculosis. Our results corroborate these data, since the *IFNG* -200T variant showed to be associated with protection the occurrence of active TB in our study group (P = 0.033, OR= 0.18, CI= 0.03 to 1.00).

According Bream, JH et al., 2002 [50], the promoter region of *IFNG* is highly conserved, suggesting that these cytokine production variations are probably due to difference in binding

to regulatory factors instead of polymorphisms in the gene, which is consistent with our results. Only seven SNPs were found in our population, five of which were at a low frequency. The polymorphisms found with a higher frequency were the -200G>T and -599C>G, the latter being located between two putative binding sites of transcription factors. As this is not yet a SNP described in the literature, functional studies are needed to better understand their functional role. The SNP -200 is of great interest for association studies. However, this polymorphism was not found in Caucasians or Indian populations, suggesting that different selective forces may be operating in different ethnic or racial groups. These data corroborate the evidence that IFN- γ is very important in the immune response and that mutations that interfere with their production may influence the outcome of active tuberculosis as shown by authors [28,31,53-54], and therefore, a selective force lead the gene to be so conserved.

4.2. Polymorphisms within the promoter region of *TNF- α* and their association with TB

TNF- α is a proinflammatory and immunoregulatory cytokine which plays a key role in the initiation, regulation and perpetuation of host defense against infections, but is fatal in excess. As this molecule plays an important role against a variety of pathogens involving different patterns of risks and benefits, it is expected that several genetic elements are involved in its control and production.

The levels of circulating TNF- α are regulated at transcriptional and post-transcriptional levels and several polymorphisms within the promoter region of TNF- α have been associated with altered circulating levels of this cytokine.

In humans, the *TNF- α* gene is located within the complex involving the human leukocyte antigens (HLA), a highly polymorphic region on chromosome 6p21.3 and hence, many of *TNF- α* polymorphisms are in linkage disequilibrium with the HLA genes. Because of differences in the distribution of HLA alleles we might expect variations in associations between polymorphisms of *TNF- α* and various conditions in different geographical areas.

The human genome analysis showed that the level of variations in the genome is approximately one SNP/1.71Kb [55]. However, the *TNF- α* promoter has higher density of SNPs. Despite this level of variation, the regions involved in gene regulation are highly conserved in humans [56-58].

In our work, we perform the mapping of the first 800pb promoter region, through direct sequencing of the amplified PCR product in 500 DNA samples from individuals living in the metropolitan area of Rio de Janeiro, Brazil and found seven polymorphisms previously described in the literature, most of which well characterized. However, according allele frequencies, only the variants -376A, -308A, -238A and -244A were present in more than one percent (0.030, 0.087, 0.011 and 0.050) respectively. The influence of these SNPs in binding of transcription factors have not been fully explored, most of the studies are focused on the association of one or two SNPs with different diseases. The SNPs -376, -308 and -238 have been the most studied but, the results of functional studies performed so far for SNPs -308 and -238 are controversial. It is believed that the variant -308A is associated with an increased transcription rate, leading to an increased production of TNF- α [59] and the variant-238A with a

decreased rate of transcription. Regarding the SNP-376G>A different studies show that this polymorphism is located in a region of multiple interactions between proteins and DNA, and that the minor allele acts in the recruitment of proteins OCT1 for this region. According Knight et al (1999) [60] there is a significant interaction between variant -376A and the OCT-1 protein, this variant binds the proteins while the variant -376G does not. The authors report also by tests with the reporter gene system, that this mutant variant moderately increases the basal levels of TNF- α and associate the same with a relative risk of 4 to cerebral malaria. The problem is that the linkage disequilibrium is strong in this area and it is difficult to study the function of an isolated SNP. In some Caucasian populations -376A allele variant is linked to -308G and -238A [60-61], what is not observed in the African Gambia. Thus, association between the linked allelic variants on TNF- α production and diseases has been studied. According, Hajeer & Hutchinson (2001), the combined allele variants -238G, -308A and -376G are associated with high TNF- α levels [62].

A large number of studies have investigated the association between polymorphisms in the promoter region of the gene for TNF- α and tuberculosis. Results vary according to the different populations studied, finding no association [63-72] or a positive association [26, 29, 73-75]. In our analysis of the single SNP association, TB was associated with the -376G>A. In this case, we observed an association of the minor allele -376A with the outcome of susceptibility *per se* the occurrence of active TB ($p=0.035$, OR 3.57, IC 0.95 < 15.72) and an increased relative risk for the occurrence of extrapulmonary TB ($p=0.038$, OR 2.68, IC 1.22 < 5.86). The association of this allele variant with the occurrence of TB and an increased relative risk for the development of extrapulmonary TB is intriguing. Given the influence of this allele with increased expression of TNF- α , one would expect an association with the protection. One possible explanation for this observation may be the small sample size in the stratified groups. The large confidence intervals (CI) for both outcomes could be a reflection of the small sample size.

The PCR-sequencing approach (gold standard) used for the mapping these genes practically discard the possibility of genotyping errors and all mutants found for all SNPs evaluated were confirmed twice by new PCR and resequencing. Another possibility would be due to the strong linkage disequilibrium observed in this region of the gene promoter of TNF- α . It is possible that other allelic variant (eg, 238A), as opposed to the functional role of variant -376A is canceling the same level of control of gene expression.

An important aspect of this study relates to the ethnic characteristics of the studied population. Brazilian population is characterized by mixture of ethnicities and the results obtained here contribute for a global understanding of the influence of genetic factors in TB outcomes. Usually, most of the studies on this field are made with ethnically homogeneous populations. A study conducted by Baena et al., 2002 [76] clearly shows the importance of ethnic difference in the association study of SNPs in TNF- α promoter with disease. According authors, the -857 SNP is a marker for Amerindians. In that study, SNPs in TNF- α promoter were also used to identify markers of ancestry, understanding that this region was well characterized previously with primates and humans. Several studies [77-81] have shown that some polymorphisms as -238; -244 and -308 are in association with the HLA genes and in addition, the SNPs -308 [77]; -863 and -857 [81], are markers of Caucasians. The -238 SNP was found in three populations

studied, although it has also been found in Caucasians [78] and Asian [80]. Instead, the -376 SNP was not detected in any of the non-African analyzed and were found with the -238.

These data demonstrate the importance of taking into account the "background" of the frequencies of SNPs of TNF- α in studies of gene-disease association.

Association studies of genetic factors with infectious diseases are difficult to conduct because of the multi factorial nature of these diseases that includes host, pathogen and environmental variables in different proportions for each disease. This multi factorial nature of TB stresses the importance to look for haplotypes in the association studies. The fact that our population is so mixed allowed us to find mutations that do not exist in other populations, such as the -308, for example, which is relatively rare in Asians and American Indians. Data from the genotyping of a large number of SNPs for different samples revealed that the human genome has a block structure haplotype [82-83] and configuration of a haplotype sometimes is more important than a single SNP genotype to determine phenotype [84]. Moreover, the construction of a haplotype block is useful for identifying SNPs that isolates would not influence the phenotype [85].

5. Conclusions

In conclusion, this study showed that the proximal part of the promoter region of *IFNG* is highly conserved, as seen in previous publications and the identified SNPs were in very low frequency. The -200T allele variant was associated with protection occurring active TB, and pulmonary TB. In addition, this variant was also associated with latent infection. Concerning *TNF- α* , the high genetic variability was confirmed, but only the -376G>A SNP showed an association with susceptibility *per se* to TB occurrence and increased risk for the occurrence of extrapulmonary TB.

The data presented here shows the reality of a population with characteristics of high ethnic miscegenation, provides the different SNPs identified enabling the realization of real sample calculation for any association studies that may be idealized with these targets and other conditions for this population and finally, provides haplotype that can be used in other studies of association with other diseases.

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