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Partial Mapping of the IL-10 Promoter Region: Identification of New SNPs and Association with Tuberculosis Outcome in Brazilians

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1. Introduction

Tuberculosis (TB) is one of the oldest infectious diseases which affect humankind. According to the World Health Organization (WHO), in 2009 the TB global burden reached 9.4 million of incident cases, 14 million of prevalent cases and 1.3 million deaths. Most cases were in the South-East Asia and Western Pacific regions. Concerning HIV-TB co-infection, 11-13% of the incident cases were HIV-positive, been the African continent accounted for approximately 80% of these cases (World Health Organization and Global Tuberculosis Programme 2010). Several factors are involved in susceptibility to active disease after infection by *Mycobacterium tuberculosis* including environmental and host genetic markers. Today, the search for functional genetic markers in target genes and association studies with different TB outcomes has been common in the literature (Amim et al, 2007, Calado et al, 2006). Several genes encoding different cytokines, receptors and transporters molecules are involved in the host immune response against *M. tuberculosis*, therefore playing crucial roles in TB susceptibility. The interleukin-10 (IL-10), one of the most important Th2 cytokine involved in the immunoregulation. It is a homodimeric molecule of 37kDa consisting of two monomers of 18.5kDa that interacts with the heterodimeric receptor complex to modulate the biological activities of several cells lineage such as T-cells and the myeloid cells, including monocytes, dendritic cells and macrophages. Interleukin 10 inhibits activation of these cells resulting in a reduced production of pro-inflammatory mediators, including different cytokines and chemokines (Groux et al, 2003; David et al, 2008, Lin et al, 2003; Westendorp et al, 1997).

Variability in IL-10 production has a hereditary component of approximately 75% and this variation is mainly due to polymorphisms within the promoter region of the IL-10 gene (Shin et al 2005; Eskdale et al, 1997).

The human IL-10 coding gene spans about 4.7 kb on chromosome 1q31-32 and contains five exons. It is a highly polymorphic gene, with sixty described single nucleotide polymorphisms (SNPs), spreaded along the promoter, coding and intronic regions (Ensembl Genome Browser April 2011, ENSG00000136634). According to the literature data, the genetic diversity of the IL-10 gene expressed by allele frequencies, varies depends on the ethnicity of the studied population (Moraes et al, 2003). Based in the admixture genetic background of Brazilians, the aim of this work was to map the proximal region of the regulatory sequence (promoter region) of the IL-10 coding gene to look for new SNPs and to evaluate the possible association between IL-10 gene polymorphisms and different TB outcomes after infection with *M. tuberculosis* in Brazilians.

2. Material and methods

2.1 Selection of subjects and sample collection

Four hundred and ninety two consanguineously unrelated individuals from Rio de Janeiro were enrolled in this study. They comprised 221 patients with culture-confirmed active TB (who had been treated at the University Hospital Complex: Thoracic Institute/Clementino Fraga University Hospital from Federal University of Rio de Janeiro-UFRJ and 271 close healthy contacts of TB cases, with no previous TB history and with information of Tuberculin Skin Test (TST) response, (TST+ and TST-) from the same Hospital. This study was approved by the ethics committee of Oswaldo Cruz Foundation.

After a written informed consent a volume of 5 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 IL-10 genotyping

The partial mapping of the promoter region of IL-10 coding gene was performed by PCR amplification followed by direct sequencing of a 1500pb region upstream of the transcription site. Two sets of primers, EF: 5` CTGTGCCTCAGTTTGCTCAC 3`, ER: 5´ACTCTGCTGAAGGCATCTCG3´, IF 5´GCAATTTGTCCACGTCACTG 3´ and IR 5´TTGGTTGAACATGAACTTCTG 3´ were used for amplification and sequencing of a DNA fragment of 1001pb (fragment 1) and one set, EF 5´ TTCCCCAGGTAGAGCAACAC3´ and ER5´GGCACATGTTTCCACCTCTT3´ for amplification and sequencing of a second DNA fragment of 565pb (fragment 2). Primers were design by using the Primers3 software (http://frodo.wi.mit.edu/cgi-bin/ primer3/primer3 www.cgi).

The PCR-mediated DNA amplification of the fragment 1 was performed from 100 ng of genomic DNA in a reaction mixture containing 200ng of each primer (IL10frag1) EF and (IL10frag1) EF, 0,2mM of dNTPs, 2,5mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Invitrogen by Life Technologies, USA) The cycling conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min., 70°C for 1 min. and 72°C for 1 min with a final

extension at 72°C for 5 min. For amplification of the fragment 2, 100ng of genomic DNA were used in a similar mixture except for the primers (IL10frag2) EF and (IL10Frag2) ER. Amplification conditions were also the same used for fragment 1 except for the annealing temperature of 63,3°C. 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 PCR and re-amplification for three times followed by re-sequencing.

2.3 Computational analysis

The SNPs identification in each individual sample was achieved after alignment with the reference sequences AF418271 and Z30175 for the fragments 1 and 2 (http://www.ncbi.nlm.nih.gov/GenBank). We have defined the DNA sequence of the transcription starting site of the human IL-10 gene from the first nucleotide immediately preceding the A of the ATG taken position -1 (Eskdale et al, 1997), through SeqScape v.s2.6 software (Applied Biosystem www.appliedbiosystems.com). Haplotype reconstruction were achieved through the of Software PHASE v.s2.1.1, development by Yongtao Guan and Matthew Stephens from University of Chicago department of human genetics and department of Statistics.

2.4 Statistical analysis

Pair-wise linkage disequilibrium was also tested for the loci studies. The Hard-Weinberg equilibrium using x^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. All tests were performed at the 0.05 level of significance. Epi Info version 3.5.1 2008, (Centers for Disease Control and Prevention, USA) regarding the value of 5% ($p < 0.05$) as threshold for statistical significance in the association study of haplotypes between the numbers observed and expected separately in patients and contacts.

3. Results

Two epidemiological model of study were adopted in this work and the study population was stratified according the different models. Expected genotype frequencies were calculated from respective single allele frequencies and were consistent with Hard Weinberg Equilibrium using x^2 test.

3.1 SNPs profile of the human IL-10 promoter among Brazilian subjects

Using the direct PCR sequencing approach, analysis of the 492 DNA samples enrolled in this study revealed the presence of thirteen SNPs within *IL-10* promoter region, seven of which not reported yet and located at positions (-537G>T, -633G>A, -637G>C, -750C>T, -840A>C, -1162A>G/T and -1189C>T) respectively. The six remaining SNPs found, already deposited in GenBank-Entrez SNP database, were located at positions (-1117G>A, reported as -1082, -886G>A, -854C>T , reported as -819, -692G>A, -627C>A, reported as -592

Locus	Genotype	Subjects (N=492)	Absolute Frequency	Allele Frequency
-1189*	CC	483	0,982	**0,0090**
	CT	9	0,018	
	(fa) T	9	-	
-1162*	AA	485	0,986	**0,0060 (G)** **0,0030 (T)**
	AG	2	0,004	
	AT	3	0,006	
	GG	2	0,004	
	(fa) T	3	0,006	
	(fa) G	4	0,008	
-1117	GG	55	0,112	**0,6780**
	GA	207	0,421	
	AA	230	0,467	
	(fa) A	667	0,888	
-886	GG	464	0,943	**0,0295**
	GA	27	0,055	
	AA	1	0,002	
	(fa) A	29	0,057	
-854	CC	198	0,402	**0,3700**
	CT	224	0,455	
	TT	70	0,142	
	(fa) T	364	0,597	
-840*	AA	484	0,984	**0,0080**
	AC	8	0,016	
	(fa) C	8	-	
-750*	CC	473	0,960	**0,0213**
	CT	17	0,034	
	TT	2	0,004	
	(fa) T	21	0,038	
-692	GG	475	0,965	**0,0170**
	GA	17	0,035	
	(fa) A	17	-	
-637*	CC	487	0,989	**0,0050**
	CG	5	0,011	
	(fa) G	5	-	
-633*	GG	490	0,996	**0,0020**
	GA	2	0,004	
	(fa) A	2	-	
-627	CC	192	0,390	**0,3770**
	CA	229	0,465	
	AA	71	0,144	
	(fa) A	371	0,610	
-537*	GG	491	0,998	**0,0010**
	GT	1	0,002	
	(fa) T	1	-	

Locus	Genotype	Subjects (N=492)	Absolute Frequency	Allele Frequency
-464	GG	479	0,973	**0,0130**
	GT	13	0,026	
	(fa) T	13		

Table 1. Genotype and allele frequencies of SNPs within *IL-10* promoter in Brazilians from Rio de Janeiro.

and -464G>T. Upon genotype and allele frequency analysis, the three more frequent SNPs were the ones located at positions -627C>A, -854C>T and -1117G>A (Table1). Analysis of linkage disequilibrium (LD) showed that only two, SNPs -627C>A and -854C>T out of the thirteen SNPs identified in the total sample were in LD.

3.2 Haplotypes characterization

Haplotypes	*-1189*	*-1162*	*-1117*	*-886*	*-854*	*-840*	*-750*	*-692*	*-637*	*-633*	*-627*	*-537*	*-464*	*Alleles*	Frequency
1	C	A	G	G	C	A	C	G	C	G	C	G	G	265	0,270
2	**T**	A	G	G	C	A	C	G	C	G	C	G	G	2	0,002
3	C	**T**	G	G	C	A	C	G	C	G	C	G	G	2	0,002
4	C	A	**A**	G	C	A	C	G	C	G	C	G	G	274	0,280
5	C	A	G	**A**	C	A	C	G	C	G	C	G	G	28	0,028
6	C	A	**A**	G	**T**	A	C	G	C	G	C	G	G	10	0,010
7	C	A	**A**	G	C	**C**	C	G	C	G	C	G	G	2	0,002
8	C	A	G	G	C	A	**T**	G	C	G	C	G	G	6	0,006
9	C	A	**A**	G	C	A	**T**	G	C	G	C	G	G	2	0,002
10	C	A	G	G	C	A	C	**A**	C	G	C	G	G	2	0,002
11	C	A	**A**	G	C	A	C	**A**	C	G	C	G	G	2	0,002
12	C	A	G	G	C	A	C	G	**G**	G	C	G	G	2	0,002
13	C	A	G	G	C	A	C	G	C	G	**A**	G	G	1	0,001
14	C	A	**A**	G	C	A	C	G	C	G	**A**	G	G	11	0,011
15	C	A	G	G	**T**	A	C	G	C	G	**A**	G	G	2	0,002
16	C	A	**A**	G	**T**	A	C	G	C	G	**A**	G	G	311	0,316
17	**T**	A	**A**	G	**T**	A	C	G	C	G	**A**	G	G	7	0,007
18	C	**T**	**A**	G	**T**	A	C	G	C	G	**A**	G	G	1	0,001
19	C	A	**A**	G	**T**	**C**	C	G	C	G	**A**	G	G	6	0,006
20	C	A	G	G	C	A	**T**	G	C	G	**A**	G	G	1	0,001
21	C	A	**A**	G	**T**	A	**T**	G	C	G	**A**	G	G	10	0,010
22	C	A	G	G	C	A	C	**A**	C	G	**A**	G	G	2	0,002
23	C	A	G	**A**	C	A	C	**A**	C	G	**A**	G	G	1	0,001
24	C	A	**A**	G	**T**	A	C	**A**	C	G	**A**	G	G	10	0,010
25	C	A	**A**	G	**T**	A	C	G	**G**	G	**A**	G	G	2	0,002
26	C	A	**A**	G	**T**	A	C	G	C	**A**	**A**	G	G	2	0,002
27	C	A	G	G	C	A	C	G	C	G	C	**T**	G	1	0,001
28	C	A	**A**	G	C	A	C	G	C	G	C	G	**T**	7	0,007
29	C	A	G	G	C	A	**T**	G	C	G	C	G	**T**	2	0,002
30	C	A	**A**	G	**T**	A	C	G	C	G	**A**	G	**T**	3	0,003
31	C	A	**A**	G	C	A	C	G	**G**	G	**A**	G	**T**	1	0,001
32	C	**G**	**A**	G	C	A	C	G	C	G	C	G	G	6	0,006

Table 2. Haplotypes description and frequencies

3.3 Association of the IL-10 SNPs and TB outcomes

Frequencies of the *IL-10* polymorphisms in patients affected by active tuberculosis (n=221) and healthy controls TST+ (n=271) were compared among stratified groups to evaluate the possible association of the identified SNPs and the outcomes of susceptibility *per ser* to the occurrence of active TB, and disease severity. Table 3 shows that the most studied and well characterized SNPs at positions -1117, -854, -627, (also reported respectively as -1082, -819 and -592), did not show any association with the studied TB outcomes. Additionally, any of the remaining 10 SNPs showed to be associated with TB occurrence at neither genotype nor allele level.

Locus	Genotype	Pacients N=221	Controls TST+ N= 126	χ^2	*p*-valor	OR
-1189	CC	212	126	5,26	0,022	#
	CT	9	0			
-1162	AA	217	125	1,16	0,763	#
	AG	1	0			
	AT	2	1			
	GG	1	0			
-1117	GG	24	12	1,20	0,547	#
	GA	100	51			
	AA	97	63			
-886	GG	208	123	2,23	0,135	0,39
	GA	13	3			
-854	CC	89	45	1,67	0,432	#
	CT	100	66			
	TT	32	15			
-840	AA	213	126	ND	ND	ND
	AC	8	0			
-750	CC	221	114	ND	ND	ND
	CT	0	10			
	TT	0	2			
-692	GG	215	121	0,41	0,521	1,48
	GA	6	5			
-637	CC	216	126	ND	ND	ND
	CG	5	0			
-633	GG	221	125	ND	ND	ND
	GA	0	1			
-627	CC	85	44	0,95	0,619	#
	CA	102	65			
	AA	34	17			
-537	GG	220	126	ND	ND	ND
	GT	1	0			
-464	GG	211	123	1,02	0,311	0,514
	GT	10	3			

Table 3. Genotype distribution of the *IL-10* SNPs among TB patients and healthy controls (TST+)

Locus	Genotype	Pacients N=221	Controls (TST+/TST-) N= 271	χ^2	*p*-valor	OR
-1189	CC	212	271	**ND**	**ND**	**ND**
	CT	9	0			
-1162	AA	217	269	0,621	0,892	#
	AG	1	1			
	AT	2	1			
	GG	1	1			
-1117	GG	24	31	1,699	0,428	#
	GA	100	107			
	AA	97	133			
-886	GG	208	256	0.931	0.638	#
	GA	13	14			
	AA	0	1			
-854	CC	89	109	0,025	0,988	#
	CT	100	124			
	TT	32	38			
-840	AA	213	271	**ND**	**ND**	**ND**
	AC	8	0			
-750	CC	221	252	**ND**	**ND**	**ND**
	CT	0	17			
	TT	0	2			
-692	GG	215	260	0,659	0,417	1,51
	GA	6	11			
-637	CC	216	271	**ND**	**ND**	**ND**
	CG	5	0			
-633	GG	221	269	1,637	0,200	#
	GA	0	2			
-627	CC	85	107	0,299	0,861	#
	CA	102	127			
	AA	34	37			
-537	GG	220	271	ND	ND	#
	GT	1	0			
-464	GG	211	268	**5,528**	**0,019**	**4,23**
	GT	10	3			

Table 4. Genotype frequencies of the *IL-10* SNPs among TB patients and healthy controls (TST+/TST-)

Genetic diversity of the IL-10 gene, mainly in the promoter region has been studied in several populations and many studies have been associated polymorphisms in this region with differential IL-10 production. Among the sixty already described SNPs, the ones at positions -1117 G>A, -854C>T, -627C>A, also described as (-1082, -819 and -592) are the better characterized and taken as haplotypes are related to IL-10 production. The allele G at -1117, and haplotypes containing this allele have been associated with high IL-10 production, while the allele A and the haplotype ATA have been associated with low IL-10 production. Even so, association studies have been shown conflicting results depending on several factors,

including differences in laboratory experiments and ethnicity of the studied populations. As a result, different kinds of associations with immune response and diseases outcomes are commonly reported. Here, no association was found of any between the already described SNPs within IL-10 promoter with TB. This result corroborates at least in part with previous association study of these well characterized SNPs with leprosy, in which, only the only the -854C>T (-819) showed associated with leprosy susceptibility in Brazilians residents in Rio de Janeiro (Santos et al, 2002). No data concerning TB association are available in Brazil.

Locus	Genótipo	TBP (N=139)	TBE (N=43)	χ^2	*p*-valor	OR
-1189	CC	136	39	**4,532**	**0,033**	**0,215**
	CT	3	4			
-1162	AA	137	42	3,854	0,278	#
	AG	0	1			
	AT	1	0			
	GG	1	0			
-1117	GG	19	2	2,619	0,270	#
	GA	62	21			
	AA	58	20			
-886	GG	130	42	1,089	0,297	#
	GA	9	1			
-854	CC	61	16	1,193	0,551	#
	CT	58	22			
	TT	20	5			
-840	AA	134	42	0,010	0,565	0,640
	AC	5	1			
-692	GG	134	43	ND	ND	ND
	GA	5	0			
-637	CC	137	41	1,577	0,209	0,290
	CG	2	2			
-627	CC	60	14	1,802	0,406	#
	CA	59	23			
	AA	20	6			
-537	GG	139	42	ND	ND	ND
	GT	0	1			
-464	GG	134	39	2,274	0,132	0,363
	GT	5	4			

The influence of demographic characteristics such as gender and age in the studied outcomes were also evaluated, no significant difference was found for gender or age. (data not shown).

Table 5. Genotype distribution of the IL-10 SNPs among patients with pulmonary and extrapulmonary tuberculosis (TBP and TBE)

When genotype association was evaluated by comparison of the frequencies of TB patients against healthy controls without stratification by TST response (TST+/TST-), a significant increased frequency of the heterozygous -464 GT, showed to be associated with susceptibility *per se* to the occurrence of active TB (OR 4,23, *p* value 0,019). An interesting finding was the observation of the SNPs -750C>T and -633G>A, only the group of health

controls and on the contrary, the SNPs at positions -1189C>T, -840A>C, -637C>G and -537G>T, present only in the group of TB patients (Table 4).

Assessment of the possible evaluation of the *IL-10* SNPs found with the outcome of disease severity, were achieved by comparing the frequencies found in the group of pulmonary TB patients (TBP) and the group of extrapulmonary TB patients (TBE). Table 5 shows a significantly higher frequency of the allele variant -1189T in the group of patients with extrapulmonary TB in comparison with pulmonary TB suggesting an association of this allele variant with the outcome of severity. No statistically significant differences between the groups were observed for the other SNPs evaluated.

After haplotyping reconstruction and identification of thirty two different haplotypes, only the three more frequent, respectively, haplotype 1 (CAGGCACGCGCGG), haplotype 4 (CAAGCACGCGCGG) and haplotype 16 (CAAGTACGCGAGG) with frequencies of 27, 28 and 31.6 % respectively were considered for the association study. No haplotype association was found for any of the evaluated outcomes.

4. Conclusions

In this study, using a PCR direct sequencing approach, seven new SNPs and six already described were identified, in a region of one thousand and fifty hundred base pairs, proximal to the transcription starting site of IL-10 coding gene.

Evaluation of the possible association of the identified SNPs with different TB outcomes by comparison of allelic, genotype and haplotype frequencies between groups showed that none of the new SNPs were associated neither with susceptibility nor with severity. However, an interesting finding is that three of the new SNPs were present only among patients and one, only in the group of controls. Although it can suggest an association with susceptibility *per se* to TB occurrence and resistance to TB respectively, it is not conclusive. The functional role of these SNPs should be elucidated and sample size should also be increased.

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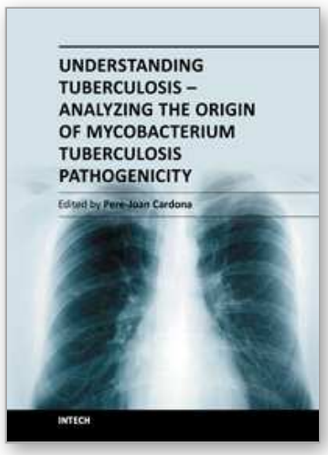

Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity

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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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