MBL2 gene polymorphisms and susceptibility to tuberculosis in a northeastern Brazilian population

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\begin{abstract}

The innate immune system represents the first line of host defense against pathogens. Genetics factors regulating the immune responses play a role in the susceptibility to infectious diseases, such as tuberculosis (TB). We analyzed MBL2 promoter and exon 1 functional single nucleotide polymorphisms (SNPs) in a group of 155 TB patients and 148 healthy controls in order to evaluate their influence on the onset of infection and TB development. There was no association between MBL2 – 550 HL promoter polymorphisms and susceptibility to develop TB, but heterozygous – 221 Y/X genotype was significantly more frequent in pulmonary TB patients than controls. Moreover, MBL2 exon 1 O allele, was significantly associated with susceptibility to TB development in general \((p = 0.023, \text{ OR} = 1.61, 95\% \text{ CI } 1.05–2.49)\) and pulmonary TB \((p = 0.0008, \text{ OR} = 2.16, 95\% \text{ CI } 1.35–3.46)\); C allele at codon 57, as well as A/C genotype, were significantly more frequent in TB patients than in controls. Our results indicate that MBL2 polymorphisms, especially at codon 57, could be considered as risk factors for TB development.

\end{abstract}

1. Introduction

Among all the infectious diseases affecting humans, tuberculosis (TB) caused by \textit{Mycobacterium tuberculosis} remains one of the most lethal (\textit{WHO}, 2011). Based on tuberculin test, epidemiologists estimate that one-third of the world population (2.2 billion people) is infected with the bacillus (Ducati \textit{et al.}, 2006). In Brazil, 80,000 cases are registered annually with incidence rate of 37.1/1,00,000 inhabitants, according to data from the Brazilian Health Ministry (Brazil, 2008).

The risk of infected individuals to develop the active form of the disease during their lifetime ranges between 5% and 10%. In these individuals, the chances of illness depend on the immune system’s ability to prevent the multiplication of dormant \textit{M. tuberculosis} (Oliveira \textit{et al.}, 2004).

Innate immunity is the first line of host defense. At some time in the cycle of TB infection, most infected humans, who are immunocompetent, will show the presence of the mycobacteria and begin to generate an immune response, destroying macrophages containing bacilli. This process will result in the presentation of mycobacterial antigens to the host immune system, leading to the generation of a specific immune response against \textit{M. tuberculosis} (Clark-Curtiss and Haydel, 2003; Ducati \textit{et al.}, 2006).

Previous studies have demonstrated the association of several genes with the susceptibility to TB, such as human leukocyte antigen (HLA), natural resistance-associated macrophage protein 1 (NRAMP1), vitamin D receptor (VDR), interleukin-1 (IL1), interleukin-12B and interleukin-12 receptor (IL-12Rb1) (Singh \textit{et al.}, 1983; Bellamy \textit{et al.}, 1998, 1999; Goldfeld \textit{et al.}, 1998; Greenwood \textit{et al.}, 2000; Wilkinson \textit{et al.}, 2000; Remus \textit{et al.}, 2004; Morris \textit{et al.}, 2011). A linkage analysis on sib-pairs conducted in Africa (Bellamy \textit{et al.}, 2000) has mapped TB susceptibility loci at chromosomes 15q11–13 and Xq26, although another genome-wide scan, performed in the north of Brazil, did not replicate those findings (Miller \textit{et al.}, 2004).

Certain innate immunity proteins, such as the mannose binding lectin (MBL), can recognize the mannose on pathogens’ surface, promoting both the opsonization and activation of the complement system. MBL deficiency has been associated with an
increased frequency of various infections, including sepsis, aspergillosis, meningococcal disease and invasive pneumococcal infections.

In TB context, the role of MBL is controversial. Some studies showed that MBL deficiency protects against TB (García-Laorden et al., 2006; Cosar et al., 2008; Denholm et al., 2010; Liu et al., 2010); on the other hand, MBL deficiency was also associated with susceptibility to TB infection (Selvaraj et al., 2006; Alagarasu et al., 2007; Capparelli et al., 2009).

MBL2 gene (10q11.2–q21) encodes for the mannose binding lectin and presents several polymorphisms, six of which are known for their functional effect. Three are located at exon 1: the first one, rs5030737 is a C>T transition at codons 52 (CGT>TGT) that results in Arg52Cys substitution (where the variant allele is also known as “D” allele), rs1800450 is a G>A transition (known as “B” allele) at codons 54 (GGC>GAC) resulting in Gly54Asp substitution, and rs1800451 is a G>A transition (known as “C” allele) at codon 57 (GGA>GAA) resulting in Gly57Glu substitution. (Madsen et al., 1994). These SNPs together are identified as “AO” polymorphisms, were the “A” wild-type allele, indicate no variant alleles at codons 52, 54 and 57 (C, G and G nucleotide respectively) and the “O” mutant allele, indicate the presence of one or more mutant alleles in either codons 52, 54 and 57 (T, A and A nucleotide, respectively). The presence of a O allele, in heterozygosis correlate for their functional effect. Three are located at exon 1: the first two letters indicate the variants of the promoter region, namely HOF/SUS, all located at metropolitan area of Recife, Pernambuco, Brazil.

This group was composed of 119 pulmonary TB (84 males and 35 females, mean age 28 years ± 10.4) and 36 extra-pulmonary tuberculosis patients (17 males and 19 females, mean age 31 years ± 16.9). The diagnosis was based on clinical symptoms and radiographic findings, along with bacteriological confirmation (culture, smear and/or polymerase chain reaction) as described by the American Thoracic Society (2000).

As control group, we enrolled 148 healthy individuals (81 females, 67 males; mean age 25 years ± 2.42), unrelated to patients, with negative Mantoux test, showing no symptoms of tuberculosis or previous history of the disease. All patients and control subjects were matched for ethnicity, HIV-negative and not under immunosuppressive medication. We tried to choose the best controls in terms of similarity of exposition to TB patients, by enrolling healthy individuals from the same areas (metropolitan Recife, Pernambuco, Brazil), where patients have been collected. HIV infection was exclusion criteria when enrolling TB patients, since MBL2 polymorphisms, being also related with susceptibility to HIV infection, could represent a confounding factor.

Written and informed consent was obtained form the patients or their parents (in case of minor age) and the CPqAM/FIOCRUZ Ethics Committee (CEP Registration – 55/05) approved the study. Patients underwent a standardized clinical-epidemiological questionnaire. Data were stored and subsequently processed using the Statistical Package for Social Sciences (SPSS – version 10.0 for Windows).

2.2. MBL2 genotyping

Genomic DNA was extracted from whole blood using QIAamp DNA Blood Kit, according to manufacturer instructions (QIAamp DNA Blood Midi Kit, Qiagen).

MBL2 promoter and exon 1 polymorphisms (GenBank accession: rs11003125, rs7096206, rs5030737, rs1800450, rs1800451) have been genotyped by direct sequencing with the following primers: 5’–GCCAGTTTGTGGACTAC–3′ and 3’–CTCTATATCCCCAGCAGT–5′, using the Big Dye Terminator kit 3.1 (Applied Biosystems). Sequencing reactions were run on the ABI 3130 genetic Analyzer (Applied Biosystems, Foster City, CA, USA); sequences were handled using the 4Peaks (http://mekentosj.com/4peaks/) and Codon-Code Aligner (http://www.codoncode.com/aligner/) software.

The haplotypes and combined genotypes were computed using Arlequin version 3.01 software (available at http://cmpg.unibe.ch/software/arlequin3/) and identified by a specific nomenclature, where the first two letters indicate the variants of the promoter region (“HL” and “XY” variants) and the third letter indicates the combination for the three polymorphisms in exon 1 (“AO” variant) (Garred et al., 1997). The SNP at position +4 has been genotyped but not considered in this study because of very low relevance in the variation of serum MBL levels. (Bouwman et al., 2006)

2.3. Statistical analysis

Chi-square test was used to verify the Hardy–Weinberg equilibrium and the Fisher’s exact test was performed for pair-wise comparison of allele, genotype and haplotype frequencies using contingency tables as appropriate, and only p values <0.05 were considered as significant. All the statistical analyses were carried out using the open-source R package. (R Development Core Team, 2012) available at http://www.r-project.org site. When calculating odds ratio (OR) in Tables 1 and 2, the alleles and corresponding homozygous genotypes with major frequency in the control group have been selected as reference (OR = 1) and the other ORs have been presented relative to that reference (Fisher’s exact test, 2 × 2 contingency tables, degrees of freedom = 1).
The power analysis was performed with the “G-power” software (version 3.0.5, http://wwwpsycho.uni-duesseldorf.de/abteilungen/aap/gpower3/), post hoc goodness of fit $\chi^2$ test, with an “–error” probability of 0.05.

The possible presence of population stratification bias has been gauged according to Lee and Wang (2008), considering an "–error" probability of 0.05.

For the promoter "XY" variant, although no differences were seen in allelic frequencies, the $\chi^2$ test, with an "–error" probability of 0.05.

When $\lambda_{\text{specific}}$ was considered for the 57 C allele might be due to the confounding effect of a population stratification bias, and the $\chi^2$ test, with an "–error" probability of 0.05.

In addition to analyzing the exon 1 polymorphisms at codon 52, 54 and 57 together as O alleles, we then considered the three polymorphisms singularly (B–D variant alleles, Table 2). No significant differences were found when we compared the frequencies of polymorphisms at codons 52 ("AD" variant) and 54 ("AB" variant) between TB patients and controls, as well as between pulmonary and extra-pulmonary TB and controls. The analysis of codon 57 showed that the mutant C allele and the A/C genotype were significantly more frequent in TB patients than controls and both associated with an increased risk of TB; the same association was observed in pulmonary TB patients but not in extra-pulmonary. The C/C mutant genotype was never found in any of the 155 patients, and any of the controls (Table 2).

In order to exclude that the association of the 57 C allele might be due to the confounding effect of a population stratification bias, we evaluated the potential bias in our study, according to the method described by Lee and Wang (2008). Considering an incidence rate of TB in the Brazilian population variable between 30/1,000,000 and 92/1,000,000 (Hijjar et al., 2001) and an allelic frequency of codon 57 for the Brazilians ranging from 0.003 to 0.24, as reported by Boldt et al. (2006), the potential confounding rate ratio (U) was 2.49, which is less than the OR we found for the codon 57 SNP (OR = 2.74). This indicates that the association found between $MBL2$ codon 57 SNP and TB cannot be ascribable to population stratification bias alone.

We also considered the $MBL2$ combined promoter and exon 1 genotypes, classified according to Bouwman et al. (2006) as high type (A/O plus O/O $p = 0.029, OR = 1.73, 95\% CI 1.04–2.89$), indicating an association with susceptibility to TB development.

When the specific form of TB was considered, we found that the O allele, A/O and A/O plus O/O genotypes were all significantly more frequent in pulmonary (but not in extra-pulmonary) TB patients than in controls.

In Table 1, $MBL2$ gene polymorphisms frequencies among TB infected subjects (classified as pulmonary or extra-pulmonary TB) and healthy controls (HC) from Recife, Pernambuco, Brazil.

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<tr>
<th>Alleles</th>
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**3. Results**

The results of $MBL2$ promoter region and exon 1 genotyping in TB patients and controls are shown in Table 1. All polymorphisms were in Hardy–Weinberg equilibrium in both patients and control groups.

$MBL2$ promoter "HL" SNP showed no difference in allelic or genotype frequencies between TB patients globally considered and healthy controls, as well as between each of the two TB subgroups (pulmonary and extra-pulmonary) and controls.

For the promoter "XY" variant, although no differences were seen in allelic frequencies, the $\chi^2$ test, with an "–error" probability of 0.05.

When $MBL2$ exon 1 "AO" variants were analyzed, the O allele was significantly more frequent in TB patients in general (23%) than in healthy controls (16%), and associated with increased susceptibility to TB development ($p = 0.036; OR = 1.82; 95\% CI 1.02–3.30$).

The possible presence of population stratification bias has been gauged according to Lee and Wang (2008), considering an "–error" probability of 0.05.

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4. Discussion

Host genetic factors can determine differences in the susceptibility and/or resistance to infections, as well as in the clinical patterns of diseases. In our study we showed that MBL2 “XY” promoter polymorphism and the “AC” polymorphism at codon 57 are involved in the susceptibility to TB development in a northeastern Brazilian population. A significant association was found for MBL2 C allele and A/C genotype at codon 57, and for the Y/X promoter genotype, with the pulmonary but not extrapulmonary form of TB disease.

So, we can assume that there is no association between the presence of these variations and the development of extra-pulmonary TB and that MBL2 variant can influence the risk only of the pulmonary form; however, since MBL2 frequencies were not significantly different between pulmonary and extra-pulmonary TB patients, the lack of association in the extra-pulmonary group could be due to the small sample size of this group (36 patients). Calculations of statistical power indicate that our study, although having a power >95% to detect a medium/large effect of MBL2 variants in TB, may have instead failed to detect a smaller effect (power <30% for w = 0.01).

MBL2 polymorphisms have been associated with low levels of MBL in serum. Individuals homozygous for MBL2 mutation have almost undetectable levels of MBL (<10 ng/ml); average levels are reduced to approximately 350 ng/ml in heterozygotes, while MBL concentration reaches over 1600 ng/ml in wild type homozygous individuals (Davies et al., 2001). The decrease in concentration of circulating MBL has been associated with recurrent infections in childhood and possibly in adults. However, the effect of low MBL levels on TB has been controversial: some studies suggested that O/O genotype may be associated with susceptibility to TB, whereas others estimated a relation with increased susceptibility (Denholm et al., 2010).

Alagarasu et al. (2007) and Selvaraj et al. (2006), have previously suggested that O/O genotype may be associated with susceptibility to TB, since they observed a significant increase in the frequency of O/O genotype in TB patients than controls. Additionally, Capparelli et al. (2009), reported higher frequencies of the O/O genotype among patients (22.3% vs. 3.5% in controls) in an Italian population. In our study, the O/O genotype frequency distribution was similar between patients and controls, but the O allele, as well as the A/O genotype, were significantly more frequent in TB patients, (pulmonary TB), than healthy controls; the homozygous A/A genotype among patients (22.3% vs. 3.5% in controls) in an Italian population. In our study, the O/O genotype frequency distribution was similar between patients and controls, but the O allele, as well as the A/O genotype, were significantly more frequent in TB patients, (pulmonary TB), than healthy controls; the homozygous A/A genotype among patients (22.3% vs. 3.5% in controls) in an Italian population. In our study, the O/O genotype frequency distribution was similar between patients and controls, but the O allele, as well as the A/O genotype, were significantly more frequent in TB patients, (pulmonary TB), than healthy controls; the homozygous A/A genotype among patients (22.3% vs. 3.5% in controls) in an Italian population.
2010). MBL2 variants, either structural alleles (codons 54 and 57) in Gambian children (Bellamy et al., 1998) and South African adults (Hoal-Van Helden et al., 1999), or full promoter haplotypes responsible for low MBL production, have been shown to be protective against tuberculosis. Søborg et al. (2003) demonstrated a significantly decreased frequency of individuals with the low-expressing MBL genotype in Caucasian patients compared to control subjects. The same tendency was also observed in patients of other ethnic origin. The authors hypothesized that heterozygosity for MBL2 variant alleles, responsible for low serum MBL levels, was associated with protection against clinical TB. Studies in Danish patients (Søborg et al., 2003) and Turkish children (Solgun et al., 2011) showed no association between MBL2 polymorphisms at the codons 54 and 57 and susceptibility to pediatric TB.

There is no doubt about the difficulty in comparing results from studies conducted in different populations, even when the same allele or haplotype are analyzed and the same study design is used. In addition to this, several studies investigated the role of MBL in TB in the context of HIV co-infection and thus with the confounding aspect of immunosuppression. As reported in the methods section we decided to eliminate this bias excluding patients with HIV infection we decided to eliminate this bias excluding patients with HIV co-infection from our study. Since the frequency of MBL2 polymorphism, as well as the incidence rates of tuberculosis, are known to differ from Recife and the one from Belém do Pará studied by Araújo et al. (2012) are from Brazil, there are considerable differences in the ethnic composition. Alves-Silva et al. (2000) had shown, by studying the mitochondrial genome of different regions of Brazil, that the Northern population (Belém do Pará) is a combined mixture of the genome of Native Americans (54%), Africans (15%) and Europeans (31%), while the Northeastern population is comprised of 22% Native American, 44% African and 34% European gen-

OR = odds ratio; C.I. = confidence intervals; Ref = reference.
authors admit, is not directly valid in human disease. Thye et al. findings are in contrast with our results, indicating association of MBL2 SNP at codon 57 with TB susceptibility. Once again, allele C frequencies are quite different between our population and Ghanaian, and moreover we don’t exactly know to what extent our patients were infected by M. africanaum or M. tuberculosis isolates. The putative protective MBL2 haplotype LYQC, which is virtually unique to sub-Saharan Africa and occurs there at high frequencies, might have been selected because it confers protection from clinical TB caused by M. africanaum/M. bovis. The pattern of variation in the gene may represent a past adaptation to pathogens, with selection maintaining polymorphisms that optimized the fitness of the carriers in these environments. Widespread and ancient diseases such as tuberculosis, were found to be negatively associated with the MBL2 “low-secretor haplotypes” and protection against these diseases has been taken as evidence for the hypothesis that natural selection drove these MBL2 haplotypes to their actual high frequency. It is possible, as suggested by Boldt et al. (2006) that stochastic evolutionary factors erased much of the ancient imprint left by natural selection for chastic evolutionary factors erased much of the ancient imprint left by natural selection for

References


