Association of MBL2, TGF-β1 and CD14 gene polymorphisms with lung disease severity in cystic fibrosis*

Associação entre os polimorfismos dos genes MBL2, TGF-β1 e CD14 com a gravidade da doença pulmonar na fibrose cística

Elisangela Jacinto de Faria, Isabel Cristina Jacinto de Faria, José Dirceu Ribeiro, Antônio Fernando Ribeiro, Gabriel Hessel, Carmen Sílvia Bertuzzo

Abstract

Objective: To identify associations between genetic polymorphisms (in the MBL2, TGF-β1 and CD14 genes) and the severity of the lung disease in patients with cystic fibrosis (CF), as well as between the presence of ΔF508 alleles and lung disease severity in such patients. Methods: This was a cross-sectional cohort study, based on clinical and laboratory data, involving 105 patients with CF treated at a university hospital in the 2005-2006 period. We included 202 healthy blood donors as controls for the determination of TGF-β1 and CD14 gene polymorphisms. Polymorphisms in the MBL2 and TGF-β1 genes at codon 10, position +869, were genotyped using the allele-specific PCR technique. The C-159T polymorphism in the CD14 gene was genotyped using PCR and enzymatic digestion. Results: Of the 105 CF patients evaluated, 67 presented with severe lung disease according to the Shwachman score. The MBL2 gene polymorphisms were not associated with disease severity in the CF patients. Analysis of the T869C polymorphism in the TGF-β1 gene showed an association only between TC heterozygotes and mild pulmonary disease. Although patients presenting the TT genotype of the C159T polymorphism in the CD14 gene predominated, there was no significant difference regarding lung disease severity. Conclusions: There was an association between the TC genotype of the T869C polymorphism (TGF-β1) and mild pulmonary disease in CF patients. The CD14 gene, the TT genotype seems to be a risk factor for pulmonary disease but is not a modulator of severity. We found no association between being a ΔF508 homozygote and presenting severe lung disease.

Keywords: Cystic fibrosis; Polymorphism, genetic; Severity of illness index; Mannose-binding lectin; Transforming growth factor beta.

Resumo


Descritores: Fibrose cística; Polimorfismo genético; Índice de gravidade de doença; Lectina de ligação a manose; Fator transformador de crescimento beta.

* Study carried out in the Department of Medical Genetics, Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.
Correspondence to: Elisangela Jacinto de Faria. Caixa Postal 6111, Cidade Universitária Zeferino Vaz, CEP 13083-970, Campinas, SP, Brasil.
Tel 55 19 3252-2603. E-mail: elliiss@yahoo.com.br
Financial support: None.

Association of MBL2, TGF-β1 and CD14 gene polymorphisms with lung disease severity in cystic fibrosis

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by more than 1,600 mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, located on the long arm of chromosome 7. These mutations are divided into six classes. There are classical and atypical CF phenotypes, depending principally on the class and type of mutation. The incidence rate is 1/2,500 newborns, whereas that of CF patients is 1/25.[1-3]

Population studies involving large numbers of CF patients confirm the genotype-phenotype relationship, mainly for pancreatic manifestations. Therefore, little is known regarding the genetic characteristics of the genotype-phenotype relationship in pulmonary manifestations. It is known that even individuals homozygous for a mutation of higher prevalence (ΔF508) present greater variability in the impairment and evolution of pulmonary disease.[4]

Although environmental influences can interfere with pulmonary clinical manifestations, the possibility of an additional genetic variation, such as the presence of modifying genes, has been described, contributing to the final clinical expression in each patient. Some authors have reported that polymorphisms in genes other than CFTR can modify pulmonary disease severity in CF.[5]

A study in monozygotic twins has shown a higher concordance in relation to pulmonary disease severity when compared with the severity in dizygotic twins, suggesting a strong genetic contribution to the variability of pulmonary disease severity in CF patients.[6]

Modifying genes, with the exception of the CFTR gene, can influence phenotype severity in CF patients through a number of mechanisms, being able to modulate the phenotype alternating the conduction of chloride; to regulate the splicing and the expression of the CFTR gene; and to modulate the susceptibility to bacterial infection and inflammatory response in the lungs. In addition, lung disease in CF patients can be modified by genes associated with mucociliary clearance, as well as by those associated with damage to and repair of the epithelial tissue.[7]

The concept of multiple genetic modifiers in Mendelian diseases, such as CF, is different from the concept of multiple genetic variants in non-Mendelian diseases, such as asthma. In complex genetic diseases, such as asthma, multiple genetic variants interact among themselves and the environment, causing the disease. Nevertheless, CF, being a Mendelian disease, is caused by mutations in the CFTR gene and there are genetic variations not connected to the CFTR gene, which may be unfavorable or favorable, and which modify phenotype severity together with environmental factors. In fact, genetic polymorphisms, whether or not they have effects in healthy subjects, can be modifiers in CF.[8]

Identifying the consequence of the action of modifying genes will allow better understanding of physiopathological aspects and of the genotype-phenotype relationship, as well as maximizing the treatment of patients with CF.[9]

Among modifying genes, the following can be found:

- **MBL2**: Located on the q11.2-q21 arm of chromosome 10, MBL2 encodes the mannose-binding lectin (MBL) protein. It is a plasmatic protein with an important role in the innate defense system, which constitutes the first activation component of the complement system lectin pathway and acts in the neutralization of pathogenic microorganisms by an independent antibody mechanism.[9] Its deficiency has been correlated with decreased pulmonary function in CF patients.[10]

- **TGF-β1**: The TGF-β1 gene has been mapped to chromosome 19q13.1-q13.3. This gene is expressed in endothelial cells, hematopoietic cells and cells of related tissues. The TGF-β1 gene encodes the protein of TGF-β1, which is a member of a family of growing and differentiation factors, with multiple functions in a variety of different organic systems. The TGF-β1 protein is notable for its capacity of modulating a variety of cellular functions, including cell proliferation, differentiation and in vivo and in vitro apoptosis.[10] Underexpression and overexpression of this protein can both cause damage to the respiratory tract.

- **CD14**: The CD14 gene is located on chromosome5q31.1, with 3,900 bp. It presents two exons and encodes a protein of 375 amino acids, being expressed at the border of the macrophage, monocyte...
and neutrophil membranes. It functions as a receptor for lipopolysaccharides, components of the external membrane of gram-negative bacteria. It is a constitutive element of the cell wall of *Pseudomonas aeruginosa*, which has high immunogenic power. Underexpression of CD14 has been related to the early colonization of bacteria, including *P. aeruginosa*, in the lungs.

The objective of the present study was to determine how strongly lung disease severity in patients with CF correlates with polymorphisms of exon 1 (codons 52, 54 and 57) and the promoter region (haplotypes HY, LY and LX) of the MBL2 gene, with T869C polymorphism in the TGF-β1 gene and with the C-159T polymorphism in the CD14 gene. We also evaluated the relationship between ΔF508 alleles and lung disease severity in CF patients.

### Methods

This was a cross-sectional clinical and laboratory cohort study involving patients treated between 2005 and 2006 at the Cystic Fibrosis Outpatient Clinic of the *Universidade Estadual de Campinas* (Unicamp, Campinas State University) Department of Pediatrics and Hospital de Clínicas. We included all patients under follow-up treatment and who had been diagnosed with CF, confirmed based on clinical history and on at least two sweat tests with chlorine values equal to or above 60 mEq/L conducted through sweat stimulus by iontophoresis with pilocarpine, as well as on identification of genetic mutation.

We evaluated 105 CF patients, 67 of whom presented the clinical classification of lung disease. We included 202 healthy blood donors as controls for the polymorphisms in the TGF-β1 and CD14 genes.

The study was approved by the Research Ethics Committee of the Unicamp School of Medical Sciences, and all of the legal guardians gave written informed consent.

The clinical criteria analyzed included pulmonary manifestations, digestive manifestations and the Shwachman score (SS). Laboratory evaluation included pulmonary function tests, determination of sodium/chlorine levels in sweat, chest X-ray and HRCT scan of the chest. The SS evaluates physical activity, physical examination findings, nutrition and the radiologic profile. For each item, the maximum score is 25 points; lower scores translating to poorer clinical status. The total score is graded as very mild (86-100), mild (71-85), moderate (56-70), severe (41-55) and extremely severe (40 or less). All patients were previously genotyped for the CFTR gene by the team of the Molecular Genetics Laboratory of the Hospital de Clínicas. The DNA was extracted through the PCR technique, and specific regions were amplified so that the following mutations could be analyzed: ΔF508, G542X, N1303K, G551D and R553X.

For the MBL analysis, DNA extraction from peripheral blood leukocytes was conducted. After DNA extraction, we sequenced specific primers, through which various amplification reactions were conducted. Each had an initiator capable of detecting an allele or group of alleles. Using the sequence-specific PCR primers, we genotyped 105 individuals for known mutations in the H and L promoter region at the position −550 (G-C)—located at 550 bp before the start of the transcription site, where the guanine-to-cytosine substitution occurs—and in the X and Y promoter region, in the −221 (G-C) position—located at 221 bp before the transcription start site, where the guanine-to-cytosine substitution occurs. The −550 and −221 polymorphisms in the promoter region form the HY, LY and LX haplotypes.

The codons 52, 54 and 57, located on exon 1, give rise to three variable alleles (designated D, B and C, respectively). The regular allele has been called A, and the variable D, B and C alleles are classified as O. The point mutations in the D, B and C alleles occurred, respectively, in the nucleotides 223 (C-T)—cytosine-to-thymine substitution—230 (G-A)—guanine-to-adenine substitution—and 239 (G-A)—guanine-to-adenine substitution.

For the analysis of the TGF-β1 gene, DNA was extracted from peripheral blood leukocytes. Following DNA extraction, we identified the TGF-β1 gene polymorphism, located on codon 10, position +869 (T-C)—thymine-to-cytosine substitution—through the technique called amplification refractory mutation system.

For the CD14 gene analysis, we conducted DNA extraction from peripheral blood leukocytes. After DNA extraction, the CD14 polymorphism (C-159T, cytosine-to-thymine substitution) was genotyped.
Table 1 - Methods, sequence-specific primers and restriction enzymes used, as well as fragments generated by the polymorphisms of the MBL2, TGF-β1 and CD14 genes.

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Method</th>
<th>Primers</th>
<th>Amplified fragment, bp</th>
<th>Restriction enzymes and fragments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBL2 gene, codon 52 D</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>268</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene, codon 52 not D</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>268</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene, codon 54 B</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>278</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene, codon 54 not B</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>278</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene, codon 57 C</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>290</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene, codon 57 not C</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>290</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Control MBL2 gene</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>431</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene promoter region, allele H</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>316</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene promoter region, allele L</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>316</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene promoter region, allele Y</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>443</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>MBL2 gene promoter region, allele X</td>
<td>PCR-SSP</td>
<td>5'-CTGCACCCAGATTGAGGACACAG-3'</td>
<td>440</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>TGF-β1 gene (T869C)</td>
<td>PCR-ARMS</td>
<td>General sense primer:</td>
<td>241</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TCCGTGGGATACTGAGACAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense primer C:</td>
<td>241</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GCAGGCTACAGACGACGAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense primer T:</td>
<td>241</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-AGCACGCTACAGACGAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Internal control primer 1:</td>
<td>429</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GCTCTCCCAACACTACTG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Internal control primer 2:</td>
<td>429</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TGCTGCAACGATGGTTGTTGAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14 gene (C-159T)</td>
<td>PCR + RE</td>
<td>5'-GCCCTCTGACAGTTTATGTAAATC-3'</td>
<td>497</td>
<td>Avall, 353 and 144</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TGCTGCAACGATGGTTGTTGAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SSP: sequence-specific primers; ARMS: amplification refractory mutation system; and RE: restriction enzyme.

The methods, primer sequences and restriction enzymes used, as well as the size of the fragments generated by MBL2, TGF-β1 and CD14 gene polymorphisms, are described in Table 1.\(^{(15-17)}\)

The analysis of the results and associations between the variables in CF patients and those in the control group were made using the chi-square test and ORs. The difference between the groups was considered statistically significant.
Table 2 - Comparison between the presence of two ΔF508 alleles and the absence of ΔF508 alleles in terms of the severity of lung disease.

<table>
<thead>
<tr>
<th>Lung disease</th>
<th>Two alleles, n (%)</th>
<th>No alleles, n (%)</th>
<th>( \chi^2 )</th>
<th>p</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe</td>
<td>1 (5)</td>
<td>6 (46.1)</td>
<td>5.71</td>
<td>0.01</td>
<td>16.29 (1.43-787.09)</td>
</tr>
<tr>
<td>Moderate</td>
<td>11 (55)</td>
<td>4 (30.8)</td>
<td>1.87</td>
<td>0.17</td>
<td>0.36 (0.06-1.93)</td>
</tr>
<tr>
<td>Mild</td>
<td>8 (40)</td>
<td>3 (23.1)</td>
<td>1.02</td>
<td>0.31</td>
<td>0.45 (0.06-2.63)</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( \chi^2 \): chi-square test.

Table 3 - Genotypic comparison between CF patients and the control group for polymorphism T869C in the TGF-β1 gene.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Study, n (%)</th>
<th>Control, n (%)</th>
<th>( \chi^2 )</th>
<th>p</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>17 (16.2)</td>
<td>43 (21.3)</td>
<td>1.14</td>
<td>0.28</td>
<td>0.71 (0.37-1.38)</td>
</tr>
<tr>
<td>TC</td>
<td>83 (79.04)</td>
<td>132 (65.3)</td>
<td>6.18</td>
<td>0.01</td>
<td>2.00 (1.11-3.61)</td>
</tr>
<tr>
<td>CC</td>
<td>5 (4.76)</td>
<td>27 (13.4)</td>
<td>5.48</td>
<td>0.01</td>
<td>0.32 (0.11-0.92)</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>202</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( \chi^2 \) = 7.68; p = 0.02. \( \chi^2 \): chi-square test.
The control sample is not in HWE ($\chi^2 = 18.72; p = 0.00008$).

In the genotypic comparison, there was a significant difference between the group of CF patients and the control group regarding C159T polymorphism in the CD14 gene. The TT genotype was found to be a risk factor in the sample, but not a modulating factor of lung disease severity ($p = 0.001; OR = 4.36; variation, 1.68-12.16$; Table 5).

**Discussion**

Among COPDs, asthma and CF are phenotypically manifested as consequent to a genetic and an environmental component, which determine the severity and the clinical course over the lifetime of patients with these diseases.

In CF and asthma, we identified mutations in 1 and in more than 100 genes, respectively, characterizing the identification of many phenotypes in these two COPDs. We demonstrated, therefore, that the phenotypical complexity of asthma is higher than that of CF. Nevertheless, whereas the association between polymorphisms and phenotypical manifestations has been widely studied in asthma, there have been few studies in CF.

After an extensive review of the literature, we can state that this is the first study in Brazil to determine the association between polymorphisms of the MBL2, TGF-β1 and CD14 genes and lung disease severity in children and teenagers with CF.

In the present study, for polymorphisms in which the control sample was not found to be in HWE, the probable explanation comes from the fact that, in the HWE guidelines, an ideal population, with no selective pressure, is recommended. In the case of the polymorphisms studied, since they influence mechanisms related to inflammation, it is possible that certain genotypes suffer from a selective pressure and, consequently, the genotypic distribution has not met the HWE criteria.

In CF, the pulmonary component can be influenced by genetic and environmental factors, as well as by modifying genes other than the CFTR gene.\(^{(18)}\)

In contrast to the findings of other studies, in the analysis of pulmonary disease severity and of the presence of ΔF508 alleles, we identified fewer ΔF508/ΔF508 homozygotes among patients with severe lung disease, showing a lack of association between being ΔF508 homozygous and presenting greater lung disease severity.

In our sample of CF patients, MBL2 gene polymorphisms were not associated with lung disease severity. One possible explanation for our results is the fact that most patients were younger than 15 years of age. Some authors have shown that MBL deficiency is related to lung disease severity only in CF patients older than 15 years of age.\(^{(19)}\) In such patients, the growth hormone can significantly affect the level of circulating MBL. That study revealed significant age- and physical development-related differences among CF patients in terms of MBL and pulmonary function.\(^{(19)}\)

Various authors have reported that only CF patients whose genotype is OO (homozygous for polymorphisms in exon 1 of the MBL2 gene), which is related to the low production of MBL protein, present a decrease in pulmonary function.\(^{(20,21)}\) The same was not observed in another study in which the two MBL2 gene genotypes—AO (heterozygote) and OO (homozygote)—were associated with a decrease in pulmonary function.\(^{(22,23)}\)

It has been shown that children diagnosed with CF colonized by *P. aeruginosa* and who are MBL deficient have more severe pulmonary dysfunction in comparison with those presenting intermediate or high levels of circulating MBL.\(^{(24)}\) The authors have shown that these modulating...
protein), although there were no differences in relation to lung disease severity.

In one study, the CD14-159CC polymorphism was found to be associated with the early colonization of airways by *P. aeruginosa* in children with CF. These children presented decreased plasma levels of the soluble CD14 protein, together with an inappropriate pro-inflammatory response. Although children with high plasma levels of the soluble CD14 protein might be relatively protected against early colonization by *P. aeruginosa*, when becoming colonized, they may have a more intense inflammatory response.

Since ethnic and racial differences are common in polymorphic systems, inducing the expression of a clinical phenotype in different populations, it is possible that different results would be found in other populations and racial groups.

The results obtained in the present study allow us to conclude that many questions remain regarding the function of the modifying genes in CF in different populations. Therefore, multicenter studies, evaluating a larger number of patients in each mutation class, are necessary for understanding the effects of modifying genes in CF.

**Acknowledgements**

The authors would like to thank the members of the multidisciplinary team of the Cystic Fibrosis Outpatient Clinic of the State University at Campinas *Hospital das Clínicas*.

**References**


---

**Table 5** - Genotypic comparison between the samples from patients and those from the control group for C159T polymorphism in the CD14 gene.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Study, n (%)</th>
<th>Control, n (%)</th>
<th>$\chi^2$</th>
<th>p</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>16 (15.2)</td>
<td>8 (4)</td>
<td>10.68</td>
<td>0.001</td>
<td>4.36 (1.68-12.16)</td>
</tr>
<tr>
<td>CT</td>
<td>67 (63.8)</td>
<td>131 (64.9)</td>
<td>0.03</td>
<td>0.85</td>
<td>0.96 (0.57-1.62)</td>
</tr>
<tr>
<td>CC</td>
<td>22 (21)</td>
<td>63 (31.1)</td>
<td>3.62</td>
<td>0.05</td>
<td>0.58 (0.32-1.05)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>105</strong></td>
<td><strong>202</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\chi^2 = 13.87; p = 0.0009. \chi^2$: chi-square test.
Association of MBL2, TGF-β1 and CD14 gene polymorphisms with lung disease severity in cystic fibrosis


About the authors

Elisangela Jacinto de Faria
PhD in Medical Sciences. Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.

Isabel Cristina Jacinto de Faria
PhD in Medical Sciences. Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.

José Dirceu Ribeiro
Tenured Professor. Department of Pediatrics, Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.

Antônio Fernando Ribeiro
Tenured Professor of Pediatric Gastroenterology. Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.

Gabriel Hessel
Tenured Professor of Pediatric Gastroenterology. Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.

Carmen Silvia Bertuzzo
Tenured Professor. Department of Medical Genetics, Universidade Estadual de Campinas – Unicamp, State University at Campinas – School of Medical Sciences, Campinas, Brazil.