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Study of the effect of DNA polymorphisms in the mannose-binding lectin gene (*MBL2*) on disease severity in Slovak cystic fibrosis patients

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Abstract. Lung infections are the leading cause of morbidity and mortality in cystic fibrosis (CF). Mannose-binding lectin (MBL) is a key factor in innate immunity. We therefore investigated whether *MBL2* gene variants are associated with pulmonary function or susceptibility to *Pseudomonas aeruginosa* and *Burkholderia cepacia* infection in Slovak patients affected with CF. DNA polymorphisms in exon 1 and the promoter region were typed by single base primer extension assay in 91 patients and 100 healthy controls. The concentrations of MBL protein were determined in 34 patients by a sandwich enzyme-linked immunosorbent assay, and spirometric and microbiological data were collected from medical records. In this study we found that *MBL2* genotypes were associated neither with earlier acquisition of *P. aeruginosa* or *B. cepacia* nor with reduced pulmonary function among patients. Although *MBL2* genotypes were associated with the MBL2 protein serum level, results were statistically significant only for polymorphisms in exon 1, with $p = 0.0008$. The role of the *MBL2* gene in lung disease severity in CF patients represents a very complex phenomenon where both genetic and environmental factors play an important role in addition to that of the *MBL2* gene. Understanding this complexity requires further studies based on a broader scale of genetic factors involving both a whole-genome approach and a larger patient cohort.

Key words: Cystic fibrosis — Mannose-binding lectin-polymorphism — Modifier gene — Lung function

Abbreviations: CF, cystic fibrosis; FEV_1, forced expiratory volume in 1 second; MBL, mannose-binding lectin.

Introduction

Cystic fibrosis (CF) is the most common lethal inherited disease among Caucasians. The leading cause of mortality in 90% of CF patients is respiratory insufficiency due to chronic inflammation caused by bacterial colonization of the respiratory tract. The most important pathogens are *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Carlsson et al. 2005). Pulmonary symptoms are highly variable among patients, and even among those in the same family.

It is assumed that the severity and progression of pulmonary dysfunction in CF is modulated by secondary genetic factors called CF modifiers (Cutting 2005; Knowles 2006). One of the first genes implicated as a pulmonary modifier in CF was the mannose-binding lectin 2 gene (*MBL2*) (Garred et al. 1999). MBL2 protein is an important mediator component of the innate immune defence system, which functions as an opsonin and complement activator. It is assigned to the family of proteins called collectins, because these contain

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collagen-like regions and lectin domains, and they bind to carbohydrate structures from a wide range of pathogenic bacteria, viruses, fungi and parasites through the lectin domain. MBL2 is synthesised in the liver by hepatocytes, secreted into the blood and circulates as dimers or hexamers composed of subunits containing three identical polypeptides (Petersen et al. 2001; Yarden et al. 2004).

The human *MBL2* gene is derived from a single gene on chromosome 10q11.2-q21 (*MBL1* is an inactive pseudogene) (Garred et al. 1999). It has been shown that *MBL2* variant alleles causing low MBL2 serum levels are associated with an increased risk of different types of infections, primarily occurring in children (Summerfield et al. 1997), but also in adults (Summerfield et al. 1995).

The following three single nucleotide polymorphisms causing independently low serum levels of MBL2 are in exon 1 of the *MBL2* gene; allele D at codon 52, allele B at codon 54, and allele C at codon 57 (Madsen et al. 1994; Yarden et al. 2004; Dorfman et al. 2008). These amino acid changes are considered to affect the tertiary structure of the collagen region of the MBL2 protein. Heterozygosity or homozygosity for these polymorphisms result in little or no functional MBL2 protein, and hence these alleles have been named 0 (null) alleles, whereas the normal allele has been named A (Madsen et al. 1994; Garred et al. 1999; Yarden et al. 2004; Dorfman et al. 2008). Additionally, the Y/X promoter polymorphism in codon -221 has a significant effect on the MBL2 serum level (Madsen et al. 1995; Garred et al. 1999; Yarden et al. 2004; Carlsson et al. 2005; Dorfman et al. 2008).

The aim of this research is to study the presence of structural *MBL2* variant alleles and the promoter variant, with respect to MBL2 protein level and consequent influences on development of pulmonary disease in Slovak CF patients.

Materials and Methods

In this study, 91 Slovak CF patients and 100 randomly selected healthy people were included. Clinical diagnosis of CF was performed at the Centre of Cystic Fibrosis (University Hospital with Policlinic, Bratislava) and departments of clinical and medical genetics in Slovakia.

Of the 91 patients, 68 were homozygous for the p.F508del mutation, while 23 patients were compound heterozygous for the mutation p.F508del with these other severe mutations: g.CFTRdele2,3 (5), p.G542X (5), p.N1303K (3), g.2184insA (2), p.R553X (2), g.3659delC (1), g.1898+1G>A (1), p.W1282X (1), g.605insT (1), g.4108delT (1), p.E831X+p.R851X (1).

The median diagnostic age of patients aged between 1 month and 19 years was 20.7 months while the median age of patients from 1.2 to 40.0 years at the time of *MBL2* gene variant genotyping was 13.8 years. The patient group consisted of 47 females and 44 males.

The FEV_1 (%) value, signifying the forced expiratory volume in 1 second, was chosen to measure lung function (Aurora et al. 2000). FEV_1 predicted values were calculated according to the Knudson coefficient (Knudson et al. 1983) and were measurable in 73 patients. Spirometer measurements were not performed on patients under 6 years of age.

Chronic bacterial infections with *Pseudomonas aeruginosa* were recorded in 65 patients and with *Burkholderia cepacia* in 26 patients.

DNA samples were isolated using the Puregen^{TM} DNA purification kit (Qiagen, Germany) and the SNaPshot method in multiplexed PCR reaction, also referred to as "minisequencing", was employed in *MBL2* gene polymorphism analysis (Quintáns et al. 2004; Yarden et al. 2004). Yarden et al's method was modified in the following steps:

1) Amplification of target genomic DNA sequence by PCR. The PCR reaction was performed in a volume of 25 µl containing 1 × buffer (0.1 mol/l Tris-HCl, 0.015 mol/l $MgCl_2$, 0.5 mol/l KCl, 1.7 g/l BSA, 1% Triton X-100), 0.2 mmol/l dNTP (Invitrogen, USA), 2 mmol/l $MgCl_2$ (Invitrogen, USA), 1 U/µl Taq DNA polymerase (Invitrogen, USA), 0.4 µmol/l primers (Sigma, Germany) (Table 1) and 200 ng/µl DNA. The mixture was then incubated in an XP-Thermal Cycler (Bioer Technology Co., China) using the following amplification profile: denaturation at 94°C for 4 min, 32 cycles at 94°C for 1 min, 62°C for 40 s, 72°C for 2 min, and a final extension step at 72°C for 7 min. The length of PCR product was 782 bp which was verified by gel electrophoresis on 1.5% agarose gel.
2) Purification of PCR product before extension reaction by Exonuclease I/Shrimp alkaline phosphatase (USB, USA).
3) SNaPshot extension reaction by SNaPshot^{TM} (Applied Biosystems, USA) SNP detection kit and 4 unlabelled single nucleotide extension primers (Sigma, Germany) for *MBL2* SNPs (Table 1).
4) Purification of PCR product following the extension reaction was conducted with Shrimp alkaline phosphatase (Fermentas, USA).
5) The mixture for capillary gel electrophoresis contained 9.15 µl Hi-Di^{TM} formamide (Applied Biosystems, USA), 0.25 µl GeneScan^{TM} 120 LIZ^{TM} size standard (Applied Biosystems, USA) and 0.6 µl SNaPshot product. After denaturation, the mixture was resolved on an ABI PRISM 3100 Avant genetic analyzer (Applied Biosystems, USA).

Statistical analysis

For analysis and date evaluation the GeneMapper v3.7 software was used. One-way ANOVA with an LSD post-hoc test was performed for statistical analysis of quantitative parameters, plus the Chi-squared test for testing associations between quantitative parameters. *p*-values less than 0.05 were considered significant. Data was analyzed using XLStatistics

Table 1. Sequences of primers for amplification of the *MBL2* gene fragment and sequences of single nucleotide extension primers for *MBL2* SNPs

Primer	Nucleotide sequence 5′→3′
YX-F	5′-CCTTCCTCTTTGGATCACCA-3′
YX-R	5′-CAGGCAGTTTCCTCTGGAAG-3′
AB (875 g/a)	5′-CGGCTTCCCAGGCAAAGATGGGCGTGATG-3′
AC (884 g/a) R	5'-AAAAAAAAAAAAAAAACCTGGTTCCCCCTTTTCT-3'
AD (868 c/t)	5'-AAAAAAAAAAAAGCATCAACGGCTTCCCAGGCAAAGATGGG-3'
YX (425 g/c)	5′-AAAAAAAAAAAAAAAAAAACAATGCAGGGTCCCATTTGTTCTACTCGGAC-3′

Primers were modified from the original protocol Yarden et al. (2004). The concentration of YX-F and YX-R primers was 0.4 μmol/l, and of other 4 SNP primers 0.2 μmol/l. The anneling temperature of first two primers was 62°C and the lenght of PCR product 782 bp. F, forward primer; R, reverse primer.

5.0 and Microsoft Excel 2007 and are presented as mean (or ± the standard deviation).

Genotypes were verified by direct sequencing using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, USA).

Concentrations of MBL2 protein were determined in 34 patients by a Sandwich enzyme-linked immunosorbent assay (ELISA) on microtitre plates coated with monoclonal anti-MBL antibodies.

Results

MBL2 gene variants and genotypes

Genotyping was performed separately for the exon 1 variants and the promoter polymorphism. In exon 1 variants, the AA genotype represents patients with only wild-type variants, genotypes AB, AC and AD were commonly designed as A0, where A represents the wild-type allele with 0 for alleles B, C or D, and the 00 genotype specified compound heterozygotes for non-wild-type alleles (BC, BD, CD) or homozygotes for the non-wild-type alleles (BB, CC, DD). The only homozygote for the BB polymorphism was detected in one control subject. Polymorphism in the promoter region was classified as follows: YY were homozygotes for wild-type allele, YX heterozygotes and XX homozygotes for non-wild-type allele (Table 2).

Comparison of genotype frequencies between patients and controls showed no significant differences for the exon 1 variants ($p = 0.438$) or for the promoter polymorphism ($p = 0.666$, Table 2). Likewise, combining these polymorphisms into joined genotypes (AA-YY, AA-YX, AA-XX, A0-YY, A0-YX, 00-YY, 00-YX) did not show significant differences between patients and controls ($p = 0.744$, Table 3).

Spirometric data and MBL2 gene variants

The FEV_1 value efficiently correlates with lung disease prognosis in CF (Aurora et al. 2000). The average age of patients at spirometric measurement was 14 years, and the average FEV_1 value was 77.9% (± 22.44%). The FEV_1 values were evaluated as follows: 1) ≥80% normal value, 2) 60–79% mild type, 3) 45–59% moderate severe type, 4) <45% severe type. Although, we found more AA patients with FEV_1 above 80% than A0 and 00 patients, these results were not statistically significant ($p = 0.802$, Table 4). For the promoter polymorphism, we observed no differences between lung function and Y genotypes (Table 4).

In the next step, the patients were subdivided into groups, according to the level of MBL2: 1) high producers, genotypes AA-YY and AA-YX (≥1000 μg/l), and 2) low producers, genotypes AA-XX, A0-YY, A0-YX, 00-YY, and 00-YX (<1000 μg/l) (Yarden et al. 2004). The results were similar to those for polymorphisms in exon 1, where more patients were found with FEV_1 above 80% in the group of high producers than in low producers, but neither of these results was statistically significant ($p = 0.18$, Table 4).

Analysis of other spirometric data evaluated in this study besides FVC (forced vital capacity), such as PEF (peak

Table 2. Genotype frequencies of *MBL2* gene variants in Slovak patients with CF and controls

Genotypes	Polymorphisms	CF patients		Controls	
		n	(%)	n	(%)
AA		58	63.7	66	66
A0	AB	17	18.7	18	18
	AC	4	4.4	5	5
	AD	7	7.7	9	9
00	BB	0	–	1	1
	BC	1	1.1	0	–
	BD	2	2.2	1	1
	CD	2	2.2	0	–
YY		62	68.1	62	62
YX		27	29.7	34	34
XX		2	2.2	4	4
Total		91		100	

Exon 1: $p = 0.438$; promoter: $p = 0.666$.

Table 3. Genotype frequencies of all 4 polymorphisms in CF patients and controls

Genotypes	CF patients		Controls	
	n	(%)	n	(%)
AA-YY	36	39.5	34	34
AA-YX	20	22	29	29
AA-XX	2	2.2	3	3
A0-YY	22	24.2	26	26
A0-YX	6	6.6	6	6
00-YY	4	4.4	2	2
00-YX	1	1.1	0	–
Total	91	100	100	100

$p = 0.744$

expiratory flow rate) and MEF (maximum expiratory flow rate), showed no significant association with particular *MBL2* genotypes, therefore the results of these statistical analyses are not presented. Similarly, the association analysis of Cl^{-2} ion concentration with *MBL2* genotypes exhibited no statistically significant results.

MBL2 protein level

Data on the MBL2 serum level was available in 34 CF patients (Table 5). The average value was 1967.5 μg/l, with a median of 1363.2 μg/l. Our results show that high levels of MBL2 (≥1000 μg/l) are associated with genotype AA and low levels (<1000 μg/l) with genotypes A0. These results are statistically significant ($p = 0.0008$). The average value of MBL2 in patients with AA the genotype was 2734.12 μg/l (median 1901.6 μg/l), while in patients with genotype A0 it was 364.7 μg/l (median 89.0 μg/l) ($p = 0.0008$). Protein levels were also compared with the promoter polymorphism and these results were, also statistically insignificant (YY had an average value of 2075.94 μg/l with median 1691.5 μg/l and YX had 1740.9 μg/l with a median of 1270.6 μg/l) ($p = 0.14$).

MBL2 genotypes and bacterial infection

The majority of the patients (71.4%) had experienced positive cultures for *P. aeruginosa*. No significant correlation was found between genotypes and the age of onset of the first *P. aeruginosa* infection, and on average, patients with AA, A0, and 00 genotypes were infected at about 10 years of age. Significant differences were, however, found in MBL2 levels in infected patients with *P. aeruginosa*. Patients with the AA genotype had protein levels of 2764.29 μg/l (median 1901.6 μg/l), while patients with A0 and 00 genotype had only 371.4 μg/l (median 89.0 μg/l) ($p = 0.0008$).

Similar results were observed for *B. cepacia* infection. Patients with the AA genotype had a protein level of 1712.87 μg/l (median 1271.8 μg/l) while A0 patients had a significantly lower level at 233.6 μg/l (median 27.7 μg/l) ($p = 0.001$).

In this study we also assessed the correlation of analyzed DNA polymorphisms with other clinical symptoms such as aspergillosis, increased immunoglobulin levels, hepatopathy, pancreatic insufficiency, meconium ileus, hypo-albuminaemia, cystic fibrosis related diabetes mellitus, atopy, body mass index, cardiomyopathy, pneumothorax, and nasal polyps. As these results did not show any statistical significance, they are not presented herein.

Discussion

Lung disease, the major life-limiting complication of CF, is poorly correlated with types of mutations in the disease-causing *CFTR* gene. Emerging data suggests a multi-factorial modulation of lung disease severity including genetic, environmental, and stochastic factors (Cutting 2005). In recent years, many genes have been studied as candidate modifier genes in CF. One of the first of these was the *MBL2* gene, an important mediator component of the innate defence system. Variants in the *MBL2* gene showed an association with multiple symptoms of CF in some studies but not in others (McDougal et al. 2010).

No significant differences in the frequencies of genotypes in the *MBL2* gene were found in our study, and similar results have been collaborated in other works (Garred et al. 1999; Yarden et al. 2004; Dorfman et al. 2008; Faria et al. 2009).

Although the majority of candidate gene studies have documented worse lung disease with insufficient *MBL2* genotypes (Garred et al. 1999; Yarden et al. 2004; Dorfman

Table 4. Distribution of *MBL2* genotypes according to FEV_1 in CF patients ($n = 73$)

		AA (%)	A0, 00 (%)	YY	YX, XX	High	Low
FEV_1 (%)	≥80	27 (55.1)	9 (37.5)	23 (48.94)	13 (50)	27 (57.45)	9 (34.62)
	60–79	11 (22.45)	9 (37.5)	14 (29.78)	6 (23.08)	10 (21.28)	10 (38.46)
	45–59	8 (16.33)	2 (8.33)	5 (10.64)	5 (19.23)	7 (14.89)	3 (11.54)
	≤45	3 (6.12)	4 (16.67)	5 (10.64)	2 (7.69)	3 (6.38)	4 (15.38)
n		49	24	47	26	47	26

High producers are AA-YY, AA-YX; low producers are AA-XX, A0-YY, A0-YX, 00-YY, 00-YX ($p = 0.18$).

Table 5. MBL2 protein level in CF patients (n = 34)

MBL2 (μg/l)	Genotypes			
	AA-YY	AA-YX	A0-YY	A0-YX
≤100	1	1	3	3
100–1000	1	0	3	1
≥1000	14	6	1	0

Grey color in table indicates the number of patients deviating from the expected value. AA genotype should be associated with higher values, while A0 genotype with lower values. p = 0.0008.

et al. 2008; Chalmers et al. 2011), a few studies showed no effect (Carlsson et al. 2005; Collaco and Cutting 2008), while Collaco and Cutting (2008) reported reduced lung function with high or intermediate producers.

MBL2 deficiency has been associated with a more rapid decline in pulmonary function (Dorfman et al. 2008), and, although some association was observed in our cohort between genotypes in *MBL2* gene and spirometric data, these results were not statistically significant. Spirometric data is not always reliable, and it can vary with age, sex and environmental factors. Other airway symptoms, such as sinusitis and nasal polyps have also been reported to affect lung function (Carlsson et al. 2005).

The A0 and 00 *MBL2* genotypes are known to result in low MBL protein level (Muhlebach et al. 2006), and this was also confirmed in our results. This is in contrast to the promoter polymorphism which does not affect the protein level. This observation can be explained by the fact that homozygosity for any of the structural mutations or their compound heterozygozity prevents oligomerization of MBL2, while homozygosity for the X allele reduces production of the MBL2 oligomer (Dorfman et al. 2008). Disease association studies involving MBL have often been conducted at the genotype level instead of measuring the MBL protein concentration, but ideally, both genotype and protein data should be analyzed (Muhlebach et al. 2006). In our group of 23 patients with AA genotype, only 3 had a lower protein level than 1000 ng/ml, and in 11 A0 patients only 1 had a protein level higher than 1000 ng/ml (Table 5). These results indicate an association between genotypes and protein levels. Results of the MBL protein level average values corresponded with polymorphisms in exon 1.

In some studies, analysis of the age of contraction of first infection with *Pseudomonas aeruginos* showed that MBL2 deficiency was significantly associated with earlier onset of infection (Summerfield et al. 1995, 1997; Dorfman et al. 2008; Chalmers et al. 2011). However, other studies have substantiated our results that there is no effect of MBL2 variation on colonization or on the age of acquisition (Garred et al. 1999; Yarden et al. 2004; Collaco et al. 2008).

McDougal et al. (2010) found that *MBL2* genotypes corresponding to low levels of MBL2 were associated with earlier *P. aeruginosa* infection than the genotypes which corresponded to high levels of MBL. In our cohort, A0 patients with bacterial infection had a lower protein level than AA patients, but clinical symptoms and pulmonary function did not reflect these findings. This may be explained by the fact that the MBL protein is synthesized exclusively in the liver and therefore does not provide a protective role against *P. aeruginosa* colonization, since the MBL protein reaches the inflammation site quite late (Ezekowitz et al. 1988).

In conclusion, varying results achieved so far in the study of the relationship of the *MBL2* gene to lung disease severity in CF patients indicate that this represents a very complex phenomenon. Besides the *MBL2* gene itself, other factors, both genetic and environmental, play an important role. Understanding this complexity requires further studies based on a broader scale of genetic factors, with the whole-genome approach and a more extensive cohort of patients.

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