Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2012; 37 (1) ; 87–93.
doi: 10.5505/tjb.2012.70288

Short Communication [Kısa Duyuru]

Yayın tarihi 30 Mart, 2012 © TurkJBiochem.com
[Published online 30 March, 2012]

KOAH hastalarında mannoz bağlayan lektin (MBL) gen polimorfizmi ve serum MBL derişimi arasındaki ilişki

[Mannose binding lectin (MBL) gene polymorphism and relationship between serum MBL concentrations in COPD patients]

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Registered: 11 April 2011; Accepted: 24 October 2011
[Kayıt Tarihi : 11 Nisan 2011; Kabul Tarihi : 24 Ekim 2011]

ABSTRACT

Aim: We aimed to assess mannose-binding lectin (MBL) gene polymorphisms and serum MBL concentrations in a sample of Turkish chronic obstructive pulmonary disease (COPD) patients as well as in cigarette smokers. Furthermore, we looked for the possible correlations of serum MBL concentrations with pulmonary function tests.

Materials and methods: Forty COPD patients and 40 healthy volunteers were included. The subjects were thereafter divided into 2 groups according to smoking status. Circulating MBL concentrations were assessed by ELISA and MBL gene polymorphisms were assessed by real time PCR method. Spirometry was performed to all subjects except healthy nonsmokers.

Results: In the whole study population MBL gene frequencies were found 82.5%(66/80) for A/B genotype, 15%(12/80) for D/D genotype and 2.5%(2/80) for B/B genotype. Circulating MBL concentrations were found 2103±1311 ng/ml and 2324±1001 ng/ml in smoker and nonsmoker COPD patients, respectively, whereas they were 1746±1142 ng/ml in smoker and 2040±879 ng/ml in nonsmoker controls. No statistical difference was found between the study groups for serum MBL concentrations. Serum MBL concentration correlated positively with cigarette smoking (r=0.280, p=0.030) and negatively with pulmonary functions (FEV_1 (r=-0.246, p=0.058).

Conclusion: To our knowledge, no previous study has been performed in healthy Turkish population to detect the MBL gene polymorphisms. A/B genotype was the most frequent MBL variant in our study population; however serum MBL concentrations were not found compatible with MBL deficiency. We believe these results need further investigation which includes larger series to evaluate whether serum MBL concentration is a risk factor for COPD.

Key Words: COPD, cigarette smoking, MBL, MBL variant, early markers, innate immunity

Conflict of interest: Authors declare no conflict of interest

ÖZET

Amaç: Bu çalışmada bir grup Türk Kronik Obstruktif Akciğer Hastalığı (KOAH) hastası ve sigara içen örneklemde mannoz bağlayan lektin (MBL) gen polimorfizmi ve serum MBL konsantrasyonun saptanması amaçlanmıştır. Aynı zamanda, MBL konsantrasyonunun solunum fonksiyon testleri ile olası ilişkisi de incelenmiştir.

Gereç ve Yöntem: 40 KOAH hastası 40 sağlıklı gönüllü çalışmaya dahil edildi. Hastalar ve gönüllüler sigara içme durumlarına göre 2 gruba bölündü. Dolaşımsal MBL konsantrasyonu ELISA metoduyla, MBL gen polimorfizmi de gerçek zamanlı PCR metoduyla çalışıldı.. Spirometre sağlıklı sigara içmayenler dışında tüm çalışma grubuna uygulandı.

Bulgular: Tüm çalışma grubunda MBL gen frekansı A/B genetipi için %82.5 (66/80), D/D genotipi için %15 (12/80) ve B/B genotipi için %2.5 (2/80) bulundu. Dolaşımsal MBL konsantrasyonu sigara içen KOAH hastalarında 2103±1311 ng/ml, içmeyenlerde 2324±1001 ng/ml saptanırken, sigara içen gönüllülerde 1746±1142 ng/ml, içmeyenlerde 2040±879 ng/ml bulundu. Serum MBL konsantrasyonlarında gruplar arasında farklılık yoktu. Serum MBL konsantrasyonu sigara içimi ile pozitif (r=0.280, p=0.030), solunum fonksiyon testleri ile negatif (FEV_1 (r=-0.246, p=0.058) ilişkili saptandı.

Sonuç: Bilgilerimiz dahilinde, sağlıklı Türk popülasyonunda MBL gen polimorfizmini saptamaya yönelik bir çalışma yapılmamıştır. Çalışma grubumuzda en sık varyant genotip A/B saptandı, ancak serum MBL konsantrasyonları MBL eksikliği ile uyumlu değildi. Bu sonuçların, daha fazla olgu içeren çalışmalarla doğrulanması, serum MBL konsantrasyonunun KOAH'da risk faktörü olup olmadığının değerlendirilmesini sağlayacaktır.

Anahtar Sözcükler: KOAH, sigara içimi, MBL, MBL varyantları, erken belirteçler, doğal bağışıklık

Çıkar çatışması: Yazarlar çıkar çatışması bulunmadığını beyan eder.

Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a preventable and treatable disease characterized by poorly reversible airflow limitation. Airflow limitation is usually progressive and associated with an abnormal response of the lungs to noxious particles or gases that eventually results in chronic airway inflammation [1]. COPD is fifth leading cause of death worldwide [2]. Therefore, diagnostic and therapeutical procedures against COPD need to be improved. Genetic variations are the potential risk factors of COPD development and gene polymorphisms which make individuals susceptible to COPD have been under research [3]. Previous reports pointed on the association of functional polymorphisms of mannose binding lectin-2 (MBL) -2 gene with COPD [4, 5].

MBL is a member of collectin proteins family [6], whose structure is similar to other best known collectins such as respiratory system surface surfactant proteins A and D [7]. It is a carbohydrate binding protein synthesized by hepatocytes which binds repeating mannose components of the cell wall [8]. As soon as MBL attaches a microorganism, conformational changes occur in MBL multimers. It is one of the major pathogen recognizing molecules of the innate system and is regarded as an acute phase reactant whose production increases with any inflammatory stimulus without any specifity [9, 10].

Currently, it has been understood that MBL binds to the endothelium and activates the complement system by an alternative pathway called C1 independent MBL pathway leading to tissue damage. MBL complement activity depends on the relation of MBL with associated serine proteases called "MBL-associated serine proteases"; MASP [1]. In 1989, it was shown that opsonization defect was related with low levels of mannose binding protein [11] and at the same year the gene coding the MBL protein was discovered [12]. MBL2 gene is located on 10^{th} chromosome over the 10q11.2–21 area [9]. There are 5 single nucleotide polymorphisms affecting the concentration of serum MBL levels [10]. It has been reported that single mutations occurring in 52^{nd}, 54^{th} and 57^{th} codons of exon 1 region known as D, B and C variants respectively, are related with low serum MBL concentrations [13]. These mutations result in the structural deformation of the proteins which eventually leads functional deficiency of MBL [14]. The wild type MBL allele is A. Exon 1 mutations including glycine–aspartic acid substitution in codon 54 is B variant, glutamic acid-glycine substitution in codon 57 is C variant, and arginin–cystein substitution in codon 52 is D variant [15, 16]. Heterozygous individuals for these mutations have a substantial decrease in MBL serum concentration.

Given the possible association of MBL polymorphism with susceptibility to infection and inflammation, in the present study we investigated serum MBL concentrations and MBL genotypes in a sample of Turkish COPD patients, smokers and healthy controls. We also looked for the possible associations of pulmonary function tests with serum MBL concentrations.

Material and Methods

Between April 2008 – 2009, 40 COPD patients who referred to our pulmonary diseases outpatient clinic and 40 healthy controls from family members of hospitalized patients were recruited. The participants were chosen and grouped consecutively into four equal groups according to smoking status as smoker and nonsmoker COPD patients and controls. Inclusion criterion for COPD patients was; being in the stable state (no exacerbations in the last 6 weeks). COPD was defined as a history of smoking more than 20 pack-years and a FEV_1/ forced vital capacity (FVC) ratio of less than 70%, 20 minutes after salbutamol administration [17]. Inclusion criterion for the control group was the absence of COPD confirmed by history, physical evaluation and/ or spirometry where necessary. Exclusion criteria for both COPD patients and control group were; having other inflammatory diseases (inflammatory bowel disease, rheumatologic diseases, vasculits), acute infections, respiratory diseases other than COPD. The study was approved by the human-research review board and all the participants provided written informed consent.

Demographic features and medical history including smoking status of the study population were recorded. Nonsmokers were defined as never smokers or ex smokers. Those with a history of less than 10 years of smoking or who quitted smoking at least 20 years ago were accepted as ex smokers. COPD patients (group 1 and 2) and smoker controls (group 3) underwent pulmonary function tests. Venous blood samples were obtained from all the participants.

Pulmonary function tests

PFTs were performed with Jaeger Master Screen Pneumo V452İ device by a single technician. FEV_1, FVC, FEV_1/ FVC were measured according to ATS criteria [17].

MBL polymorphism

DNA Extraction Procedure: Commercial "High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) " was used for leukocyte genomic DNA extraction from patient peripheral blood with anticoagulated with EDTA specimens. MBL polymorphism was assessed by real time PCR technique. This process was applied with Roche Applied Science Light Cycler 1.5 Instrument (Roche Instrument Center AG, Rotkreuz, Switzerland). A DNA sample obtained from Coriell DNA bank, with a known AB heterozygous variant (NA6993) exon 1 codon 54 was used as a positive control. Mannose binding lectin primers; forward primer: 5'-CATCAACGGCTTCCCAGGC-3', reverse primer:

5'-TGGGCTGGCAAGACAACTATTAG-3', detection probe: 5'-TTCTTCCTTGGTGCCATCACGCCCA-3' probe and anchor probe: 5'-CAGCCCAACACGTACCTGGTTCCCCCT-3' were purchased from TIB MOLBIOL GmbH (Eresburgstraße 22-23 D-12103 Berlin). PCR master mixture was prepared as: 0,5 pmol/µl (µM) for primers for each capillary, 0,15 pmol/µl (µM) for probe concentrations, 3mM for Mg^{+2} concentrations, 2µl for Light Cycler® FastStart DNA Master HybProbe (Roche Diagnostics, Germany Catalog no: 03 003 248001) enzyme mixture and 2 µl for last reaction volume. In real time PCR protocol, after pre-incubation at 95^0C for 10 minutes, amplification program was done up to 45 cycles, as 10 seconds at 95^0C, 20 seconds at 60^0C, 15 seconds at 72^0C. Single reading was performed at 60^0C. Afterwards, continuous fluorescent reading with $0,1^0$C/second temperature increase was applied for one cycle as 0 second at 95^0C, 30 seconds at 40^0C and 0 second at 95^0C. Lastly, 30 seconds waiting period was assigned for the device to cool down. Data were analyzed by Light Cycler Software 3.5.3 (Roche Diagnostics GmbH, Mannheim, Germany) and A, B, C, D variants were studied. Melting peak temperature (T_m, C^0) analysis of MBL2 exon 1 allel A, B and D were found to be 66.6, 61 and 62.2 C^0, respectively and this data was also compared with the literature, Melting peaks of our specimens and positive control heterozygous AB samples were compared and the MBL variants were determined [18].

Serum MBL assessments

Serum MBL concentrations were analyzed by enzyme immune absorbent assay (ELISA) method by commercial available reagent "MBL Oligomer ELISA Kit" (Bio Porto Diagnostics, Gentofte, Denmark). "MBL oligomer deficient serum " (Bıo Porto Diagnostics, Gentofte, Denmark) containing MBL <1 arbitrary unit (AU) for per ml was used as negative control and MBL standard serum " (Bio Porto Diagnostics, Gentofte, Denmark) containing 1000 AU MBL per ml was used as positive control. Intra-assay variation coefficient (%CV) values for the reagent were 3.6%at 2279 ng/ml, and 3.8%at 28,4 ng/ml concentrations, while inter-assay CV were 9.2%for 2310 ng/ml, and 4.3%for 29,7 ng/ml concentrations. Working range of the reagent was between <2 ng/ml and 4443 ng/ml. Washing was done by automatic ELISA washer Biotek ELx50 (Biotek Instruments, USA) and absorbance was read by automatic micro plate reader at 450 nm. Specimen concentrations were calculated using KC junior software from the specimen absorbances.

Statistical Analysis

Data were analyzed by SPSS 10.0 package program. Groups were compared by unpaired t tests and Mann Whitney u test for continuous variables. Normality assumption was checked by Kolmogorov Smirnov test. Homogeneity of variances was checked by Levene test. Chi-square analysis and Fisher's exact test were used for categorical data. Relation between continues variables was analyzed using Pearson's correlation test. A p value, <0.05 was considered as statistically significant.

Results

The demographic data of the subjects are depicted at Table 1. MBL polymorphism variants found in our study population and the distribution of the variants according to the study groups as well as serum MBL concentrations are shown in Table 2. We did not find any statistical significant difference (p = 0.424) within the groups for serum MBL concentrations.

Cut off values for the MBL deficiency is controversial in the current literature. In our study we analyzed MBL polymorphisms for different cut off levels of 50 ng/ml, 100 ng/ml, 200 ng/ml and 300 ng/ml and calculated the odds ratios (OR) and 95%confidence intervals (CI). Having a D/D variant was not found to be associated with statistically significant low serum MBL concentration risk with respect to A/B variant for all the cut off values. Only 2 of the study participants had B variant, so B variant was excluded from the statistical analyses. We did not find any statistical significant difference for MBL allele frequencies among the study groups according to different cut off thresholds (Table 3).

Having A/B variant was not found a statistically significant risk for low levels of serum MBL with respect to D/D genotype when cut off values of 50 ng/ml (p = 0.590) (OR = 1.9, %95 CI = 0.182-20.059), 200 ng/ml (p = 0.184) (OR = 2.81, %95 CI = 0.61-12.89) and 300 ng/ml (p = 0.324) (OR = 2.11, %95 CI = 0.47-9.30) were considered. In the present study, 300 ng/ml which has the lowest "p value" was accepted as the threshold concentration for MBL deficiency and 14 subjects had serum MBL levels lower than 300 ng/ml (Table 3).

We found mild correlations between serum MBL concentrations and pulmonary function tests as well as smoking intensity. A mild statistical significant negative correlation was observed with serum MBL concentrations and FEV_1/FVC (r = -0.264, p = 0.041). There was also a negative correlation between serum MBL concentrations and FEV_1 (r = -0.246, p = 0.058) but this relation was insignificant. Smoking intensity (pack/years) was also shown to be positive correlated with serum MBL concentrations (r = 0.280, p = 0.030).

Discussion

Chronic lung inflammation is the major underlying pathology in COPD, and markers of either systemic or airway inflammation are valuable as potential predictors of disease development, progression, severity and acute exacerbations of COPD. There are established markers for COPD such as α1-antitrypsin gene, however further research is needed to implement new cost-effective and feasible biomarkers in clinical practice [3].

Table 1: Demographic features and functional parameters of the study population

	Nonsmoker			Smoker		
	COPD (n = 20)	Control (n = 20)	p value	COPD (n = 20)	Control (n = 20)	p value
Age (years) *	64.6 ±7.4	47.4 ±6.3	<0.001*	58.6 ±12.0	29.7 ±7.2	<0.001†
Gender (n) (female/male)	2/18	10/10	0,006†	0/20	9/11	<0,001††
Smoking (package/year)	0	0		52.2 ±24.7	11.9 ±10.4	<0.001†
Steroid therapy (n)	15			13		
FEV_1 (%) *	52.1 ±17.7	-		52.6 ±19.3	100.2 ±8.1	<0.001†
FEV_1 (L) *	1.5 ±0.6	-		1.7 ±0.8	3.7 ±1.1	<0.001†
FVC (%) *	67.7 ±17.3	-		70.6 ±21.7	103.5 ±10.4	<0.001†
FEV_1/ FVC*	58.1 ±12.5	-		56.9 ±11.0	82.2 ±6.3	<0.001†

COPD: Chronic obstructive pulmonary disease, FEV1: Forced expiratory volume in 1 second, FVC: Forced vital capacity
*Values are expressed as mean ±Standard deviation (SD), p <0.05 was considered significant.
† Student's t test
†† Chi square test

Table 2. Prevalence of mannose binding lectin polymorphism variants and serum mannose binding lectin concentrations in the study groups.

	Nonsmoker			Smoker		
	COPD n (%)	Control n (%)	p value	COPD n (%)	Control n (%)	p value
A/B Variant	15 (75.0)	18 (90.0)	0.3*	18 (90.0)	15 (75.0)	0.4**
D Variant	5 (25.0)	2 (10.0)		1 (5.0)	4 (20.0)	
B Variant	0 (0.0)	0 (0.0)		1 (5.0)	1 (5.0)	
Total	20 (100.0)	20 (100.0)		20 (100.0)	20 (100.0)	
Serum MBL (ng/ml), mean ±SD	2103.4 ±1311.0	2040.2 ±879.3	0,8***	2324.1 ±1001.2	1746.8 ±1142.3	0,09***

COPD: Chronic obstructive pulmonary disease, MBL: Mannose binding lectin
*Fisher's exact test **Chi square test ***Student's t test

Table 3: Serum mannose binding lectin (MBL) concentration of the study population according to MBL polymorphism

MBL polymorphism	Serum MBL concentration				
	Mean ±SD	≥50 (%)	≥100 (%)	≥200 (%)	≥300 (%)
A/B variant (n = 66))	2189.0 ±1045.3	95.5	92.4	89.4	86.4
D/D variant (n = 12)	1642.7 ±1056.9	91.7	83.3	75.0	75.0
B/B variant (n = 2) *	50.0	0.0	0.0	0.0	0.0
Total	2053.6 ±1094.2	100.0	100.0	100.0	100.0
p value	0.049**	0.4***	0.2***	0.1***	0.3***

COPD: Chronic obstructive pulmonary disease, MBL: Mannose binding lectin
Values are shown as mean ±Standard deviation (SD)
*B variant was excluded from the statistical analyses
Mann Whitney U test * Fisher's exact test

Currently, MBL has been regarded as an acute phase reactant [9] and the relationship of serum MBL levels with COPD is an area of interest. Therefore we aimed to study MBL variants and the relationship of serum MBL levels with smoking and COPD. To our knowledge, this is the first study in a Turkish sample of COPD patients to examine the polymorphisms in the gene encoding MBL. We hypothesized to observe variant MBL alleles and MBL deficiency levels, in smokers and COPD patients; however we found relatively high serum MBL concentrations and a different distribution of MBL alleles from the previous literature in the whole study population. We also determined a mild correlation between the serum MBL concentrations and smoking amount as well as pulmonary functions. However, we did not observe any MBL genotype more frequent in COPD patients than the control group, besides MBL deficiency was not found in COPD patients and smokers.

Yang et al. have studied MBL2 polymorphism and serum MBL2 concentration in a group of COPD patients and smoker controls [4]. A/A genotype frequency was found 73.5%in COPD patients and 73.1%in smoker individuals, whereas A/B genotype frequency was 22.5%, 25.0%and B/B genotype frequency was 4.0%, 1.9%in COPD patients and smoker controls, respectively. They have shown a statistically significant relation with serum MBL2 concentration and MBL genotype in COPD patients. Those having A/B and B/B genotypees (30-246 ng/ml) had serum MBL concentrations 10 times lower than the ones with A/A genotype (398-1355 ng/ml). In a research investigating MBL deficiency haplotypes worldwide, A/B genotype frequency was reported 13.6%in European countries and 25%in East Asian countries [19]. In our study, MBL polymorphism was studied in 66 participants and most of them (82.5%) had A/B genotype, while 15%had D/D genotype and 2.5%had B/B genotype. The high frequency of A/B genotype as well as absence of wild tip A/A genome observed in our study is different from the results of the previous literature. This result was unexpected for us and might be due to the features of our study population and small number of participants since the study was conducted as a pilot program. It was not possible to compare the serum MBL levels of three genomes determined in our study population because the number of participants with B/B variant was not adequate for statistical evaluation and therefore, B/B variant was excluded. When serum levels of MBL in A/B and D/D genotypes were compared no significant difference were demonstrated. We also showed that study participants having A/B genotype had similar serum MBL concentrations (<50-3558 ng/ml) regardless of COPD presence and smoking.

Serum MBL concentrations in healthy population has been reported to be between 800-1000 ng/ml [20] and a cut off value of <500 ng/ml or MBL function <0.2 U/µl C4 deposition have been defined for determining MBL deficiency [21, 22]. MBL deficiency was shown to be associated with increased susceptibility of infections [22, 23]. Recent observations have determined increased mortality in pneumococcal pneumonia [24] and predisposition for Legionellosis in patients with low MBL levels [25]. On the other hand, elevated serum MBL levels might play a role in inflammatory process through the activation of complement [26]. There are some reports demonstrating elevated plasma MBL levels and complement activity in allergic patients although it has not been clarified whether these results are related with genotypic features or disease pathology [27, 28]. However, some studies have suggested that genetically reduced MBL levels increase the susceptibility for COPD and asthma [4, 29, 30], while some others demonstrated the risk of COPD and asthma in MBL deficient patients were not increased [31, 32]. Eagan et al., compared 415 COPD patients with 231 controls MBL deficiency was not found more common in COPD patients. It was also determined that the severity of the disease or the exacerbation frequency and recurrent lower respiratory infections were not associated with MBL deficiency [32]. In our study, we found mean serum MBL concentrations 2103 ±1311 ng/ml in nonsmoker and 2324 ±1001 ng/ml in smoker COPD patients, while it was 1746 ±1142 ng/ml in smoker and 2040 ±879 ng/ml in nonsmoker controls. Although, smoker controls seemed to have lower levels of MBL, it did not reach statistical significance ($p = 0.424$). After analyzing 100, 200, 300 ng/ml, we established a cut off value of 300 ng/ml for MBL deficiency in the present study. In previous literature MBL variants have been reported to be related with low levels of serum MBL [4, 33]. We also expected to observe low serum MBL levels for A/B, B/B and D/D genotypes and we determined low (<50 ng/ml) serum MBL concentrations in two individuals having B/B genotype similar to the literature. However, in participants with other MBL variants serum levels were determined to be elevated. These high levels of serum MBL found in our study might be related with features of our study population or with inflammatory process due to COPD and smoking. However, it is difficult to make an accurate comment because of the small number of the study population. It has been reported that, MBL concentrations could vary tenfold among the subjects carrying the same haplotype and for every time genotypic features were not predictive for serum MBL levels [34].

The relationship of MBL genotypes and pulmonary functions has also been investigated [35-37]. Wang et al reported no difference in MBL2 gene polymorphism and serum MBL concentration in a group of asthmatics and healthy controls [34]. They also could not show any association between MBL variants and levels with pulmonary functions. However, there are studies reporting significant negative or positive correlations between the MBL variants and the pulmonary function test parameters [36, 37]. Similarly, we found a mild negative correlation with serum MBL levels and $FEV_{1,}$

FEV_1/FVC. Smoking which is the most important risk factor in COPD was also determined to be correlated with serum MBL levels in a manner that as the intensity of smoking increased the serum levels of MBL elevated. These findings establishing the relation of MBL with smoking may pioneer further studies investigating the value of MBL as a marker of COPD inflammation.

Our study had some limitations one of which was the smaller age of the control group with respect to COPD patients. However, when we looked at the current literature we saw that in most of the case control studies the age of the groups have not been matched and it has been reported that serum MBL concentrations did not change with age and gender [38, 39]. Other limitations of our study are the small number of study population which we think this study may be accepted as a pilot study.

In conclusion, this is the first study, demonstrating MBL polymorphism in Turkish population. Different distributions of MBL variants were observed and MBL deficiency was not detected in COPD patients and smokers. The correlations found between serum MBL concentration with pulmonary function tests and smoking need to be further investigated in larger series of COPD patients to evaluate whether serum MBL concentration is a risk factor for COPD progression and exacerbations.

Acknowledgement

This study was funded by the Scientific Research Projects Committee of Celal Bayar University

Conflict of interest: Authors declare no conflict of interest

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