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ORIGINAL ARTICLE

Mannose-binding lectin gene polymorphism and its effect on short term outcomes in preterm infants☆

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Abstract

Objective: Mannose-binding lectin, which belongs to the collectin family, is an acute-phase reactant that activates the complement system. This study aimed to investigate the effect of *MBL2* gene polymorphism on short-term outcomes in preterm infants.

Method: Infants of <37 gestational weeks who were admitted to the neonatal intensive care unit during a two-year period were enrolled in this prospective study. The neonates were categorized into two groups according to their *MBL2* genotypes. Normal *MBL2* genotype was defined as *MBL2* wild-type (AA genotype), whereas mutant *MBL2* genotype was defined as *MBL2* variant-type (AO/OO genotype). The relationship between *MBL2* genotype and short-term morbidity and mortality was evaluated.

Results: During the two-year study period, 116 preterm infants were enrolled in this study. In *MBL2* variant-type, mannose-binding lectin levels were significantly lower and incidences of mannose-binding lectin deficiency (MBL level < 700 ng/mL) were higher ($p < 0.001$). In this group, the prevalence of respiratory distress syndrome and mortality was significantly higher ($p < 0.001$, $p = 0.03$ respectively). In the *MBL2* wild-type group, the prevalence of necrotizing enterocolitis (NEC) was higher ($p = 0.01$). Logistic regression analyses revealed that *MBL2* variant-type had a significant effect on respiratory distress syndrome development (odds ratio, 5.1; 95% confidence interval, 2.2–11.9; $p < 0.001$).

Conclusions: *MBL2* variant-type and mannose-binding lectin deficiency are important risk factors for respiratory distress syndrome development in preterm infants. Additionally, there is an association between *MBL2* wild-type and NEC. Further studies on this subject are needed.

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PALAVRAS-CHAVE
Lectina ligante de manose;
Prematuro;
Síndrome do desconforto respiratório

Polimorfismo do gene da lectina ligante de manose e seu efeito em desfechos de curto prazo em bebês prematuros

Resumo

Objetivo: A lectina ligante de manose (MBL, do inglês *mannose-binding lectin*), que pertence à família das colectinas, é um reagente de fase aguda que ativa o sistema complemento. Este estudo teve como objetivo investigar o efeito do polimorfismo do gene *MBL2* em desfechos de curto prazo em prematuros.

Método: Este estudo prospectivo incluiu crianças com menos de 37 semanas de gestação admitidas na unidade de terapia intensiva neonatal durante dois anos. Os neonatos foram categorizados em dois grupos de acordo com os genótipos do *MBL2*. O genótipo normal do gene *MBL2* foi definido como *MBL2* do tipo selvagem (genótipo AA), enquanto o genótipo mutante do gene *MBL2* foi definido como o gene variante (genótipo AO/OO). Foi avaliada a relação entre o genótipo *MBL2* e a morbidade e mortalidade em curto prazo.

Resultados: Durante o período de dois anos, 116 bebês prematuros foram incluídos neste estudo. Os níveis de lectina ligante de manose foram significativamente menores nos variantes do *MBL2* e as incidências de deficiência de lectina ligante de manose (nível de MBL < 700 ng/mL) foram maiores ($p < 0,001$). Nesse grupo, a prevalência de síndrome do desconforto respiratório (SDR) e a mortalidade foram significativamente maiores ($p < 0,001$, $p = 0,03$, respectivamente). No grupo *MBL2* do tipo selvagem, a prevalência de enterocolite necrosante foi maior ($p = 0,01$). Análises de regressão logística revelaram que os genes variantes do *MBL2* apresentaram um efeito significativo no desenvolvimento da síndrome do desconforto respiratório (*odds ratio*, 5,1; intervalo de confiança de 95%, 2,2–11,9; $p < 0,001$).

Conclusões: As variantes do *MBL2* e a deficiência de lectina ligante de manose são importantes fatores de risco para o desenvolvimento da síndrome do desconforto respiratório em neonatos prematuros. Além disso, existe uma associação entre *MBL2* do tipo selvagem e a enterocolite necrosante. Mais estudos são necessários sobre esse assunto.

Introduction

Mannose-binding lectin (MBL) is an acute-phase reactant that activates the complement system. It belongs to the collectin family of proteins, which includes lung surfactant protein A (SP-A) and SP-D.[1] MBL plays a key role in first-line immune responses as a component of innate immunity.[2] Because adaptive immunity is underdeveloped in preterm infants, innate immunity gains higher importance.[2,3] The *MBL2* gene is located on the long arm of chromosome 10, and mutant *MBL2* alleles occur as a result of three single-point mutations in this gene (B, C, and D). Although functional MBL levels are low in heterozygous polymorphisms, MBL levels in homozygous polymorphisms are so low that they may not even be determinable.[4,5] MBL activates the complement system by binding to mannose or sugar motifs, which are found in many microorganisms, and plays an important role in innate immunity and inflammation.[2,6] In newborns, an increase in sepsis frequency is observed when MBL levels are low.[2,3,7]

The mortality and morbidity rates in preterm infants are higher than those in term infants. As gestational week and birth weight decrease, the risk of complications increases. In preterm infants, complications are observed in the early (neonatal period) and late periods (after discharge). Although the survival rate of most preterm infants has improved because of advances in medical care, the incidence of short-term complications remains relatively stable. Short-term complications increase the risk of long-term sequelae.[8,9]

In recent years, many studies have been conducted on the importance of MBL during the neonatal period, and most of these are associated with sepsis. In sepsis, proinflammatory and anti-inflammatory cytokine ratio is vital in terms of defense against infectious agents. The imbalance in this ratio is manifested by increased morbidity and mortality during the neonatal period.[5,6] Increased cytokine levels have a significant role in the pathophysiology of morbidities such as respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), and retinopathy of prematurity (ROP).[10] This prospective study aimed to investigate the association of *MBL2* polymorphism with short-term outcomes in preterm infants.

Materials and methods

All preterm infants of <37 gestational weeks who were admitted to the neonatal intensive care unit (NICU) of the Uludag University Medical School during a two-year period were enrolled in this prospective study. The neonates were categorized into two groups according to their *MBL2*

genotypes. Normal *MBL2* genotype was defined as *MBL2* wild-type (AA genotype), whereas mutant *MBL2* genotype was defined as *MBL2* variant-type (AO or OO genotype). The exclusion criteria included refusal of parental consent, infants with major congenital abnormalities, and those undergoing a major surgical procedure.

Gestational age, birth weight, gender, mode of delivery, Apgar score at 1 and 5 min, prenatal demographics, antenatal steroid administration, premature rupture of membranes, history of chorioamnionitis and durations of invasive mechanical ventilation, total supplemental oxygen, central catheterization, and total parenteral nutrition were recorded. The presence of neonatal morbidities such as RDS, late-onset sepsis (LOS), IVH, NEC, BPD, ROP, and the mortality data of the preterm infants were recorded.

RDS was diagnosed based on clinical findings (tachypnea, retractions, nasal flaring, and cyanosis) or radiological findings (reticular granular pattern or air bronchograms). All neonates underwent the same management according to the NICU protocols and as recommended by European consensus guidelines on the management of neonatal respiratory distress syndrome in preterm infants.[11,12] Neonatal sepsis was defined as the presence of clinical signs of sepsis with a positive blood culture. Blood cultures were analyzed using the fully automated BACTEC method in a BACTEC 9240 device (Becton Dickinson, Heidelberg, Germany). LOS was determined by the time at which sepsis occurred between 4 and 30 days after birth.[13] IVH was evaluated by cranial ultrasound examinations, which were performed by the same pediatric radiologist and diagnosed using the Papile classification system.[14] NEC was diagnosed according to clinical and radiographic findings and classified according to modified Bell's criteria.[15] BPD was classified into three groups in terms of BPD severity depending on the duration and level of supplemental oxygen and mechanical ventilatory support at 36 weeks postmenstrual age.[16] ROP was classified according to the International Classification of Retinopathy of Prematurity.[17]

The MBL levels and gene polymorphisms were assessed within 3 h in most infants and within 24 h after birth in all infants. The blood samples for the measurement of MBL levels were collected in a test tube and these blood samples were centrifuged within thirty minutes after obtaining. After the centrifugation process, serum of the samples were immediately stored at −80 °C until analysis.

Blood samples were analyzed using enzyme-linked immunosorbent assay. PCR and restriction fragment length polymorphism were used for *MBL2* genotyping. Serum MBL levels were measured using an immunoassay kit (Oligomer ELISA kit; Antibody Shop, Copenhagen, Denmark) according to the manufacturer instructions. The lowest detectable MBL concentration was 10 ng/mL. For the definition of the functional MBL deficiency, this study used two different cut-off values of MBL concentration. An MBL level < 700 ng/mL was determined as deficiency and <150 ng/mL as severe deficiency.[1–3,18] DNA was extracted from the blood samples using a commercially available kit (Puregene, Gentra, MN, United States), and *MBL2* genotyping was performed using these samples. DNA samples were maintained at −20 °C until use. All genotypes were detected using PCR and restriction enzyme digestion. Exon 1 of *MBL2* was amplified by PCR. The primer sequences were 5′-GTA GGA CAG AGG GCA TGC TC-3′ and 5′-CAG GCA GTT TCC TCT GGA AGG-3′. In all, a 349-bp PCR product was digested with *BanI* and *MboII* for codon 54 and codon 57, respectively. The normal allele (allele A) was cut into two fragments with *BanI*, 260 and 89 bp. The variant allele B (rs1800450) and allele D (rs5030737) remained uncut. *MboII* cleaved the variant allele C (rs1800451) into 270 and 79 bp fragments. The fragments were visualized using electrophoresis on 2% agarose gel. At electrophoresis, the dual band at the restriction site was defined as a heterozygous mutation, whereas the single band was defined as a homozygous mutation. As stated, the normal structural *MBL2* allele was named A, whereas alleles B, C and D (mutation in codons 54, 57 and 52) were named O. A representative gel electrophoresis of the *MBL2* exon 1 codon 54 polymorphisms is shown in Fig. 1.

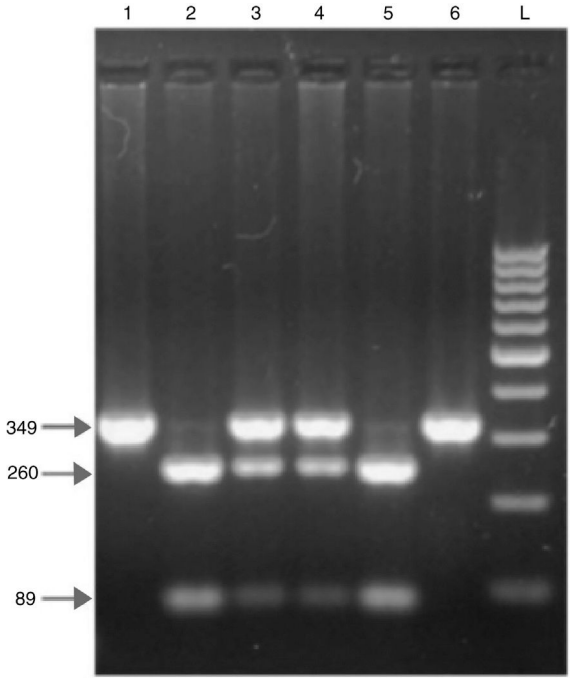

Figure 1 DNA fragments on agarose gel electrophoresis after restriction enzyme digestion of exon 1 of the mannose-binding lectin (*MBL2*) gene codon 54. In all, 349 bp PCR product was digested with *BanI* for codon 54 polymorphism. The normal allele (allele A) is cut into two fragments with *BanI* (lanes 2 and 5), 89 and 260 bp. The variant allele (allele O) remains uncut (lanes 1 and 6). Both uncut and digested fragments are seen in AO heterozygote (lanes 3 and 4). L: 100 bp DNA ladder.

This study was approved by the Ethics Committee of Uludag University Medical School and conformed to the standards set by the Declaration of Helsinki (15.01.2013-1/20). All parents provided informed consents prior to the inclusion of their children in the study.

Statistical analysis

Statistical analysis was performed using SPSS v. 20.0 software (SPSS Inc., Chicago, IL, United States). The results are presented as median (interquartile range) for the variables showing non-Gaussian distribution and mean ± standard deviation for data showing normal distribution. Student's

Table 1 Neonatal and maternal characteristics of the study population.

	MBL2 wild-type	*MBL2* variant -type	*p*
	(*n* = 69)	(*n* = 47)	
GA at birth, median in weeks (range)	30 (29–33)	31 (29–33)	0.6[a]
Birth weight, g (mean ± SD)	1539 ± 574	1459 ± 556	0.5[b]
Sex, n (%)			
Male	40 (58)	29 (62)	
Female	29 (42)	18 (38)	0.7[c]
SGA, n (%)	13 (19)	14 (30)	0.1[c]
Cesarian delivery, n (%)	58 (84)	36(77)	0.3[c]
Apgar score, median (range)			
Minute 1	7 (5–8)	6 (4–7)	0.1[a]
Minute 5	8 (7–9)	8 (7–9)	0.1[a]
Antenatal steroid, n (%)			
None		33 (48)	30. (64)
Single course		18 (26)	9.(19)
Repeat course		18 (26)	8. (17)
Maternal preeclampsia, n (%)	17 (25)	15 (32)	0.4[c]
Maternal infection, n (%)	3 (4)	4 (9)	0.4[c]
PPROM, n (%)	13 (19)	6 (13)	0.4[c]
Chorioamnionitis, n (%)	4 (6)	2 (4)	0.7[c]
MBL levels, (ng/mL) median (range)	993 (257–1812)	10 (10–473)	**<0.001**[a]
MBL deficiency, (MBL level <700 ng/mL), n (%)	30 (44)	45 (96)	**<0.001**[c]

Values with significance are presented in bold.
MBL2, mannose-binding lectin; GA, gestational age; SGA, small for gestational age; PPROM, preterm premature rupture of membranes.
[a] Mann–Whitney *U* test.
[b] Student's *t*-test.
[c] Chi-squared test.

t-test was used for group comparisons of normal distributions, and the Mann–Whitney *U* test was used for group comparisons of non-normal distributions. The chi-squared test and Fisher's exact test were used for the comparison of categorical variables. Logistic regression analysis was performed to investigate the effect of *MBL2* genotype on RDS. The analysis included factors that were demonstrated in the literature to have an effect on RDS: gestational age, birth weight, sex, antenatal steroid administration, and *MBL2* genotype were included in the analysis. A *p*-value of <0.05 was considered statistically significant.

Results

Overall, 131 preterm infants were included in this study. Ten were excluded because of blood sample insufficiency, four because of major congenital abnormalities, and one because of major surgery. In the final analysis, a total of 116 preterm infants were included: 69 with *MBL2* wild-type (AA genotype) and 47 with *MBL2* variant-type (AO/OO genotype). Overall, the rate of *MBL2* variant-type in preterm infants was 41%. MBL levels were significantly lower and MBL deficiency and severe deficiency were higher in *MBL2* variant-type than in *MBL2* wild-type ($p < 0.001$). Table 1 shows the demographic features of the study population. Codon 57 and 52 polymorphisms were not detected in any of the 116 preterm infants during the genetic evaluation. *MBL2* codon 54 genotype and allele frequencies were 59% for *MBL2* wild-type (AA genotype) and 41% for *MBL2* variant-type (AO and OO genotype).

Evaluation of short-term morbidity based on *MBL2* genotype revealed that RDS and mortality rates were significantly higher in the *MBL2* variant-type group ($p < 0.001$, $p = 0.03$; respectively). NEC was found to be more prevalent in the *MBL2* wild-type group ($p = 0.01$). There was no difference between the *MBL2* wild-type and variant-type groups in terms of IVH, BPD, ROP, and LOS (Table 2). Consideration of short-term morbidity based on MBL levels revealed that RDS was significantly higher in both the MBL deficient and severely deficient groups ($p < 0.001$). NEC was found to be more common with normal levels of MBL ($p = 0.002$). There was no significant difference in infants with or without MBL deficiency with respect to IVH, BPD, ROP, LOS, and mortality (Table 3). Further, because univariate analyses revealed that RDS development was more common in the *MBL2* variant-type, the effect of gestational age, birth weight, gender, antenatal steroid use, and *MBL2* genetics, which are factors that may affect RDS development, was investigated by logistic regression analysis. *MBL2* variant-type was found to be an independent factor for the development of RDS (OR: 5.1, 95% CI: 2.2–11.9, $p < 0.001$).

Discussion

It was observed that MBL levels were lower in preterm infants with *MBL2* variant-type than in those with *MBL2* wild-type. RDS was significantly more common in the *MBL2*

Table 2 Frequency of early neonatal outcomes according to mannose binding lectin genotypes.

	MBL2 wild-type($n=69$)	*MBL2* variant-type($n=47$)	p[a]
RDS, *n* (%)	21 (30)	31 (66)	**<0.001**
IVH, (Papile grade 3–4), *n* (%)	3 (4)	2 (4)	0.9
NEC, (>grade 1), *n* (%)	9 (13)	0 (0)	**0.01**
BPD, (grade 2–3), *n* (%)	16 (23)	6 (13)	0.2
ROP, (>stage 2), *n* (%)	10 (15)	3 (6)	0.2
LOS, *n* (%)	18 (26)	16 (34)	0.4
Mortality, *n* (%)	6 (9)	11 (23)	**0.03**

[a] Chi-squared test.
Values with significance are presented in bold.
MBL2, mannose-binding lectin; RDS, respiratory distress syndrome; LOS, late onset sepsis; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; BPD, bronchopulmonary dysplasia; ROP, retinopathy of prematurity.

Table 3 Mannose binding lectin levels in relation to early neonatal outcomes.

	MBL deficiency		Normal MBL>700 ng/mL($n=41$)	p[a]
	<150 ng/mL($n=36$)	150–700 ng/mL($n=36$)		
RDS, *n* (%)	28 (72)	23 (64)	1 (3)	**<0.001**
IVH, (Papile grade 3–4), *n* (%)	2 (5)	1 (3)	2 (5)	0.7
NEC, (>grade 1), *n* (%)	0 (0)	1 (3)	8 (20)	**0.002**
BPD, (grade 2–3), *n* (%)	5 (13)	5 (14)	12 (29)	0.1
ROP, (>stage 2), *n* (%)	3 (8)	4 (11)	6 (15)	0.06
LOS, *n* (%)	14 (36)	10 (28)	10 (24)	0.5
Mortality, *n* (%)	7 (18)	7 (19)	3 (7)	0.3

Values with significance are presented in bold.
MBL, mannose-binding lectin; RDS, respiratory distress syndrome; LOS, late onset sepsis; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; BPD, bronchopulmonary dysplasia; ROP, retinopathy of prematurity.
[a] Chi-squared test.

variant-type group and also in the MBL deficient group. Additionally, the mortality rates were higher in preterm infants with *MBL2* variant-type. In the study model, *MBL2* variant-type was a significant independent factor for RDS after adjusting for the effects of other factors. Besides, the prevalence of NEC was higher in the *MBL2* wild-type group and with normal MBL levels. It is believed that these findings will contribute toward accumulating evidence on the effect of MBL in preterm morbidities.

The collectin family and MBL play an important role in the primary immune elimination of invasive microorganisms in the innate immune response as well as in the regulation of ongoing immune responses against microbial invasion. Studies have reported an association between MBL deficiency or variant genotype as well as infection and pulmonary pathologies.[19] Pulmonary function impairment has been reported in patients with MBL deficiency and cystic fibrosis. Patients with bronchiectasis with MBL deficiency or variant-type have a higher rate of chronic microbial colonization and frequent recurrence of pulmonary problems.[19,20] In some studies, similar to the results obtained in the present study, it has been shown that MBL deficiency or variant-type cause respiratory morbidity independent of infection.[1,21] There is high sequence homology between MBL and SP-A and SP-D. The genes encoding these proteins are located on the long arm of chromosome 10 and belong to a similar lineage.[22] SP-A and SP-D are involved in the removal of many pathogens in the lungs, and although SP-A is particularly known for its immune functions, RDS is associated with decreased SP-A levels.[23] Mutant *MBL2* genetics are associated with insufficient surfactant protein-A production, which may facilitate the development of RDS. Similarly, the present study also found a significant increase in RDS prevalence and mortality in patients with mutant *MBL2* genetics. Early selective surfactant therapy in RDS has been reported to reduce pulmonary injury and mortality.[24] It is believed that during the evaluation of *MBL2* genotype at the time of delivery in premature infants with high RDS risk and $\leq$32 gestational weeks and in borderline cases with an indication for surfactant, the early administration of surfactants to patients with mutant *MBL2* genetics will reduce mortality and pulmonary morbidities.

In recent years, there has been an increased interest in the association between MBL and inflammatory morbidities. It has been reported that MBL activates the lectin pathway of complement, resulting in ischemia-perfusion damage. In patients with *MBL2* wild-type, higher MBL levels have been reported and associated with NEC, resulting in reperfusion injury after intestinal ischemia.[10] In agreement with these findings, in the present study the prevalence of NEC was higher in preterm infants with *MBL2* wild-type and normal MBL levels. However, some studies have reported that there is no association between *MBL2* genotype and NEC.[5,25] In the present work, the development of NEC may have been relatively more common because of significantly higher mortality rates in patients with *MBL2* wild-type. Because there

are debatable opinions in the literature on *MBL2* genotype and NEC development, additional studies are needed to clarify this issue.

In this study, in agreement with previous data, no correlation was found between *MBL2* genotype and MBL levels with inflammation-associated pathologies, BPD, IVH, and ROP.[5,25] The evaluation of the association between *MBL2* genotype and morbidity was the common aspect of these studies. It would be misleading to evaluate the association of *MBL2* genotype and MBL value obtained at the time of delivery with morbidities alone. Because MBL levels increases as the gestational week increases in the *MBL2* wild-type group, the evaluation of morbidity development with MBL levels obtained at different postnatal weeks may provide more accurate results to demonstrate the association between lectin pathway and inflammatory morbidities.[2] There is a clear need for extensive studies to investigate the association between MBL and inflammatory morbidities in premature infants.

Although the association of *MBL2* genotype with culture-proven sepsis has not been reported in the literature, the association between *MBL2* genotype and early clinical sepsis has been reported.[4,5,25] In contrast, the association between MBL deficiency and sepsis has been reported in many studies.[3,18] In the present study, no association was found between *MBL2* genotype and MBL levels with LOS. The authors believe that inadequate immune response to infection is observed because of low MBL levels in the early postnatal weeks in preterm infants even if the *MBL2* genotype is wild-type. Therefore, future studies should evaluate MBL values at the time of sepsis together with genotype.

This study had some limitations. Morbidity was evaluated based on only *MBL2* genotype and with the levels of MBL within 24 h after birth because the MBL levels of the infants were not reassessed during the subsequent postnatal days. Additionally, the results obtained with a limited number of cases may not reflect the overall results. The strength of this study was that it evaluated the association of *MBL2* genotype with MBL level and morbidities in preterm infants and simultaneously examined the MBL level in the first 24 h of life.

In conclusion, the presence of *MBL2* variant-type and low MBL levels are important risk factors for RDS development in preterm infants. Additionally, there is an association between *MBL2* wild-type and NEC. Considering the importance of showing that *MBL2* variant-type is an independent predictor of RDS, further prospective randomized studies on this topic are clearly required.

Conflicts of interest

The authors declare no conflicts of interest.

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