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Investigation of *MBL2* and *NOS3* functional gene variants in suspected COVID-19 PCR (–) patients

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ABSTRACT

For COVID-19 (Coronavirus Disease-2019) cases, detecting host-based factors that predispose to infection is a very important research area. In this study, the aim is to investigate the *MBL2* and *NOS3* gene polymorphisms in COVID-19 patients with lung involvement, whose first nasopharyngeal PCR results were negative. Seventy-nine patients diagnosed with COVID-19 between April-June 2020 who were admitted to a university hospital, and 100 healthy controls were included. In the first statistical analysis performed between PCR-positive, CT-negative and PCR-negative, CT-positive patients; the AB of *MBL2* genotype was significantly higher in the first group ($p = 0.049$). The B allele was also significantly higher in the same subgroup ($p = 0.001$). The absence of the AB genotype was found to increase the risk of CT positivity by 6.9 times. The AB genotype of *MBL2* was higher in healthy controls ($p = 0.006$). The absence of the AB genotype was found to increase the risk of CT positivity; also, it can be used for early detection and isolation of patients with typical lung involvement who had enough viral loads, but whose initial PCR results were negative.

KEYWORDS

COVID-19; mannose-binding lectin 2 (MBL2); endothelial nitric oxide synthase (eNOS); PCR; CT

Introduction

COVID-19 (Coronavirus Disease-2019) is a pandemic that has infected more than 58 million people since the first case was announced and has been among the major infectious events of the century [1]. Severe Acute Respiratory Syndrome Coronavirus 2 (Sars-Cov-2) virus emerges as a causative agent, and it is observed that individuals over 65 years old and patients with comorbid burden have a mortal course [2–4]. The patient population with diagnoses of hypertension, chronic respiratory disease, heart disorders, diabetes mellitus, renal failure and malignancy is defined as the most severely affected comorbidity groups [5–7].

The gold standard diagnostic method to isolate the virus is a nucleic acid amplification test (NAAT) such as the polymerase-chain reaction method (PCR) from the respiratory tract sample. There are differences in the sensitivity of PCR samples working with various body samples. In the study of Wenling Wang et al. [8], the order of sensitivity was as follows: Bronchoalveolar lavage 93%, fibrobronchoscopic brush biopsy 46%, throat culture 72%, nasal sample 63%, pharyngeal sample 32%, feces 29%, blood 1% and urine 0%. In this context, comparison of diagnostic methods based on thoracic computed tomography (CT) with PCR has also been made in the literature and it is seen that the sensitivity is higher in favor of CT [9].

In order to determine the reason why COVID-19 differs from other viral infections, and to reveal more information about this new infectious agent, many viral and host-based factors are studied. Specifically, detecting host-based factors that predispose to infection is a very important research area. In this context, two different host factors can be mentioned from the literature: Mannose binding lectin 2 (MBL2) and endothelial nitric oxide synthase (eNOS).

MBL2 is a serine protease belonging to the collectin family and is believed to be an important factor in the inherited immune system. The MBL2 protein binds to the surface of a wide range of microorganisms by its ability to recognize or function directly as an opsonin or through activation of the complement system, and it increases the phagocytosis of microorganisms by macrophages and neutrophils [10]. There are several known polymorphisms in the *MBL2* gene (10q21.1), located on the long arm of chromosome 10, in both

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the promoter and exon regions, resulting in multiple haplotypes. These genetic polymorphisms are associated with different levels of *MBL2* expression and activity [10,11]. There are studies showing that genotypes are associated with low levels of MBL2 may predispose to certain forms of infection or impaired immune response, particularly in neonates as well as adults [10,11]. Various studies on the association of *MBL2* genetic polymorphism and/or MBL2 plasma levels with severe infections, sepsis, and septic shock have shown an increased risk of developing sepsis in patients with MBL2 deficiency and a negative outcome [11,12].

Considering COVID-19 and lung involvement, eNOS, which constitutes an important endothelial protection mechanism, comes to the fore. Nitric oxide (NO) is synthesized from L-arginine by the NOS enzyme. NOS can also catalyze superoxide anion production due to the presence of substrate and cofactor. There are three main isoforms of NOS enzyme called neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). These differ in their dependence on Ca^{2+}, their expression and activity [13]. NO, an important endothelial defense system and antiviral, also comes to the fore in COVID-19 and its related comorbidities. In particular, fighting with developing inflammatory stress is vital for preventing pulmonary hypertension.

In this study, the aim is to investigate the *MBL2* and *NOS3* gene polymorphisms in COVID-19 patients with lung involvement, whose first nasopharyngeal swab PCR results were negative. The most important point of the study was the early detection and isolation of the patients with typical lung involvement, who had high viral loads, but whose initial PCR results were negative.

Methods

In this study, 79 patients diagnosed with COVID-19 between April and June 2020 who were admitted to the COVID-19 center of a university hospital, and 100 healthy individuals without any known comorbidity to create a control group were included. Healthy controls consisted of individuals who were negative for Sars-Cov-2 antibody (Sars-Cov-2 IgM, IgG) and were negative in two PCR results taken with an interval of 48 hours.

Additionally, the patient group of the previous study [14] was included in the study for statistical analysis. In addition to demographic information such as age and gender of the patients, *MBL2-rs1800450, NOS3-rs1799983 and NOS3-intron 4 VNTR* gene polymorphisms were analyzed using polymerase-chain reaction (PCR and/or restriction fragment length polymorphism (RFLP) method in DNA samples isolated from blood leukocytes at the time of diagnosis [15,16].

The *MBL2* gene is located on the chromosome 10 and comprises four exons. Inherited MBL insufficiency, which results in impaired innate immune function and enhanced susceptibility to infection, is essentially caused by three structural variants in exon 1. These three polymorphisms significantly alter the serum concentrations of MBL with the variant alleles (D, B, C) resulting from mutations in codons 52, 54 and 57, respectively. The B allele changes GGC to GAC and causes an amino acid replacement of glycine to aspartic acid (p.Gly54Asp) [17]. In humans, eNOS is encoded by a gene located on chromosome 7 q36 position that contains 26 exons and 25 introns with approximately 21kb length. Different polymorphisms have been identified in *NOS3* gene and we analyzed 2 polymorphisms of the *NOS3* gene cluster (*NOS3* exon 7 Glu298Asp *(rs1799983)* and 27-base pair repeat in intron 4 of *NOS3*) [18].

The genotype distributions and allele frequencies of the two groups were statistically compared. The institutional clinical research ethics committee approved the protocol (21/05/2020-84,539).

Case selection

This study includes only first nasopharyngeal swab PCR negative COVID-19 patients. All patients had typical COVID-19 lung involvement in their initial CT. Therefore, it is possible to define the patient group as patients diagnosed with COVID-19 who show false negativity with initial examinations before hospitalization. After hospitalization, the diagnosis of COVID-19 patients was confirmed with the PCR results of repeated upper or lower respiratory tract-derived (deep tracheal or endotracheal aspirate) samples [19].

There is also a separate statistical analysis between the patient group of previous study [14] and the patient group formed for this study in terms of *MBL2* genotype distribution: PCR-negative, CT-positive and PCR-positive, CT-negative patients with initial findings.

Isolation method

The COVID-PCR test was conducted through combined (throat-nose) swab samples. Bio-Speedy® Covidien-19 RT-qPCR Detection Kit test was applied by using Rotor-Gene Q 5 Plex Real Time PCR (Qiagen, Germany) device in line with the manufacturer's instructions.

Statistical analysis

The statistical significance of the differences between the patient groups was calculated by logistic regression analysis. The adjusted odds ratios (ORs) were calculated with a logistic regression model that checked for sex and age and are reported with 95% confidence

Table 1. Comparison of frequencies of *MBL2-rs1800450* variant between two subgroups with initial findings: PCR-negative, CT-positive and PCR-positive, CT-negative patients.

MBL2-rs1800450 Genotypes		PCR (-) CT (+) Patients n = [a] (%)	PCR (+) BT (-) Patients n = 190 (%)	OR Exp (B)	95% CI	p*
MBL2	AA	74 (93.7)	137 (72.1)	0.572*	0.152–2.159*	0.410*
	AB	2 (2.5)	43 (22.6)	6.913*	1.005–47.553*	**0.049***
	BB	3 (3.8)	10 (5.3)	1.315[&]	0.352–4.918[&]	1.000[&]
Allele						
	A	150 (94.9)	274 (72.8)			
	B	8 (4.1)	106 (27.2)	6.770[&]	3.208–14.288[&]	**0.001[&]**

[a]n = 79, *:OR (95%CI) was adjusted by age and sex, [&]Fisher's Exact Test
MBL2: Mannose binding lectin 2, PCR: Polymerase chain reaction, CT: Computed tomography, OR: Odds ratio CI: Confidence interval

intervals (CIs). Differences between the patients' group were compared using the chi-square test and the Fisher exact test when required. A p-value of less than 0.05 was accepted as significant.

Results

In the first statistical analysis performed among PCR-positive, CT-negative and PCR-negative, CT-positive patients, the AB genotype was significantly higher in the PCR-positive, CT-negative group (p = 0.049). The B allele was also significantly higher in the same subgroup (p = 0.001). The absence of the AB genotype was found to increase the risk of CT positivity by 6.9 times (Table 1.).

When the genotype and allele distributions of the *MBL2-rs1800450* polymorphism were compared in patient and control groups, it was found that there was a statistically significant difference in both genotype and allele distribution. The AB genotype was higher in healthy controls (p = 0.006). While the A allele was significantly higher in patients with detected COVID-19 (p = 0.001), the B allele was significantly higher in healthy controls (p = 0.001). (Table 2.).

Table 2. Comparison of frequencies of *MBL2-rs1800450* variant between COVID-19 (PCR-negative) patients and healthy controls.

MBL2-rs1800450 Genotypes		COVID-19 Patients n = [a] (%)	Healthy Control n = 100 (%)	OR Exp (B)	95% CI	p*
MBL2	AA	74 (93.7)	65 (65)	0.422*	0.042–4.258*	0.465*
	AB	2 (2.5)	34 (34)	0.022*	0.001–0.326*	**0.006***
	BB	3 (3.8)	1 (1)	0.256[&]	0.026–2.509[&]	0.322[&]
Allele						
	A	150 (94.9)	164 (82.0)			
	B	8 (5.1)	36 (18.0)	4.116[&]	1.854–9.137[&]	**0.001[&]**

[a]n = 79, *:OR (95%CI) was adjusted by age and sex, [&]Fisher's Exact Test.
MBL2: Mannose binding lectin 2, OR: Odds ratio CI: Confidence interval

Table 3. Comparison of frequencies of *NOS3-rs1799983* gene variants between COVID-19 (PCR-negative) patients and healthy controls.

NOS3-rs1799983 Genotypes		COVID-19 Patients n = [a] (%)	Healthy Control n = 100 (%)	OR Exp (B)	95% CI	p*
	GG	49 (62.0)	63 (63)	0.805*	0.048–13.397*	0.880*
	GT	29 (36.7)	36 (36)	0.878*	0.052–14.942*	0.928*
	TT	1 (1.3)	1 (1)	0.788[&]	0.049–12.797[&]	1.000[&]
Allele						
	G	137 (80.4)	164 (81.0)			
	T	31 (19.6)	38 (19.0)	0.961[&]	0.576–1.630[&]	0.893[&]

[a]n = 79, *:OR (95%CI) was adjusted by age and sex, [&]Fisher's Exact Test
NOS: Nitric oxide synthase, RFLP: Restriction fragment length polymorphism, OR: Odds ratio CI: Confidence interval

Table 4. Comparison of frequencies of *eNOS-intron 4 VNTR* gene variants between COVID-19 (PCR-negative) patients and healthy controls.

NOS3-intron 4 VNTR Genotypes		COVID-19 Patients n = [a] (%)	Healthy Control n = 100 (%)	OR Exp (B)	95% CI	p*
	BB	59 (74.7)	68 (68)	1.3.88[&]	0.719–2.682[&]	0.407[&]
	AB	18 (22.8) 8	28 (28)	0.758*	0.380–1513*	0.432*
	AA	2 (2.5)	4 (4)	0.621*	0.108–3.563*	0.593*
Allele						
	B	136 (86.1)	164 (82.0)			
	A	22 (13.9)	36 (18.0)	1.357[&]	0.762–2.416[&]	0.316[&]

[a]n = 79, *:OR (95%CI) was adjusted by age and sex, [&]Fisher's Exact Test
NOS: Nitric oxide synthase, VNTR: Variable number tandem repeat, OR: Odds ratio CI: Confidence interval

When the genotype and allele distributions of *NOS3-rs1799983* polymorphism were compared in patient and control groups, it was found that there was no statistically significant difference in neither genotype nor allele distribution (Table 3.).

Additionally, when the genotype and allele distributions of *NOS3-intron-4 VNTR* polymorphism were compared in patient and control groups, it was found that there was no statistically significant difference in neither genotype nor allele distribution (Table 4.).

Discussion

To the best of our knowledge, this is the first study examining the relationship between the patient group with false negativity of upper respiratory tract-related PCR samples at initial diagnosis and host genetic factors.

The *MBL2* gene polymorphism stands out in the meta-analysis from an important study by Di Maria et al. [20]. Through a meta-analysis of 32 articles, it was found in at least two studies that only the variants in *MBL2* and Myxovirus resistance protein A (MxA) played a role in SARS-CoV-related phenotypes. Two separate studies on *MBL2* genotypes found that the relationship between SARS-CoV appears to be associated with the detected polymorphisms and gene expression. Zhang et al. [21] evaluated the possible association of *MBL2* gene polymorphisms with SARS-CoV infection and disease severity in their study. The frequencies of exon 1-codon 54 and promoter 3 polymorphisms at *nt 550, 221 and 4* were determined by PCR direct sequencing in 352 patients with SARS and 392 healthy controls. Codon 54 polymorphism is associated with low *MBL2* expression in the light of information obtained from different studies [20–23].

In a recent review [24], three frequent missense mutations in *MBL2* were tested: *Gly54Asp (rs1800450), Arg52Cys (rs5030737)* and *Gly57Glu (rs1800451)*. This study highlights that Gly54Asp (rs1800450) is significantly associated with susceptibility to SARS-CoV infection, but not with disease severity [21,24]. The allele frequency per population of *Arg52Cys (rs5030737)* was positively correlated with the number of cases per country of COVID-19, but not with the mortality [24,25]. Allele frequency per population of *Gly54Asp (rs1800450)* was positively correlated with both number of cases and mortality [21,24]. The results of this review also support the hypothesis that MBL2 plays a protective role and that its inactivation is a risk factor for SARS-CoV-2 infection and that immune response plays a role in the course of the disease. We had the opportunity to examine only Gly54Asp *(rs1800450)* in our study and obtained significant results in cases with typical lung involvement, who had enough viral loads, but whose initial PCR results were negative.

In a case-control study including data of 569 SARS patients and 1188 healthy controls from 2005 [26], different *MBL2* gene polymorphisms were evaluated. The distribution of *MBL2* gene polymorphisms was found to be significantly different between SARS patients and the control group; the frequency of haplotypes associated with low or missing MBL serum levels was higher in SARS patients than in healthy controls. Serum MBL levels were also significantly lower in SARS patients than in healthy controls. However, no relationship was found between *MBL2* genotypes associated with low serum MBL levels and SARS-associated mortality.

As a summary of other studies that investigate the effect of the *MBL2* genotype on gene expression [27,28], it is reported in the literature that *MBL2* expression in the AB genotype decreased by 1:10, while there was no expression in the BB genotype. In the previous study conducted by Medetalibeyoglu et al. [14], the lower MBL2-associated BB genotype was observed more in COVID-19 patients; but in this study, the fact that the AB genotype and B allele were higher in the healthy controls starts an important discussion. Although patients with initial negative PCR results were included in this study, obtaining PCR positivity with repeated samples or respiratory tract samples obtained from the lower respiratory tract (deep tracheal aspirate or endotracheal aspiration) constitutes another important point of discussion. The higher incidence of the AB genotype and B allele in healthy controls and the higher incidence of the AB genotype and B allele in the PCR-positive, CT-negative group may be associated with upper respiratory tract viral clearance. The high viral clearance of patients with high MBL levels and the difficulty in obtaining nucleic acids may be causing this situation.

A report by Takahashi et al. [29] contains important data regarding the relationship between MBL2 and autoimmune or autoinflammatory disorders. The codon 54-gene polymorphism of 147 systemic lupus erythematosus (SLE) patients and 160 healthy controls were examined and the serum concentration of MBL was measured. The frequency of homozygosity for the codon 54 BB genotype was 6% (9/147) in patients with SLE and was significantly higher than controls ($p = 0.0294$). Patients homozygous for the B allele (BB) tend to have a higher risk of infection during treatment. C3 and CH50 levels were found to be significantly correlated with serum MBL concentration in AA homozygous SLE patients. During SLE treatment, after the initiation of immunosuppressive therapy, it was observed that MBL concentration decreased significantly in 7 of 14 patients. COVID-19 and lung involvement can be associated with high MBL, which is in parallel with the results of this study. In addition, after initiation of steroids that are widely used in treatment, it seems quite possible to have a decrease in MBL level and clinical response. C3 and CH50 levels can also be used in clinical practice because they represent a high level of MBL at the point of detection of lung involvement and treatment preferences.

Another important study related to MBL2 and organ damage has also been reported in SLE patients and it was found that there is a relationship between the formation of autoantibodies against C1q and MBL2 and renal involvement [30,31]. Similar to C1q, the presence of autoantibodies against MBL2 in patients with SLE contributes to the development of the disease. SLE patients have more IgG anti-MBL antibodies than healthy controls. They are more common in proliferative lupus nephritis, especially during active disease [31]. High levels of MBL are also associated with lung injury in patients with cystic fibrosis [32]. When the relationship between MBL2 and Behcet's disease (another autoinflammatory disease with a complex clinical picture), is examined [33], it was found that high

MBL levels are associated with Behcet's associated polymorphisms, while low MBL levels are negatively correlated with Behcet's disease. In inflammatory bowel diseases (IBD), another important autoinflammatory picture, the MBL level and its relationship to autoinflammation have been studied, and it has been reported that MBL deficiency may be protective against ulcerative colitis (UC) [34]. It is necessary to emphasize the fact that the relationship between MBL levels and autoinflammation/inflammatory syndromes is controversial. Contrary to the previous COVID-19 study [17], significantly higher incidence of the AB genotype and B allele in healthy controls may be related to the different density of the virus in the respiratory tract or differences in viral clearance in the upper respiratory tract. High MBL associated with AA genotype may cause higher incidence of false negativity but milder course of disease. In patients with genotypes associated with high MBL levels, it may be difficult to detect virus because of high viral clearance and it may cause lung disease with autoinflammatory/autoimmune patterns. Similarly, patients with genotypes associated with low MBL levels may have the chance to detect virus easier and may have a lung injury directly related to the viral involvement. In order to elaborate on this theory, new studies are needed.

NO, which is an important inhibitor of the replication of DNA and RNA viruses, is among the viral protection factors. NO, which is also considered as an important protection factor for COVID-19, decreases with age and plays a potential mortal role in this infection in elderly patients [13,35]. In this study, no significant difference was found between COVID-19 and healthy controls. However, NO seems to continue to be an important factor in this infection with its potential properties.

The most important limitation of this study is the size of the patient population. In statistical analysis, although AB genotype and B allele of ***MBL2*** were found significantly higher in healthy controls; the lack of similar results for the BB and AA genotype is also attributed to the limited patient population. The second important limitation is that concurrent MBL serum levels have not been measured in this study.

Conclusions

This study reveals very important results in terms of anti-cytokine and anti-inflammatory treatment preferences of COVID-19, which are among the new treatment options. Not only AB genotype and B allele of *MBL2* were found to be significantly higher in healthy controls and the absence of the AB genotype was found to increase the risk of CT positivity, but also it can be used for early detection and isolation of patients with typical lung involvement, who had enough viral loads, but whose initial PCR results were negative. This result will be the most important contribution point of this study and will lead to new studies in terms of host genetic factors and COVID-19.

Abbreviations

COVID-19: Coronavirus Disease-2019
Sars-CoV2: Severe Acute Respiratory Syndrome Coronavirus 2
PCR: Polymerase Chain Reaction
CT: Computed Tomography
MBL2: Mannose Binding Lectin 2
NOS: Nitric Oxide Synthase
NO: Nitric Oxide
RFLP: Restriction Fragment Length Polymorphism
OR: Odds Ratio
CI: Confidence Interval
MxA: Myxovirus resistance protein A
SLE: Systemic Lupus Erythematosus
IBD: Inflammatory Bowel Diseases
UC: Ulcerative Colitis
DNA: Deoxyribonucleic Acid
RNA: Ribonucleic Acid

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Declarations

Ethics Approval and Consent to Participate

Ethical committee approval was received (Istanbul University, Faculty of Medicine, Approval number: 21/05/2020-84,539) and the patients and control subjects gave informed consent before the beginning of the study. The experimental procedures were based on the Declaration of Helsinki and relevant institutional regulations.

Patient Consent for Publication

An informed consent obtained as written forms from all of our patients to publish.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors contributed to the editing of the manuscript. S.P., T. T., Y.O, A.M., M.P., U.I.A., G.Y.S. and N.S. collected the patients' data. I.S., S.P., M.K., S.M. wrote the main manuscript text and I. S. prepared tables. All authors reviewed the manuscript.

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