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**RESEARCH**

# Effects of TLR4 and TLR7 genes on the immune system in COVID-19 patients

## COVID-19 hastalarında bağışıklık sistemi üzerinde TLR4 ve TLR7 genlerinin etkisi

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**Abstract**

**Purpose:** This study aimed to investigate the effects of TLR4 and TLR7 gene polymorphisms on the immunity of COVID-19 patients.

**Materials and Methods:** A total of 210 COVID-19 patients were divided into two groups. Group 1: COVID-19 patients experiencing severe respiratory complications requiring intensive care unit admission (n=107). Group 2: COVID-19 patients with mild SARS-CoV-2 infection who have been recovered without requiring any treatment or hospitalization (n=103). Whole blood samples (2 mL) were collected for DNA isolation and genotyping. Genotyping analyses were then conducted using the RT-PCR method to investigate the following single nucleotide polymorphisms (SNPs): TLR4 gene rs4986790 (896A/G, Asp299Gly) and rs4986791 (1196C/T, Thr399Ile), TLR7 gene rs189681811 (c.2759 G>A Arg920 Lys ) and rs149314023 (c.655G>A Val219Ile).

**Results:** TLR7 RS189681811 and TLR7 RS149314023 gene polymorphisms were not observed in any of the study groups. However, TLR4 RS4986790 and TLR4 RS4986791 gene polymorphisms were observed in both mild and severe groups with no significant differences.

**Conclusion:** No statistically significant differences in genotype distribution were found between the two groups for the SNPs examined. Future multicentered studies with larger sample sizes and a broader range of TLR SNPs could provide valuable insights and contribute to the existing literature.

**Keywords:**. SARS-CoV-2, *TLR4*, *TLR7,* polymorphism

**Öz**

**Amaç:** Bu çalışmada TLR4 ve TLR7 gen polimorfizmlerinin COVID-19 hastalarının bağışıklığı üzerindeki etkilerinin araştırılması amaçlanmıştır.

**Gereç ve Yöntem: T**oplam 210 COVİD-19 hastalarından iki grup oluşturuldu. Grup 1: Yoğun bakım ünitesine yatışlı ciddi solunum komplikasyonları yaşayan COVİD-19 bölgeleri(n=107). Grup 2: Herhangi bir tedavi veya iyileşme gerektirmeden iyileşen hafif SARS-CoV-2 enfeksiyonu olan COVİD-19 hücreleri(n=103). DNA izolasyonu ve genotipleme için tam kan örnekleri (2 mL) toplandı. Genomik DNA izole edildi. Daha sonra RT-PCR yöntemi kullanılarak SNP analizleri yapıldı. Hastalarda incelenen SNP'ler, TLR4 geni rs4986790 (896A/G, Asp299Gly) ve rs4986791 (1196C/T, Thr399Ile), TLR7 geni rs189681811 (c.2759 G>A Arg920Lys) ve rs149314023 (c.655G>A) Val219Ile).

**Bulgular:** Çalışmamızda, TLR7 RS189681811 veya TLR7 S149314023 gen polimorfizmleri çalışma gruplarında tespit edilmedi, ancak gen polimorfizmi hem hafif (n=6) hem de şiddetli (n=7) gruplarda anlamlı bir fark dışında gözlendi. TLR4 RS498679 ile TLR4 RS4986791 gen polimorfizmi hem hafif (n=8) hem de şiddetli (n=5) gruplarda iki grup arasında anlamlı bir fark olmaksızın gözlendi.

**Sonuç:** İncelenen SNP'ler için iki grup arasında genotip dağılımında istatistiksel olarak anlamlı bir fark bulunamadı. Daha büyük örneklem büyüklükleri ve daha geniş bir TLR SNP aralığı ile gelecekteki çok merkezli çalışmalar değerli sonuçlar sağlayabilir ve mevcut literatüre katkıda bulunabilir.

**Anahtar kelimeler**: SARS-CoV-2, TLR4, TLR7, polimorfizm

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## INTRODUCTION

Severe respiratory syndrome coronavirus-2 (SARS-CoV-2) causes the development of coronavirus disease 2019 (COVID-19), resulting in mild to moderate phenotypic symptoms. Patients with SARS-CoV-2 infection may exhibit high fever, cough, chest congestion, sore throat, decreased sense of smell and taste, and conjunctivitis. However, SARS-CoV-2 can cause a life-threatening illness that leads to the development of acute respiratory distress syndrome (ARDS) in 5% of patients[1-3]. Admission to the Intensive Care Unit (ICU) is sometimes necessary for patients with severe symptoms. Life-threatening complications such as ARDS, pneumonia with acute respiratory failure, sepsis, septic shock, or multiple organ dysfunction syndrome develop in 14% of cases[5]. Particularly, SARS-CoV-2 has a severe course, especially in patients with advanced age, male gender, obesity, and comorbidities such as type-2 diabetes, cardiovascular diseases, hypertension, and immune system weaknes[2,6-8]. In addition, genetic or environmental factors are all associated with severe COVID-19 infection[9].

Toll-like receptors (TLRs) are important in activating the innate and adaptive immune systems. They are responsible for recognizing the pathogen-associated molecular patterns (PAMPs) of SARS-CoV-2. Additionally, they are active in the immune system as pathogen recognition receptors (PRR). TLRs are expressed on macrophages, monocytes, dendritic cells (DCs), natural killer cells, and adaptive immunity cells (T and B cells). TLRs can be expressed on the cell surface or inside the cell[10]. TLRs have 11 types in humans: *TLR3*, *TLR7*, *TLR8*, and *TLR9* are located within the cell, whereas *TLR2*, *TLR4*, and *TLR6* are found on the outer membrane of the cell[11].

*TLR4*, *TLR7*, *TLR8*, and *TLR9* recognize viruses and activate the immune system via NF-ϰB and MAPK molecules. With the activation of the immune system, the levels of pro-inflammatory cytokines TNF-α, IL-6, and interleukin-2 receptor (IL-2R) increase in patients. The production of TNF-α, IL-1, IL-6, and IL-8, together with the increase in neutrophil count and the decrease in lymphocyte count, are associated with disease severity as a part of the immune response[12]. Some studies have reported that *TLR4* receptors cause cytokine production and lung damage due to the effect of oxidized phospholipid (OxPL) in SARS-CoV-2 infection[13]. In addition, when the virus is recognized by endosomal-located *TLR7*, the MyD88-dependent MAPK-NF-ϰB pathway is activated, which generates TNF-α and ILs, especially IL-6. The IL-6 level in serum was found to increase dramatically during the cytokine storm in SARS-CoV-2 patients[13,14].

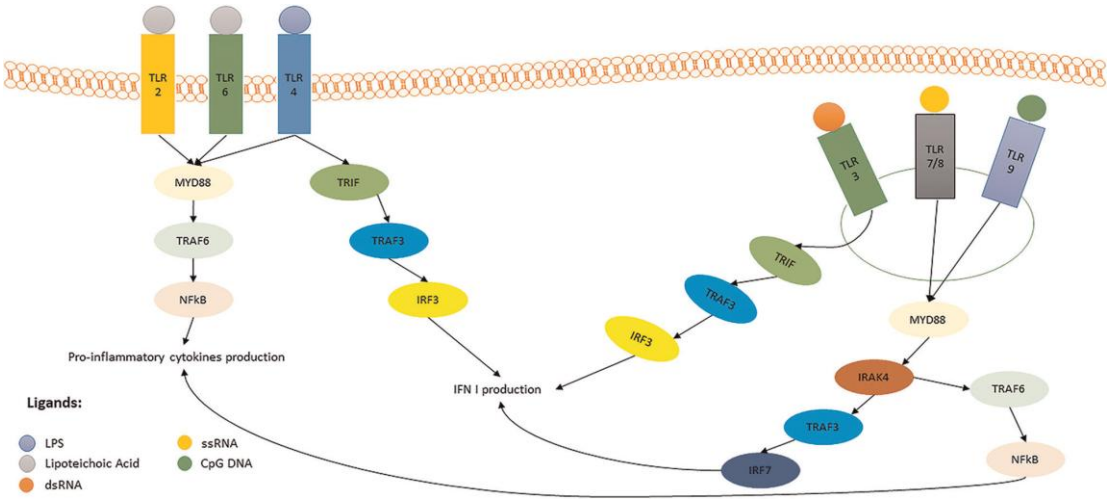


**Figure 1. Toll-like receptor signaling pathways. TLR2/6 and 4 localize in the cell membrane, and TLR3, TLR7/8, and 9 localize in the endosome surface.**

Since SARS-CoV-2 infects the human body through various mucosae such as respiratory mucosa and ocular mucosa, it is thought that the creation of a strong immune response in the mucosal membrane can provide a timely response to and prevent virus invasion. In a study conducted with this foresight, nanoparticles prepared using InAc (InAc-NPs), a TLR4 agonist, were described as an intranasal vaccine that induced a sufficiently strong immune response in mucosal areas and throughout the body[15]. As seen in this study, treatment options emerge as the key points that play an active role in the immune system are understood.

Recent studies have observed a significant association between variability and genetic polymorphism of TLR genes and COVID-19 severity. Consequently, our study aimed to investigate the effects of TLR4 and TLR7 gene polymorphisms on the immunity of COVID-19 patients. A comparative analysis was conducted on patients with severe and mild symptoms of COVID-19. The study examined the presence of TLR4 and TLR7 SNPs among both groups to investigate a possible correlation with the severity of the disease.

## MATERIALS AND METHODS

### Sample

This is a prospective cohort study that examines patients who were diagnosed with COVID-19 between December 2020 and December 2022. A total number of 210 COVID-19 patients from Tokat Gaziosmanpasa University Hospital and Tokat State Hospital were enrolled in our research. The study was conducted at the Tokat Gaziosmanpasa University Hospital Medical Genetics Laboratory.

Only individuals with a positive COVID-19 RT-PCR test were included in the study. Patients who were detected as COVID-19 positive in the RT-PCR test were divided into two separate groups: group 1 (Patients with Severe COVID-19): COVID-19 patients experiencing severe respiratory complications requiring intensive care unit (ICU) admission (n=107). Group 2 (Patients with Mild COVID-19): COVID-19 carriers infected with SARS-CoV-2, and recovered without requiring any treatment or hospitalization (n=103).

COVID-19 patients who needed outpatient or inpatient treatment were excluded from the study. The demographic characteristics are shown in Table 2.

Ethical committee approval for the study was obtained from the Tokat Gaziosmanpasa University Faculty of Medicine Ethical Board (17.02.2022/22-KAEK-040), and informed consents were obtained from all the study participants.

### Sampling, DNA isolation, and genotyping

TLR4 gene rs4986790 (896A/G, Asp299Gly) and rs4986791 (1196C/T, Thr399Ile) and TLR7 gene rs189681811 (c.2759 G>A Arg920Lys) and rs149314023 (c.655G>A Val219Ile) genotypes were examined. Whole blood samples (2 mL) were collected for DNA isolation and genotyping. Genomic DNA was isolated from 2 mL of whole EDTA blood using NucleoGene Blood DNA Extraction Kit. Then, NucleoGene qPCR Probe Master Mix was prepared. Real-time polymerase Chain Reaction (RT-PCR) was conducted using a Roche LightCycler 480 II instrument as a variation of the PCR assay to allow real-time monitoring of PCR progress(2X) (Table 1).

**Table 1. Real time PCR protocol.**

| | | |
|---|---|---|
| **Initial denaturation** | 95℃ 15 min | 95℃ 15 min |
| **Denaturation** | 95℃ 10 to 15s | 95℃, 10 to 15s |
| **Annealing** | 60℃,30 to 60s | 55 to 65℃ 30 to 60s |
| **Extension/elongation** | | 68 to 72℃, 10s |

### Statistical analysis

Statistical analyses were conducted using the IBM SPSS Statistics 20 software package. A Kolmogorov-Smirnov test was used to assess normality, and since all variables did not follow normal distribution, the Mann-Whitney U test was used to compare genotype frequencies between the mild and severe groups. $P < 0.05$ was considered statistically significant. To determine the minimum sample size required for our study, a prior power analysis was conducted using G*Power (3.1.9.7). The effect size was 0.05, the margin of error was 5%, and the power was 0.95. The results revealed that a total sample size of 176 participants (88 cases + 88 controls) would be sufficient. In the present study, 107 cases and 103 controls were recruited, which is in line with the calculated sample size.

## RESULTS

In our study, we examined a total of 210 COVID-19 patients, of which 107 with severe symptoms (53 female, 54 male; mean age ± SD: 74 ± 14.7) and 103 with mild symptoms (64 female, 39 male; mean age ± SD: 39.6 ± 14.8). Additional demographic characteristics are presented in Table 2. For the rs4986790 (896A/G, Asp299Gly) gene, the AA and AG genotype frequencies were 94% and 6% in the mild group, respectively, and 93% and 7% in the severe group. The allele frequencies of A and G were 0.97 and 0.03 in the mild group and 0.965 and 0.035 in the severe group. Similarly, for the rs4986791 (1196C/T, Thr399Ile) gene, the CC and CT genotype frequencies were 92% and 8% in the mild group, and 95% and 5% in the severe group. The allele frequencies of C and T were 0.96 and 0.04 in the mild group, and 0.975 and 0.025 in the severe group (Table 3).

As a result of our study, TLR4 RS4986791 gene polymorphisms were observed in both mild (n=8) and severe (n=5) groups without any significant difference between the two groups (p=0.239). Furthermore, TLR4 RS4986790 gene polymorphisms were observed in both mild (n=6) and severe (n=7) groups with no statistically significant difference (p=0.941). However, no TLR7 RS189681811 or TLR7 RS149314023 gene polymorphisms were detected in any of the study groups (Table 4).

**Table 2. Demographic characteristics of the study population.**

| | **Severe** (n=107) | **Mild** (n=103) |
|---|---|---|
| Age | 74±14.7 | 39.6±14.8 |
| Gender | Male: 54<br>Female: 53 | Male: 39<br>Female: 64 |

**Table 3. Frequencies of the genotypes and alleles of *TLR4* RS4986790 and *TLR4* RS4986791 genes**

| | **Severe** (n=107) | **Mild** (n=103) |
|---|---|---|
| AA genotype | 93% | 94% |
| AG genotype | 7% | 6% |
| CC genotype | 95% | 92% |
| CT genotype | 5% | 8% |
| A allele | 0.965 | 0.97 |
| G allele | 0.035 | 0.03 |
| C allele | 0.975 | 0.96 |
| T allele | 0.025 | 0.04 |

**Table 4. Comparing *TLR4* RS4986790 and *TLR4* RS4986791 gene polymorphisms between severe and mild patients**

| | **Severe** | **Mild** | **P value** |
|---|---|---|---|
| TLR4RS4986790 gene polymorphism (AG) | 7 | 6 | 0.941* |
| TLR4RS4986791 gene polymorphism (CT) | 5 | 8 | 0.239* |

*Mann-Whitney U test ($p<0.05$)

## DISCUSSION

In the present study, our objective was to compare the SNPs of TLR4 (Asp299Gly and Thr399Ile) and *TLR7* (Arg920Lys and Val219Ile) in patients with severe and mild COVID-19. No *TLR7* SNPs were detected in any of the study groups. Conversely, while *TLR4* SNPs were identified in both the mild and severe groups, the difference between the two groups was not statistically significant.

TLRs on the cell surface primarily detect microorganisms by recognizing their membrane components, such as microbial lipids and lipoproteins. TLRs recognize viral elements and activate the immune system, producing type 1 interferon and proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α. *TLR2*, *TLR3*, *TLR4*, *TLR6*, *TLR7*, *TLR8*, and *TLR9* are crucial components in the immune system response to COVID-19 infection. Moreover, it has been reported that infections caused by MERS-CoV, SARS-CoV, and SARS-CoV-2 are worsened by the cytokine storm[16,17] (16. Khadke et al., 2020; 17. Onofrio *et al.*, 2020). TLRs exert their effects on COVID-19 by increasing the production of IL-1β and other pro-inflammatory cytokines. The interaction between TLRs and viral elements can also cause fatal outcomes in COVID-19 patients[18,19].

*TLR4*, located on cell surfaces and endosomal membranes, triggers signaling pathways through multiple pathways[20-23]. Additionally, *TLR4* is found in alveolar cells and bronchial epithelial cells in the lungs. The binding between SARS-CoV-2 and *TLR4* facilitates the activation of transcription-triggering factors such as AP-1, NF-kB, and IRF. However, it has been documented that *TLR4* regulates IL-6 via NF-kB. In addition, the cytokine storm that develops TLRs contributes to organ failure and and mortality caused by viral elements [19].

Choudhury et al. reported that the spike protein of SARS-CoV-2 activates *TLR1*, 4, and 6, to a higher

extent for *TLR4*. In line with these findings, it is thought that *TLR4* antagonist molecules can be applied in the treatment of COVID-19 patients' treatment [10].

When viruses bind to endosomally located *TLR7* receptors, they initiate the MyD88-dependent MAPK - NF-ϰB signaling pathway, causing an increase in the levels of TNF-α and other ILs, including high amounts of IL-6. For these reasons, it has been observed that IL-6 levels are significantly high in the serum of SARS-CoV-2 patients diagnosed with cytokine storm[13,14]. Macrophages express *TLR4*, *TLR7*, *TLR8*, and *TLR9*, which play an active role in recognizing virus particles. TLRs also play an important role in promoting local anti-inflammatory processes, thus enhancing phagocytosis, cytokine release, and other mediators[24]. The intense release of pro-inflammatory cytokines creates a cytokine storm, ultimately resulting in organ failure and the development of SARS-CoV-2 complications[25]. Since SARS-CoV-2 enters cells through *TLR3*, 4, 7, 8, and 9 receptors, TLR antagonists are being investigated against this deadly infection[26].

*TLR7* and *TLR8* genes located on the X chromosome contribute to the production of IFN, with increased gene expression rates in women. Thus, increased production of type I IFN by *TLR7/8* provides stronger activation. This explains the more effective resistance and higher survival rates in women against SARS-CoV-2 infection. Additionally, loss-of-function variants of *TLR7* have been reported to underlie susceptibility to COVID-19 infection in men[27].

Other studies on SARS-CoV-2 suggested that *TLR4* and *TLR7/8* lead to the formation of neutrophil extracellular traps (NETs) and the activation of inflammasomes, causing acute lung injury with an excessive inflammatory effect in individuals with COVID-19[18,28].

In SARS-CoV-2, male gender, older age (>65 years), diabetes, cancer, hypertension, chronic respiratory and other underlying medical conditions such as cardiovascular disorders, and chronic obstructive pulmonary disease are major risk factors for a poor prognosis. Additional risk factors include overweight, tobacco exposure, and low SPO2. Immune-affected genes located on the X chromosome are thought to cause gender differences in responses to SARS-CoV-2. Low socioeconomic conditions are also important in the prognosis of COVID-19[29-34].

In light of the above, it is obvious that *TLR4* and *TLR7* play an important role in the immunological response to SARS-CoV-2 infection. Furthermore, the various COVID-19 symptoms and the unknown disease progression observed in some cases may be accounted for by *TLR4* and *TLR7* mutations[35,36].

In their study to investigate the relationship between *TLR4* gene rs4986790 and rs4986791 SNPs and COVID-19 severity, Taha et al. found that both SNPs were associated with the COVID-19 disease severity and progression[35]. The authors also suggested that testing for *TLR4* SNPs can provide early information about disease progression as well as the capacity of patients to benefit from *TLR4* antagonist treatment.

In our study, we detected *TLR4* gene rs4986790 and rs4986791 SNPs in both the severe and mild COVID-19 groups; however, no statistically significant difference was observed.

Fallerini et al. reported that they detected *TLR7* variants in 2% of severe COVID-19 males; however, no variants were detected among asymptomatic patients. The identified *TLR7* variants were rs189681811, rs147244662, rs149314023, rs200146658, and rs5743781. Additionally, the authors stated that there was a significant association between the detected *TLR7* variants and the disease severity[27].

In the present study, TLR7 RS189681811 and TLR7 RS149314023 SNPs were not observed in either of the study groups. Although the results of previous studies suggest that TLR4 and TLR7 play a crucial role in immune response, statistical significance was not reached in our analysis.

Among the genetic variables affecting clinical severity among COVID-19 patients, TLR gene polymorphisms, which are active in the immune system, are one of the factors that will explain the effect of the disease severity and mortality rates. These variants need to be evaluated with new studies before reaching a conclusion about their effects on COVID-19 patients. Our study's limitations were the small sample size, being a single-center study, and the inability to carry out expression studies. Moreover, additional factors contributing to severe clinical outcomes in COVID-19 patients should be considered, and patients should be meticulously identified in future studies. One of the strengths of our study was its focus on ICU patients, nevertheless, the intensity of the COVID-19 pandemic limited our

ability to access historical patient information and conduct a more comprehensive clinical evaluation.

As of yet, no other research has investigated TLR4 and TLR7 in COVID-19 patients within the Turkish population, hence limiting direct comparisons. However, future multicenter research with larger sample sizes and investigations of additional TLR4 and TLR7 gene variations may provide significant contributions to the literature.

To date, many studies have been conducted investigating the correlation between SNVs on many genes and the severity of COVID-19. In this study, the first of its kind in Turkey, we examined the *TLR4* and *TLR7* SNP variants in Turkish individuals with severe and mild COVID-19. *TLR7* SNPs were not detected in any of the study groups. However, *TLR4* SNPs were observed in both groups, although not statistically significant. Future multicentered studies with larger sample sizes and a broader range of TLR4 and TLR7 variants could provide valuable insights and contribute to the existing literature.

**Author Contributions:** Concept/Design : NA, FG; Data acquisition: NA, FG; Data analysis and interpretation: NA; Drafting manuscript: NA; Critical revision of manuscript: NA, MA; Final approval and accountability: NA, FG, SU, YT, KK, MA; Technical or material support: NA, FG, SU, YT, KK; Supervision: NA, , FG. SU, YT, KK; Securing funding (if available): n/a.
**Ethical Approval:** Ethical committee approval for the study was obtained from the Tokat Gaziosmanpasa University Faculty of Medicine Ethical Board (17.02.2022/22-KAEK-040).
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