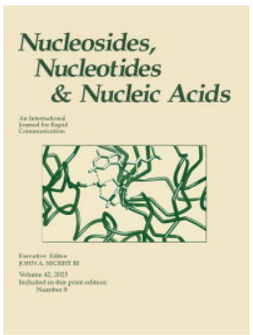

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The investigation of host genetic variants of toll-like receptor 7 and 8 in COVID-19

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ABSTRACT

Toll-like receptors (TLRs) recognize infectious agents and play an important role in the innate immune system. Studies have suggested that TLR single nucleotide polymorphisms (SNPs) are associated with poor antiviral responses against SARS-CoV-2. Therefore, we aimed to investigate the relationship of TLR7 and TLR8 (SNPs) with COVID-19 disease prognosis. A total of 120 COVID-19 patients, 40 outpatients, 40 clinical ward patients and 40 intensive care unit (ICU) patients were included in the study. TLR7 (rs179009), TLR8-129 C/G (rs3764879) and TLR8 Met1Val (rs3764880) SNPs were genotyped using the PCR-RFLP method. In female patients, individuals carrying AG genotype and G allele for TLR8 Met1Val SNP were found at a higher frequency in patients hospitalized in the ICU than in patients followed in the clinical ward ($p < 0.05$). In terms of the other two SNPs, no significant difference was found between the groups in females. Furthermore, in male patients, A allele of TLR7 rs179009 SNP was at a higher frequency in patients who have at least one comorbidity than in patients who have no comorbidity ($p < 0.05$). Our results suggest that TLR8 Met1Val SNP is important in the COVID-19 disease severity in females. Furthermore, TLR7 rs179009 SNP is important in male patients in the presence of comorbid diseases.

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Toll-like receptor; COVID-19; single nucleotide polymorphism; TLR7; TLR8

1. Introduction

Coronaviruses, a very large family of viruses, threaten public health by causing many infections including the common cold, Middle East Respiratory Syndrome (MERS) and severe acute respiratory syndrome (SARS) and towards the end of 2019, the novel coronavirus named "SARS-CoV-2" was defined as a new strain causing Corona Virus Disease 2019

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(COVID-19). World Health Organization (WHO) declares the disease as a pandemic infection as of March 2020.[1,2] While the most prominent symptoms of COVID-19 disease are fatigue, high fever, dry cough and shortness of breath, the typical feature of the patient infected with severe SARS-CoV-2 is pneumonia.[3] Although some cases with SARS-CoV2 infection develop a moderate form of COVID-19 with severe clinical manifestations, approximately 10–15% of cases develop serious illness requiring hospitalization and supportive care. Moreover, 5% of patients may need treatment in intensive care units (ICUs).[4] In severe forms of COVID-19 disease, a cytokine storm can cause acute respiratory distress syndrome (SRDS), multiple organ dysfunction syndrome (MODS), sepsis, and even death.[5]

Toll-like receptors (TLRs) in the host tissue are an important family of receptors that recognize infectious agents and play a key role in the innate immunity.[6] Ten TLRs (TLR1 to 10) have been identified in humans. TLR3, TLR7, TLR8 and TLR9 are localized in the endosomes of immune cells and sense nucleic acids. They are capable of recognizing single-stranded and double-stranded RNA, and DNA from viruses, bacteria, fungi, and parasites.[7,8] Both TLR7- and TLR8 act as the pattern recognition receptor (PRR). In humans, TLR7 can be expressed by both plasmacytoid dendritic cells (pDCs) and B cells, whereas TLR8 is mainly expressed by myeloid cells such as monocytes and neutrophils.[9] TLRs are also indirectly involved in the adaptive immune response by inducing maturation of dendritic cells, expression of co-stimulatory molecules, and due to their complex effects on the polarization Th1 and Th2 cell.[10] Numerous studies have been conducted to demonstrate the role of cell surface and intracellular TLRs in the inflammatory pathogenesis caused by SARS-CoV-2 in the development of COVID-19 disease.[11,12] Activation of TLRs by SARS-CoV-2 results in activation of the inflammasome. In COVID-19 patients, over activation of the inflammasome is also associated with poor prognosis.[13] TLR activation also leads to JAK/STAT activation, leading to macrophage activation syndrome.[14] An immune response activated by the TLR7 and/or TLR8 pathways may also cause unwanted side effects. For example, during COVID-19 disease the SARS-Cov-2 can cause a cytokine storm called COVID-19-associated hyperinflammatory syndrome.[15,16] As TLR7 is required for type-I IFN response, the role of TLR7 single nucleotide polymorphisms (SNPs) in the pathogenesis of COVID-19 may be related to increased type-I IFN response.[17]

TLR7 and TLR8 are encoded by the X chromosome,[18] and in females, one of two X chromosomes is usually inactivated. However, the TLR7 and possibly TLR8 genes in female immune cells appear to escape such silencing. As an advantage of this situation, the genetic information in both

chromosomes is expressed in females. However, because males have only one copy of the X chromosome, they show lower expression levels than females.[19] Potential to activate immune response against viruses such as SARS-CoV-2 is likely to be greater in females.[20] TLRs are considered to be a potential candidate to control of infection in the body and to develop vaccines against the virus in the early stages of COVID-19 disease.[10] In addition, studies have suggested that TLR SNPs are also associated with antiviral responses against SARS-CoV-2.[21,22] Therefore, we aimed to investigate the possible relationship of TLR7 and TLR8 SNPs on susceptibility to the acquisition of SARS-CoV-2 and COVID-19 disease prognosis.

2. Patients and methods

2.1. Study design

This is a prospective cross-sectional observational study. The adult (≥18 years) COVID-19 patients (n = 120) who confirmed by SARS-CoV-2 specific RT-PCR, with the complaints of typical symptoms of COVID-19 such as fatigue, high grade of fever, dry cough and dyspnea, and followed up between March–April 2021 were recruited from the Sivas Numune State Hospital, Sivas, Turkey.

Demographics, main clinical signs and symptoms including fever, sore throat, cough, muscle/joint pain, and admission key routine laboratory tests including complete blood count (CBC), erythrocyte sedimentation rate (ESR), chemistry analyses [blood urea nitrogen (BUN), creatinine, sodium, potassium, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK)], bilirubin, ferritin, troponin, procalcitonin, plasma D-dimer concentrations and serum C-reactive protein (CRP) levels were measured and recorded for each patient. Admission respiratory rate (RR) per minute, arterial oxygen saturation levels in room temperature (SpO_2) and chest X-ray or tomography findings, comorbid conditions including malign disorders, chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD), hypertension, diabetes mellitus (DM), obesity, cigarette smoking, pregnancy, immunocompromised diseases (HIV infection, previous corticosteroid use, organ transplantation) and outcome data (death or alive) were also recorded. Based on the oxygen (O_2) requirements, the patients were categorized into (1) Uncomplicated, non-hospitalized without respiratory distress (RR per min < 24, SpO_2 > 93% in ambient air) and patients with normal chest X-ray and/or tomography, (2) hospitalized, mild/moderate (RR < 30/min, SpO_2 level > 90% in ambient air) and patients with mild to moderate pneumonia findings on chest X-ray and/or tomography and severe (RR ≥ 30/min, SpO_2 level ≤ 90% in ambient air) patients with

severe pneumonia findings on chest X-ray and/or tomography (3) hospitalized, severe cases requiring intensive care unit (ICU) stay with respiratory distress (RR ≥ 30/min, PaO_2/FiO_2 < 300, despite of O_2 consumption 5 L/min SpO_2 level ≤ 90% or PaO_2 < 70 mmHg), and patients with severe pneumonia findings on chest X-ray and/or tomography plus hypotension (arterial systolic blood pressure < 90 mmHg), tachycardia (heart rate > 100 beats/min), acute kidney injury, elevated liver enzymes, confusion, coagulation test abnormalities, immunosuppression, elevated blood troponin, arrhythmia, and lactate > 2 mmol/L.[23] After confirmation of COVID-19 by RT-PCR, whole blood samples were collected into tubes containing EDTA (n = 1, 3 mL capacity, EDTA tubes, BD™, NJ, USA) and stored at −80 °C in a deep freeze until use. The study was approved by regional ethics committee of Sivas Cumhuriyet University (2021-01/06). All participants provided signed informed consent (IC). Patient identities were anonymized and delinked before analysis.

Adult (≥18 years) male and female COVID-19 patients who agreed to be participated in the study after explanation and sign IC were included, but patients who denied signing an IC, patients under 18-year and pregnant women were excluded from the study. All patients included in this study were followed and treated by the same clinicians according to guideline for the treatment of adult COVID-19 (SARS-CoV-2) patients of Turkish Ministry of Health (MoH), updated on October 9th, 2020.[23] All group of COVID-19 patients were treated with standard doses of oral hydroxychloroquine and favipiravir for 5 days (in hospitalized patients up to 10 days) and hospitalized patients were treated with standard medical care including fluid, oral and parenteral antibiotics, cardiovascular and respiratory support, intravenous corticosteroids and oxygen treatment when needed.[23]

2.2. Genotyping

DNA isolation was performed from 1 mL of blood taken into tubes with K_3EDTA using a commercial DNA extraction kit (Invitrogen). SNPs of the TLR7 rs179009 and TLR8-129C/G (rs3764879) and TLR8 Met1Val (rs3764880) were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Primer sequences and PCR conditions used for detection of single nucleotide polymorphisms of TLR7 and TLR8 genes have been shown in Table 1. A total volume of 25 µL with 20 pmol of each primer, 1 unit of Taq polymerase (Thermo Fisher, USA) PCR buffer, 10 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 100–150 ng genomic DNA was used for PCR reaction. Digestion products were run on

Table 1. Primer sequences and PCR conditions used for the detection of single nucleotide polymorphisms (SNPs) of TLR7 and TLR8 genes.

Gene (SNP)	Primer sequences	PCR conditions	Cycle	Restriction enzymes
TLR7 rs179009	F: 5′-TAACAACGAATAGG AAAATGC-3′ R: 5′-GTTTTAGGAAACCATCTAGCC-3′	94 °C-30 sec 54 °C-30 sec 72 °C-1 min	35	NlaIII
TLR8-129C/G (rs3764879)	F: 5′-GTGTGTGTCTGATTTGGGTTG-3′ R: 5′-TTTCTAGGCTCACACCATTTG-3′	94 °C-30 sec 53 °C-30 sec 72 °C-1 min	35	HpyCH4IV
TLR8 Met1Val (rs3764880)	F: 5′-GTGTGTGTCTGATTTGGGTTG-3′ R: 5′-TTTCTAGGCTCACACCATTTG-3′	94 °C-30 sec 53 °C-30 sec 72 °C-1 min	35	NlaIII

agarose gel at 100 V for 30 minutes, then examined on a UV trans illuminator to display DNA bands.

2.3. Statistical analysis and sample size calculation

Statistical Package for Social Sciences (IBM® SPSS®, Armonk, New York, USA, URL: https://www.ibm.com/products/spss-statistics) SPSS version 28.0 statistical program was used for the statistical analysis. Descriptive statistics were presented as frequencies, percentages for categorical variables and median [interquartile range (IQR)] for continuous variables. Continuous variables were tested firstly for normal distribution by one-sample Kolmogorov–Smirnov and Shapiro–Wilk tests. Homogeneity of variances was tested with Levene's test. In comparing the groups, the non-parametric Kruskal-Wallis with Tamhane's post-hoc test was used for continuous variables and the Freeman-Halton extension of the Fisher exact probability test for two-rows by three-columns contingency table was used for categorical variables. The Chi-square and Fisher's exact tests were used for comparison of two independent groups with categorical variables. The genotype and allele frequencies of TLR7 and TLR8 SNPs were determined by gene counting method. *P* values, odds ratios (ORs), and 95% confidence intervals (95% CIs) were calculated and used to evaluate the association between TLR7 and TLR8 SNPs and COVID-19 risk. Hardy-Weinberg equilibrium and polymorphic information content & heterozygosity were calculated with Gene-Calc software (https://gene-calc.pl/). In the statistical evaluation, *P* value less than 0.05 was considered as significant.

Sample size was calculated by using G Power 3.1. Since the main objective of this work was to compare the difference in parameters between three groups, One-Way ANOVA was set to calculate sample size. α was set at 0.05 and β was set at 0.20. The total samples size of 120 (n = 40 each group) was required to achieve power of 0.8.

3. Results

3.1. Demographics, baseline clinical characteristics and outcome data

A total of 120 RT-PCR confirmed COVID-19 patients (n = 63, female and n = 57, male) were recruited and they were divided into three groups: Outpatient clinic (Group 1; n = 40), Clinical ward (Group 2; n = 40), and ICU (Group 3; n = 40). Comparison of demographic and clinical characteristics of the COVID-19 patients is detailed in Table 2. The median (IQR) age (year) for both in clinical ward group [58.5 (52.5–73.0)] and ICU group [64.5 (59.2–75.7)] were significantly higher than that of the outpatient clinic group [48.0 (32.5–59.7)] of COVID-19 patients ($P < 0.0001$ for both comparisons). Cough (84.2%), fatigue (76.7%) and dyspnea (45.0%) were the most common symptoms among all group of cases. However, dyspnea, fatigue and another symptom sputum were significantly higher in the ICU group than that

Table 2. Demographic and clinical characteristics of the COVID-19 patients (N = 120)

Variables	Group 1 outpatient clinic (n = 40)	Group 2 clinical ward (n = 40)	Group 3 intensive care Unit (n = 40)	*P*-value
Age, median (IQR), y	48.0 (32.5-59.7)	58.5 (52.5-73.0)[a]	64.5 (59.2-75.7)[a]	**<0.0001**
Male gender	17 (42.5)	23 (57.5)	17 (42.5)	0.362
Symptoms				
History of fever	7 (17.5)	14 (35.0)	8 (20.0)	0.144
Cough	32 (80.0)	32 (80.0)	37 (92.5)	0.247
Sore throat	8 (20.0)	1 (2.5)[b]	0 (0.0)[b]	**<0.0001**
Dyspnea	0 (0.0)	15 (37.5)[c]	39 (97.5)[c,d]	**<0.0001**
Fatigue	25 (62.5)	27 (67.5)	40 (100.0)[c,d]	**<0.0001**
Sputum	0 (0.0)	3 (7.5)	22 (55.0)[c,d]	**<0.0001**
Chest pain	2 (5.0)	1 (2.5)	3 (7.5)	0.701
Loss of appetite	15 (37.5)	9 (22.5)	8 (20.0)	0.156
Headache	4 (10.0)	9 (22.5)	8 (20.0)	0.246
Nausea and/or vomiting	1 (2.5)	6 (15.0)	4 (10.0)	0.120
Myalgia and/or joint pain	12 (30.0)	13 (32.5)	0 (0.0)[c,d]	**<0.0001**
Fever, (≥38 °C)	0 (0.0)	10 (25.0)[b]	7 (17.5)[e]	**<0.001**
Comorbid diseases				
COPD	2 (5.0)	6 (15.0)	5 (12.5)	0.315
CVD	2 (5.0)	8 (20.0)	12 (30.0)[e]	**<0.01**
Hypertension	10 (25.0)	18 (45.0)	21 (52.5)[e]	**0.031**
Diabetes mellitus	4 (10.0)	11 (27.5)	20 (50.0)[c]	**<0.0001**
Obesity	0 (0.0)	2 (5.0)	4 (10.0)	0.066
Cigarette smoking	6 (15.0)	15 (37.5)[e]	4 (10.0)[f]	**<0.01**
Pregnancy	2 (5.0)	0 (0.0)	0 (0.0)	0.109
Immunosuppression*	0 (0.0)	1 (2.5)	2 (5.0)	0.217
Fatal outcome	0 (0.0)	0 (0.0)	3 (7.5)	0.135

Except for age, all data presented as *n* (%). Age data presented as median [interquartile range (IQR)]. COPD: Chronic obstructive pulmonary disease; CVD: Cardiovascular disease.

[a] $P < 0.0001$ vs. Outpatient clinic group (Kruskal Wallis test with Tamhane's posttest).

[b] $P < 0.01$ vs. Outpatient clinic group (Fisher's exact test).

[c] $P < 0.0001$ vs. Outpatient clinic group (Fisher's exact test).

[d] $P < 0.0001$ vs. Clinical ward group (Fisher's exact test).

[e] $P < 0.05$ vs. Outpatient clinic group (Fisher's exact test).

[f] $P < 0.001$ vs. Clinical ward group (Fisher's exact test).

*Steroid use, 1; Malignant disease, 1; Collagen tissue disease, 1.

of other groups (97.5%, 100.0% and 55.0%, respectively; $P < 0.0001$ for all comparisons). Further, dyspnea was also found to be higher in clinical ward group than that of the outpatient group (37.5% vs. 0.0%; $P < 0.0001$). Fever (≥38 °C), one of the most important physical signs, was statistically frequent both in Clinical ward (25.0%) and ICU patients (17.5%) than that of outpatient clinic patients (0.0%; $P < 0.01$ and $P < 0.05$, respectively). As expected, hypertension (40.8%) and DM (29.2%) were the most common comorbid diseases among all group of cases and according to outpatient clinic patient group, both comorbities were significantly higher in the ICU group patients (52.5% and 50.0%; $P < 0.05$ and $P > 0.0001$, respectively). CVD was also significant comorbid disease in the ICU group of patients (30.0%; $P < 0.05$). Fatal outcome was observed in 3 (7.5%) out of 40 patients in the ICU group and the case fatality rate (CFR) was 2.5% (3 out of 120) for this cohort. However, no statistical difference was observed among the groups ($P > 0.05$).

3.2. Baseline laboratory characteristics

Table 3 shows comparison of baseline biomarkers of 120 COVID-19 patients according to patient groups. In general, blood routine and biochemistry test results, blood CRP, D-dimer and ESR were the worst in the ICU group of patients. The median (IQR) WBC number (1×10^3/mm^3) in the ICU group [11.0 (7.6–15.8)] was significantly higher than that of both outpatient clinic [5.6 (4.4–6.5)] and clinical ward [6.5 (4.4–8.5)] groups ($P < 0.001$). The median RBC number, blood hemoglobin level and the hematocrit ratio were significantly lower both in the ICU and clinical ward group of patients than the outpatient clinic of patients ($P < 0.001$ for all comparisons). For the comparison of biochemistry test results, the median serum BUN, AST, LDH, CPK, ferritin and troponin levels and blood CRP, D-dimer and ESR all were significantly higher in the ICU group than that of the outpatient clinic group ($P < 0.001$ for all comparisons). According to patients who were hospitalized in the clinical ward, the ICU stayed COVID-19 patients had higher levels of BUN, AST, LDH, ferritin, CRP, D-dimer (one of the most important biomarker for COVID-19 patient follows) and ESR levels ($P < 0.05$ for all comparisons). However, serum median (IQR) procalcitonin levels (ng/mL) were significantly lower in the ICU group than that of the Clinical ward group of patients [0.2 (0.1–0.7) vs. 0.1 (0.1–0.2); $P < 0.001$] and also Clinical ward patients had statistically significant lower level of procalcitonin than that of the Outpatient clinic group [0.1 (0.1–0.2) vs. 0.5 (0.1–0.9); $P < 0.001$].

Table 3. Baseline biomarkers of the COVID-19 patients (N = 120)

Variable	Group 1 outpatient clinic (n = 40)	Group 2 clinical ward (n = 40)	Group 3 Intensive care unit (n = 40)	*P*-value	Normal
Blood routine					
WBC, (1×10^3/mm^3)	5.6 (4.4 - 6.5)	6.5 (4.4 - 8.5)	11.0 (7.6 - 15.8)[a,b]	**<0.0001**	4.0 – 11.0
Platelets, (1×10^3/mm^3)	211.5 (188.7 - 260.5)	185.5 (145.2 - 230.5)	231.0 (183.7 - 272.2)	>0.05	150.0 – 450.0
RBC, (1×10^6/mm^3)	5.1 (4.7 - 5.3)	4.6 (4.2 – 4.9)[a]	4.5 (4.0 - 5.0)[a]	**<0.0001**	4.7-6.1 (male); 4.2-5.4 (female)
Hemoglobin, (g/dL)	14.3 (13.6 - 15.5)	13.4 (12.3 - 14.0)[a]	12.3 (11.0 - 14.1)[a]	**<0.0001**	13.2-16.6 (male); 11.6-15.0 (female)
Hematocrit, (%)	43.0 (40.5 - 45.8)	40.3 (36.1 - 42.3)[a]	37.9 (34.4 - 42.8)[a]	**<0.0001**	38.3-48.6 (male); 35.5-44.9 (female)
Blood biochemistry					
BUN, (mg/dL)	24.6 (19.9 - 30.0)	31.3 (26.3 - 43.8)[a]	54.7 (41.4 - 78.6)[a,b]	**<0.0001**	8.0 - 23.0
Creatinine, (mg/dL)	0.8 (0.7 - 0.9)	0.8 (0.7 - 1.0)	0.8 (0.6 - 1.2)	>0.05	0.6 – 1.2
Sodium, (mEq/L)	138.5 (138 - 140.7)	137.0 (135.2 - 139.0)	137.5 (134.0 - 140.0)	>0.05	135.0 – 145.0
Potassium, (mEq/L)	4.1 (3.9 - 4.3)	4.0 (3.8 - 4.2)	4.3 (3.9 - 4.7)	>0.05	3.5 – 5.5
Total bilirubin, (mg/dL)	0.6 (0.5 - 0.7)	0.5 (0.3 - 0.7)	0.6 (0.4 - 1.0)	>0.05	1.2
AST, (IU/L)	24.0 (20.0 - 28.7)	31.0 (22.0 - 41.7)[c]	45.0 (29.2 - 57.2)[a,d]	**<0.0001**	0 – 35.0
ALT, (IU/L)	22.0 (18.0 - 28.7)	26.0 (16.5 - 36.0)	28.0 (18.2 - 48.7)[c]	**<0.05**	0 – 45.0
LDH, (IU/L)	194.0 (155.0 - 254.0)	270.0 (201.0 - 331.0)	531.5 (389.5 - 706.7)[a,b]	**<0.0001**	0 – 280.0
CPK, (IU/L)	43.5 (28.2 - 73.7)	83.0 (39.5 - 216.7)	124.0 (57.0 - 354.7)[a]	**<0.0001**	0 – 176.0
Ferritin, (ng/mL)	123.5 (65.2 - 281.7)	254.5 (156.5 - 416.5)[a]	522.0 (292.9 - 714.7)[a,b]	**<0.0001**	24.0-336.0 (male); 11.0-307.0 (female)
Troponin, (ng/mL)	3.7 (2.1 - 4.7)	7.9 (5.3 - 11.0)[a]	15.7 (9.1 - 44.9)[a]	**<0.0001**	0 – 0.04
Procalcitonin, (ng/mL)	0.5 (0.1 - 0.9)	0.1 (0.1 - 0.2)[a]	0.2 (0.1 - 0.7)[b]	**<0.0001**	0 - 0.1
CRP, (mg/L)	12.0 (5.9 - 23.5)	65.3 (16.3 - 111.1)[a]	124.2 (88.0 - 168.4)[a,b]	**<0.0001**	0 – 5.0
D-dimer, (mg/L)	639.0 (552.7 - 732.5)	724.5 (601.0 - 953.7)	1080.5 (682.7 - 5086.5)[a,b]	**<0.0001**	0 – 500.0
ESR, (mm/hr)	20.0 (15.5 - 30.7)	39.0 (30.0 - 54.0)[a]	62.5 (52.2 - 85.0)[a,b]	**<0.0001**	15 (male); 20 (female)

Data are presented as *median (IQR)*.

WBC: white blood cell; RBC: red blood cell; BUN: blood urea nitrogen, AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; CPK, creatine phosphokinase; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

[a] $P < 0.001$ vs. Outpatient clinic group.

[b] $P < 0.001$ vs. Clinical ward group.

[c] $P < 0.05$ vs. Outpatient clinic group.

[d] $P < 0.05$ vs. Clinical ward group.

3.3. Genotype and allele frequencies in COVID-19 patients

TLR7 and TLR8 genes are inherited on the X chromosome. Therefore, heterozygous alleles are not expected in males as males are hemizygous for these SNPs. Therefore, only allele frequencies were calculated in males. Moreover, male and female patients were analyzed separately. Table 4 shows the genotype and allele frequencies of TLR7 and TLR8 SNPs in both male and female COVID-19 patients. No significant difference was found between the groups in terms of 3 SNPs in males. On the other hand, in female patients, individuals carrying AG genotype (Clinical ward vs. ICU: OR (95% CI): 4.19 (1.1–15.9); $P = 0.035$) and G allele (Clinical ward vs. ICU: OR (95% CI): 4.27 (1.49–12.28); $P = 0.007$) for TLR8 Met1Val SNP were found at a higher frequency in patients hospitalized in the ICU than in patients followed in the Clinical ward. In terms of the other two SNPs, no significant difference was found between the groups in female patients.

Most of our patients had at least one comorbidity such as cardiovascular diseases (CVD), diabetes, obesity, hypertension, smoking, and pregnancy. Therefore, by comparing COVID-19 patients has at least one comorbidity and patients without comorbidity, we examined whether TLR7 and TLR8 SNPs increase the susceptibility to comorbidities of patients. We found no significant difference between the groups in terms of 3 SNPs in females. Whereas, in male patients, we found that individuals who have A allele of TLR7 rs179009 SNP was at a higher frequency in patients who have at least one comorbidity than in patients who have no comorbidity (OR; 95% CI: 5.1; 1.1–24.9); $P = 0.04$). For the other two SNPs, there was no significant difference in male patients (Table 5).

4. Discussion

The fact that it is still unclear which immune cells play a role in the detection of SARS-CoV-2 and initiating the inflammatory response encourages researchers to shed light on this issue. The severity of COVID-19 varies widely among individuals.[24] Therefore, a great deal of effort is required to understand why some people develop a mild course of disease and others require hospitalization. It is critical to elucidate the molecular mechanisms that drive the acute antiviral and inflammatory response to SARS-CoV-2 infection in order to develop treatments for severe COVID-19 disease.

In the current study, it was observed that WBC, BUN, AST, ALT, LDH, CPK, ferritin, troponin, CRP, D-Dimer, ESR values increased depending on the severity of the disease, while the values of RBC, hemoglobin, and hematocrit decreased depending on the severity of the disease. A

Table 4. Genotype and allele frequencies of TLR7 and TLR8 single nucleotide gene polymorphisms (SNPs) in COVID-19 patients (N = 120)

SNP	Genotype/ allele	Group 1 outpatient clinic (n = 40)	Group 2 clinical ward (n = 40)	Group 3 intensive care unit (n = 40)	OR (95%CI)[a]	*P-value*[a]	OR (95%CI)[b]	*P-value*[b]	OR (95%CI)[c]	*P-value*[c]
TLR7 (rs179009)	**Female**									
	AA	17 (73.9)	11 (64.7)	13 (65.1)	1		1		1	
	AG	5 (21.7)	5 (29.4)	5 (23.8)	1.54 (0.36-6.6)	>0.05	0.84 (0.19-3.7)	>0.05	1.3 (0.31-5.48)	>0.05
	GG	1 (4.3)	1 (5.9)	5 (11.1)	1.54 (0.08-27.35)	>0.05	4.23 (0.42-41.87)	>0.05	6.53 (0.67-62.98)	>0.05
	AG + GG	6 (26.1)	6 (35.3)	10 (34.9)	1.54 (0.39-6.03)	>0.05	1.41 (0.38-5.13)	>0.05	2.17 (0.62-7.55)	>0.05
	A	39 (84.8)	27 (79.4)	31 (67.4)	1		1		1	
	G	7 (15.2)	7 (20.6)	15 (32.6)	1.44 (0.45-4.59)	>0.05	1.86 (0.66-5.25)	>0.05	2.69 (0.97-7.42)	>0.05
	Male									
	A	13 (76.5)	22 (95.7)	14 (82.4)	1		1		1	
	G	4 (23.5)	1 (4.3)	3 (17.6)	0.14 (0.01-1.46)	>0.05	4.71 (0.44-49.94=)	>0.05	0.69 (0.13-3.72)	>0.05
TLR8-129 C/G (rs3764879)	**Female**									
	CC	8 (34.8)	10 (58.8)	14 (60.9)	1		1		1	
	CG	10 (43.5)	5 (29.4)	6 (26.1)	0.4 (0.09-1.65)	>0.05	0.85 (0.2-0.6)	>0.05	0.34 (0.09-1.3)	>0.05
	GG	5 (21.7)	2 (11.8)	3 (13)	0.32 (0.04-2.1)	>0.05	1.07 (0.15-7.64)	>0.05	0.34 (0.06-1.82)	>0.05
	CG + GG	15 (67.2)	7 (41.2)	9 (39.1)	0.37 (0.1-1.35)	>0.05	0.91 (0.25-3.29)	>0.05	0.34 (0.1-1.13)	>0.05
	C	26 (56.5)	25 (75.5)	34 (73.9)	1		1		1	
	G	20 (43.5)	9 (24.5)	12 (26.1)	0.46 (0.17-1.22)	>0.05	0.98 (0.35-2.68)	>0.05	0.45 (0.19-1.1)	>0.05
	Male									
	C	8 (47.1)	14 (60.9)	8 (47.1)	1		1		1	
	G	9 (52.9)	9 (39.1)	9 (52.9)	0.57 (0.16-2.03)	>0.05	1.75 (0.49-6.22)	>0.05	1.0 (0.26-3.84)	>0.05
TLR8 Met1Val (rs3764880)	**Female**									
	AA	9 (39.1)	11 (64.7)	7 (30.4)	1		1		1	
	AG	13 (56.5)	6 (35.3)	10 (43.5)	0.37 (0.1-1.39)	>0.05	2.61 (0.65-10.47)	>0.05	0.98 (0.27-3.58)	>0.05
	GG	1 (4.3)	0 (0)	6 (26.1)	NA		NA		7.71 (0.74-79.77)	>0.05
	AA + AG	14 (60.9)	6 (35.3)	16 (69.6)	0.35 (0.09-1.28)	>0.05	4.19 (1.1-15.9)	**0.035**	1.46 (0.43-4.98)	>0.05
	A	31 (67.4)	28 (82.4)	24 (52.2)	1		1		1	
	G	15 (32.6)	6 (17.6)	22 (47.8)	0.44 (0.15-1.29)	>0.05	4.27 (1.49-12.28)	**0.007**	1.89 (0.81-4.41)	>0.05
	Male									
	A	9 (52.9)	14 (60.9)	8 (47.1)	1		1		1	
	G	8 (47.1)	9 (39.1)	9 (52.9)	0.72 (0.2-2.57)	>0.05	1.75 (0.49-6.22)	>0.05	1.26 (0.32-4.86)	>0.05

Data are presented as *n* (%) of column. Chi-square and Fisher's exact tests were used for statistical analyses.
Patient Groups were compared with each other.
NA, not applicable.
[a]Group 1 vs. Group 2.
[b]Group 2 vs. Group 3.
[c]Group 1 vs. Group 3.

Table 5. Genotype and allele frequencies of TLR7 and TLR8 SNPs in COVID-19 patients associated with at least one comorbid disease (N = 120)

SNP	Gender	Genotype/ allele	Comorbidity		OR: (95% CI)	*p*-value
			Yes (n = 88)	No (n = 32)		
TLR7 (rs179009)	**Female**	AA	29 (67.4)	12 (60.0)	–	–
		AG	9 (20.9)	6 (30.0)	0.62 (0.18-2.12)	0.44
		GG	5 (11.6)	2 (10.0)	1.03 (0.17-6.08)	0.97
		AG + GG	14 (32.6)	8 (40.0)	0.72 (0.24-2.17)	0.56
		A	67 (77.9)	30 (75.0)	–	–
		G	19 (22.1)	10 (25.0)	0.85 (0.35-2.04)	0.71
	Male	A	41 (91.1)	8 (66.7)	–	
		G	4 (8.9)	4 (33.3)	5.1 (1.1-24.9)	**0.04**
TLR8-129 C/G (rs3764879)	**Female**	CC	24 (55.8)	8 (40.0)	–	–
		CG	14 (32.6)	7 (35.0)	0.66 (0.19-2.23)	0.51
		GG	5 (11.6)	5 (25.0)	0.33 (0.07-1.45)	0.14
		CG + GG	19 (44.2)	12 (60.0)	0.52 (0.17-1.55)	0.24
		C	62 (72.1)	23 (57.5)	–	–
		G	24 (27.9)	17 (42.5)	0.52 (0.23-1.14)	0.1
	Male	C	23 (51.1)	7 (58.3)	–	–
		G	22 (48.9)	5 (41.7)	1.33 (0.36-4.85)	0.65
TLR8 Met1Val (rs3764880)	**Female**	AA	19 (44.2)	8 (40.0)	–	–
		AG	19 (44.2)	10 (50.0)	0.8 (0.25-2.46)	0.69
		GG	5 (11.6)	2 (10.0)	1.05 (0.16-6.6)	0.95
		AA + AG	24 (55.8)	12 (60.0)	0.84 (0.28-2.47)	0.75
		A	57 (66.3)	26 (65.0)	–	–
		G	29 (33.7)	14 (350)	0.94 (0.42-2.07)	0.88
	Male	A	24 (53.3)	7 (58.3)	–	–
		G	21 (46.7)	5 (41.7)	1.22 (0.33-4.44)	0.75

Data are presented as *n* (%) of column. TLR: Toll-like receptor; SNP: single nucleotide polymorphism.

meta-analysis of seven studies with 1905 patients showed that increased CRP, lymphopenia, and increased LDH were significantly associated with COVID-19 disease severity.[25] Furthermore, elevations in D-dimer, CRP, LDH, troponin, ferritin, CPK and decrease in absolute lymphocyte count are seen in severe COVID-19 patients.[26] Although these laboratory findings are related to disease severity in COVID-19 patients, it has not been clearly demonstrated whether these values have prognostic value and therefore more comprehensive studies are needed.

In the present study, we found no significant difference among the 3 groups in terms of 3 SNPs in males. However, AG genotype and G allele frequencies of TLR8 rs3764880 (Met1Val) SNP in female patients were found to be higher in the ICU patients than in the Clinical ward patients. The results of this study suggest COVID-19 patients with the AG genotype and the G allele are more likely to be admitted into the ICU. However, we found no significant difference between the groups for the other two SNPs in female patients (Table 4).

Genetic and non-genetic factors interplay between the virus and the host is of major importance in determining the severity of the COVID-19 disease outcome.[27] The role various polymorphic forms of TLR7 and 8 as well as association of different infectious diseases have been reviewed.[28] In a review published in recent years, the potential roles of different TLRs

such as TLR2, 3, 4, 6, 7, 8 and 10 in eliminating SARS-COV-2 infection have been reviewed extensively.[10] Moreover, it has been reported that TLR3, TLR7, TLR8, and TLR9 expressions were upregulated in the nasopharyngeal epithelial cells from COVID-19 patients.[5] In another recent study, it has been shown that the level of antiphospholipid antibodies increased in severe COVID-19 patients is recognized by TLR7 and TLR8.[29,30] On the other hand, In the light of developments in the disease biology of COVID-19, TLRs are considered a suitable candidate to develop a therapeutic strategy against COVID-19 because they interact with SARS-CoV-2.[31,32]

Inheritance of TLR7 and TLR8 genes on the X chromosome provides a great advantage for the immune system of female patients.[33] However, there are some studies reporting that males are adversely affected by especially TLR7 mutations. For instance, it has been reported that loss-of-function mutations in TLR7 in fatal males in COVID-19 disease.[34] Thus, to perform genetic screening for TLR7 gene variants in severely affected male COVID-19 patients was recommended.[35,36]

To the best of our knowledge, SNPs of the TLR7 rs179009 and TLR8-129C/G (rs3764879) and TLR8 Met1Val (rs3764880) have so far not been studied, and a few studies are available which conducted in other viral infections in literature. Wang et al.[37] found that higher G allele of TLR7 rs179009 SNP and C allele of TLR8 rs3764879 in male patients with chronic Hepatitis C Virus (HCV) infection than the control subjects. But, they found no associations between chronic HCV infection and TLR7 and TLR8 SNPs among female patients. Another recent study found that the A allele of TLR8 rs3764880 was a risk allele for the development of chronic HCV infection in both sexes, while the C allele of TLR8 rs3764879 was a significant protective effect for only males,[38] The presence of SNP on TLR8 rs3764880 can confer a significant protective effect on HIV disease progression. Further, SNP on TLR8 rs3764880 modulates HIV-1 infection and HIV-1 viral load in male patients and SNP on TLR8 rs3764879 provides resistance to HIV-1 infection in both men and women.[39] In a tick-borne viral infection, Crimean-Congo hemorrhagic fever, the SNPs on both TLR8-129C/G (rs3764879) and TLR8 Met1Val (rs3764880) can predispose Turkish individuals to the illness.[40]

Different researchers examined different TLR7 and TLR8 SNPs in COVID-19. A study performed in the Egypt suggests that the GG genotype of the rs3853839 SNP in the TLR7 gene may increase susceptibility to COVID-19, disease severity, and poor outcome. The authors state that the mRNA expression of TLR7 is upregulated in COVID-19 patients with severe/critically ill and poor disease outcome. Moreover, they found that high serum IL-6, CRP and ferritin levels and, low number of WBC as

the prognostic biomarkers for COVID-19.[41] Another research group in Egypt investigated whether TLR3 and TLR7 SNPs predispose to COVID-19 and they found that TLR3 rs3775290 and TLR7 rs179008 SNPs might be an important risk factor for COVID-19 without efficacy on disease outcome. They also observed that male gender, low SpO2%, high levels of INR and LDH and lymphopenia were the independent predictive biomarkers for COVID-19 mortality.[42] A recent study reported that rs5744080 and rs2159377 SNPs on TLR8 gene had no detrimental effect on symptoms of COVID-19 patients who had moderate to severe respiratory symptoms.[43] The interferon (IFN) response is crucial against invading viruses,[44] and rare variants in TLR7 in severe COVID-19 patients cause a down-regulation in genes related with cytokine-mediated signal transduction of IFN-alpha and IFN-beta.[45] Because of the SNPs examined in the above-mentioned studies were distinct from ours, it is likely that there would be discrepancies between study findings.

Comorbid diseases such as CVD, diabetes, obesity and hypertension in COVID-19 are factors that highly associated with significant morbidity and mortality.[46] Therefore, in our study, patients with at least one comorbidity were compared with patients with no comorbidities. In male patients, individuals with TLR7 rs179009 A allele had a higher frequency in COVID-19 patients with comorbidity. In the presence of comorbidity in male COVID-19 patients, TLR7 rs179009 SNP can be considered as a risk factor that increases susceptibility to the disease. However, more studies are required to demonstrate the role of TLR7 rs179009 SNP in COVID-19 patients.

The main limitation of our study is relatively small sample size. However, by investigating the effect of polymorphic variants in TLR7 and 8 genes in covid19 patients of different severity, we provide an idea about possible individual differences in the immune response of TLRs to COVID19. Studies with larger sample sizes are needed to show the effect of TLR7 and TLR8 gene variations in patients with COVID19.

In conclusion, the results of this study show that AG genotype and G allele of TLR8 rs3764880 SNP in female patients can be evaluated as a risk factor with the potential to increase the severity of COVID-19 disease. Furthermore, the A allele of TLR7 rs179009 SNP was found at a higher frequency in male COVID-19 patients in the presence of comorbid diseases. Further and more comprehensive studies are needed to clarify the possible relevance of TLR7 and TLR8 SNPs to COVID-19 disease prognosis.

Disclosure statement

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Ethics statements

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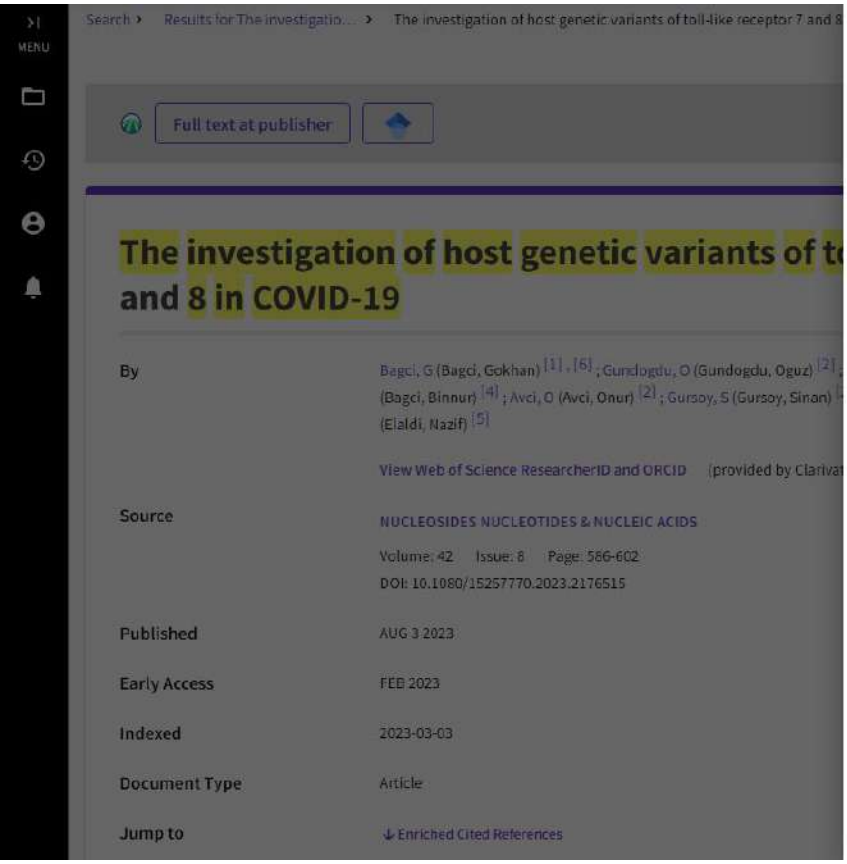

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