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Toll-like Receptor 3 c.1377C/T and -7C/A Polymorphisms Associated with COVID-19 and COVID-19 Severity

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ABSTRACT

Chinese officials have reported the novel coronavirus to the world health organization, which is called the SARS-CoV-2. Toll-like receptor 3 (TLR3) induces antiviral immune responses via the production of type I interferons and inflammatory cytokines. In this study, we aimed to examine TLR3 c.1377C/T and -7C/A polymorphisms in COVID-19 and the association between some clinical parameters. We investigated the frequencies of TLR3 (c.1377C/T and -7C/A) polymorphisms in 150 patients with COVID-19 and 171 healthy individuals as controls. We performed polymerase chain reaction (PCR) based on restriction fragment length polymorphism (RFLP). We also investigated whether TLR3 c.1377C/T and-7C/A were associated with the severity of COVID-19. In addition, CHAID tree-based classification algorithm was created to investigate the severity of the patients in our study. TLR3 c.1377C/T TT genotype frequencies were statistically significant between cases and controls (p= 0.02). For TLR3 -7C/A polymorphism, the findings showed a statistically significant difference in A allele frequencies (p= 0.03). There was a statistically significant difference in the distribution of TLR3 -7C/A CA genotype frequency (p= 0.04). Our findings suggest that TLR3 c.1377C/T and -7C/A polymorphisms may be important on susceptibility or clinical course of COVID-19.

Keywords: COVID-19, Genetic polymorphism, Severity, Toll like receptor 3, CHAID tree-based classification algorithm.

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Introduction

Coronaviruses (CoVs) have been known for many years as enveloped viruses with single-stranded RNA genomes ranging from 26 to 32 kb that may cause diseases in domestic and wild animals and humans [1]. CoVs belong to the order-Nidovirus, family-Coronaviridae, subfamily-Coronavineae [2]. In December 2019, the novel coronavirus, which was identified as SARS-CoV-2, caused pneumonia was reported to the world health organization (WHO) [3] and is named COVID-19. In February 2022, it was reported that the number of cases worldwide exceeded 424 million, and the total number of deaths approached six million [4]. Several publications have reported a high incidence of coagulation abnormalities in these patients [5]. Lymphopenia, leukocytosis, neutrophilia, thrombocytopenia, d-dimer height, c-reactive protein height, prothrombin time height, troponin increase and lactate dehydrogenase (LDH) height are the most commonly defined haematological parameters in COVID-19 [6]. The complete genome of the Wuhan-Hu-1 coronavirus, a strain of SARS-CoV-2 isolated from a COVID-19 pneumonia patient, is 29.9 kb and has a poly(A) tail at the 3' end; it has a capped structure at the 5' end [7]. Two-thirds of viral RNA encodes pp1a, pp1ab proteins, and 16 non-structural proteins, while the remaining open reading frames encode and structural and accessory proteins. The remainder of the virus genome encodes four major structural proteins, including spike glycoprotein (S), an envelope protein (E), matrix protein (M) and nucleocapsid protein (N) [8]. During transmission, virus penetrates into the cell using angiotensin-converting enzyme 2 (ACE2) [9]. ACE2 is the cell receptor for coronavirus and regulates both interspecies and human-to-human transmission [10]. The S proteins of the SARS-CoV-2 can bind to host cells with ACE2 by fusing to the membrane and releasing viral RNA. Viral RNAs are recognized by pattern recognition receptors (PRRs) as pathogen-linked molecular patterns (PAMP). TLR3, 7, 8 and 9 detect viral RNA and DNA in the endosome [11]. Until now, TLR1-TLR13 has been detected and characterized; TLR 1-9 are expressed in mice and humans, while TLR10–13 are only expressed in mice [12]. Some TLRs, such as TLR1, 2, 4, 5, 6 and 10, are expressed on the cell surface, while TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are expressed in the cell, especially in endosomes, lysosomes, and endolysosomes. TLR3 is determined in both intracellular places and the plasma membrane of human astrocytes [13]. TLR3 is nucleotide-sensing and located on chromosome 4q35 [14]. TLR3 is expressed in many cell types, such as macrophages, mast cells, natural killer cells, fibroblasts, endothelial and epithelial cells, myeloid dendritic cells, neuronal cells and astrocytes [15]. TLR3 is considered a very important receptor that recognizes

negative sense double-stranded RNA (dsRNA) from pathogenic virus [16]. In recent years, TLR3 polymorphisms have been studied in a wide range of infectious diseases, such as Hepatitis C infection [17], HPV infection [18] and Crimean Congo hemorrhagic fever disease (CCHF) [19]. However, to our knowledge, until now, TLR3 c.1377C/T and -7C/A polymorphism have not been studied in COVID-19. Thus, we aimed to investigate the frequencies of these polymorphisms and whether they were associated with the severity of COVID-19 in the present study.

Materials and Methods

We investigated association TLR3 c.1377C/T (rs3775290) and -7C/A (rs3775296) polymorphisms with SARS-CoV-2 infection in this study. Blood samples were collected from 150 COVID-19 patients. This study was approved by Sivas Cumhuriyet University Clinical Research Ethic Committee (Desicion No: 2021-02/07). Control group was composed of 171 healthy individuals whose blood had been taken during the absence of the COVID-19 outbreak (Ethic Committe Desicion No: 2009-02/5). The informed consent forms were taken from all volunteers. Firstly, DNA was extracted from blood samples of COVID-19 patients. We performed PCR-based RFLP for genotyping rs3775290 and rs3775296. The patients had no other infections and chronic disease according to hematological, biochemical and serological laboratory findings. Cases were classified as severe and non-severe in terms of severity of the disease. COVID-19 patients hospitalized in intensive unit care and asymptomatic were severe and non-severe, respectively. Healthy volunteers had no disease complaints in anamnesis, as well as their examination was normal.

TLR3 c.1377C/T and -7C/A Genotyping

Genomic DNA was extracted from blood samples in EDTA containing tubes using phenol-chloroform method. PCR-RFLP method was used for determining genotype of TLRs. In a thermal cycler (BIORAD T100); PCR was performed in a total volume of 25 ml including 1 mM of each deoxynucleotide triphosphates (dCTP, dATP, dTTP and dGTP), approximately 100 ng DNA, 10X PCR buffer (A.B.T.TM cat.: E01-01-50), 0.2 mM each of primers (Table 1), 2.5 U/µL Taq DNA polymerase (A.B.T.TM lot: W911-A911) and 1.5 mM MgCl2. PCR reaction conditions for the 35-cycle amplification were as follows: initial denaturation at 94°C for 5 minute, denaturation at 94°C for 30 second, annealing at appropriate temperature of primers in Table 1 for 30 second and extension at 72°C for 1 minute, and final extension at 72°C for 5 minute. PCR products (5mL) were visualized in a 2% agarose gel. Amplification products were cut with restriction enzymes (NEB) in the Table 1 overnight.

Table 1. Experimental conditions for genotyping of the SNPs by RFLP

Gene polymophism	SNP	Primers	Annealing temp (°C)	Restriction endonuclease
TLR3 c.1377C/T	rs3775290	5'-CCAGGCATAAAAAGCAATATG-3' 5'-GGACCAAGGCAAAGGAGTTC-3'	52	TaqI
-7C/A	rs3775296	5'-GCATTTGAAAGCCATCTGCT-3' 5'-AAGTTGGCGGCTGGTAATCT-3'	52	MboII

SNP: Single nucleotide polymorphism; temp: Temperature

The fragments and undigested products were separated with 4% agarose gel electrophoresis and observed after stained with ethidium bromide (Figure 1).

In addition, ABI PRISM 377 automatic sequencer (Applied Biosystems, Foster City, CA) was used to verify sequence of three types (homozygous wild, heterozygous and homozygous mutant) of TLRs.

Statistical Analysis

All statistical analyses were carried out using the SPSS version 25. Statistical significance of the differences in TLR3 alleles and genotypes of all groups were calculated by Pearson's chi-squared test (Table 2). Genotype and allele frequency differences were considered significant when p-values were ≤0.05. One-way ANOVA was used to compare different genotypes in terms of clinical parameters for TLR3 c.1377C/T and -7C/A polymorphisms. Tukey test was used for lettering the groups. In addition, CHAID tree-based classification algorithm was created to determine the severity of the patients in our study (Fig. 2).

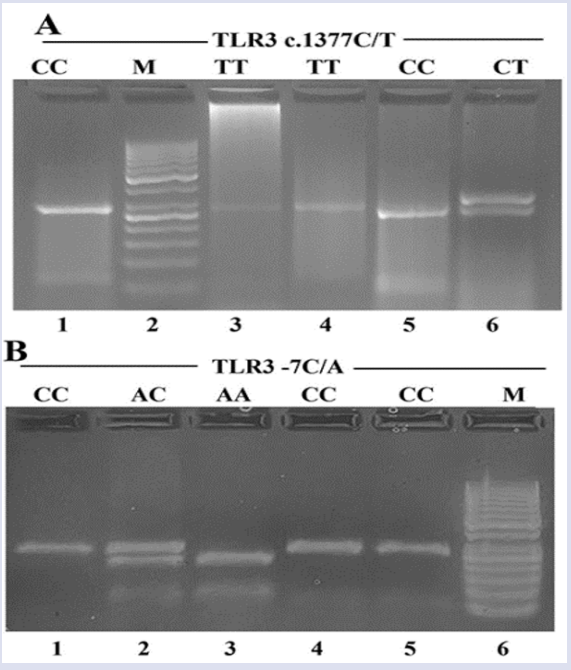

Figure 1: PCR-RFLP analysis of TLR3 c.1377C/T and -7A/C polymorphisms

A Homozygous wild genotype (CC): 275+62 bp (lane 1 and 5), heterozygous genotype (CT): 337+275+62 bp (lane 6), homozygous mutant genotype (TT): 337 bp (lane 3 and 4) for TLR3 c.1377C/T. B Homozygous wild genotype (CC): 279 bp (lane 1, 4 and 5), heterozygous genotype (CA): 279+207+72 bp (lane 2), homozygous mutant genotype (AA): 207+72 bp (lane 3) for TLR3 -7A/C. Bp: base pair, CI: Confidence interval, M: molecular weight marker (50 bp DNA ladder, Fermentas), OR: Odds ratio, PCR-RFLP: Polymerase chain reaction based restriction fragment length polymorphism. Two replicates were made all experiments

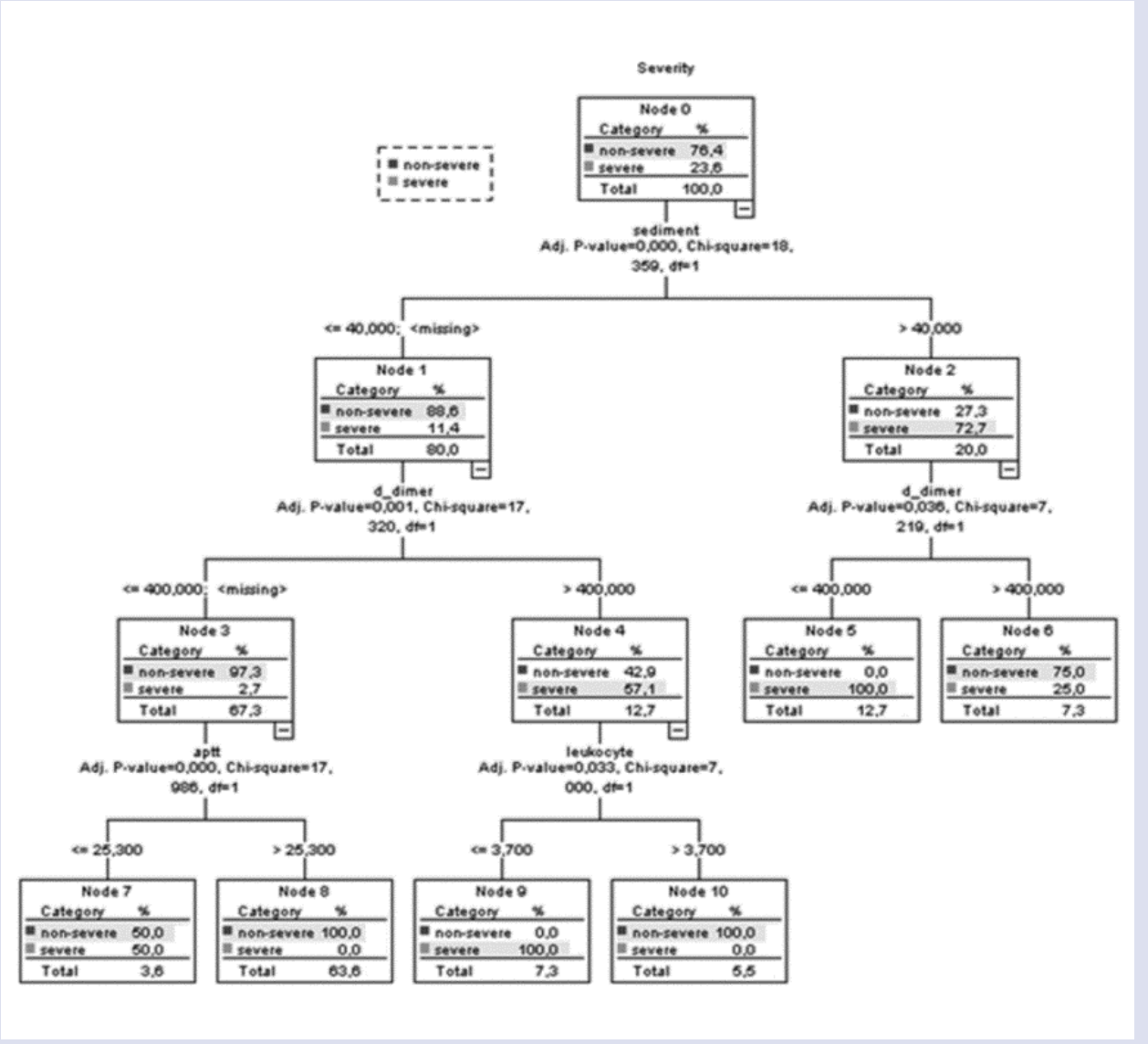

Figure 2 Determination severity of COVID-19 patients using CHAID tree-based classification algorithm Adj. p-value: Adjusted p value; activated partial thromboplastin time: Aptt; df: Degree of freedom.

Results

In this study, 150 patients with COVID-19 and 171 healthy individuals were included in our study population for TLR3 c.1377C/T (rs3775290) and -7C/A (rs3775296) polymorphisms. We compared allele and genotype frequencies of study population regarding disease severity (not demonstrated in Table). We found a statistically significant difference in TT genotype distribution between patients with COVID-19 and healthy controls (p= 0.02, OR= 2.80) in TLR3 c.1377C/T polymorphism (Table 2). There was a statistically significant difference in distribution of CA genotype (p= 0.04) and allele frequencies (p= 0.03) (Table 2) between two groups in TLR 3 -7C/A polymorphism. We also compared allele and genotype frequencies of patients with COVID-19 regarding disease severity for TLR3 c.1377C/T and -7C/A. There was no statistically significant difference between severe and non-severe groups in these polymorphisms (p> 0.05). In addition, we compared genotype and allele frequencies of case and controls in terms of gender. For TLR3 c.1377C/T, we found a statistically significant difference in male patients with TT genotype compared to control individuals (p= 0.04). We found 3.92 times higher risk in males with

TT genotype (Table 2). For TLR3 -7A/C, there was statistically significant difference in distribution A allele of females with COVID-19 (p= 0.05, OR= 0.55). The data of the present study are summarized in Table 2.

Table 2. Risk estimates and frequencies of allele and genotypes for TLR3 (c.1377C/T and -7C/A) polymorphisms in COVID-19 patients and healthy controls.

TLR3 polymorphism	Case, n (%)	Control, n (%)	P value	OR (95%CI)
c.1377C/T				
Allele				
C	203 (67.67)	248 (72.51)		
T	97 (32.33)	94 (27.49)	0.18	1.26 (0.90-1.77)
Genotype				
CC	72 (48.33)	85 (49.71)		
CT	59 (39.17)	78 (45.61)	0.63	0.89 (0.56-1.42)
TT	19 (12.5)	8 (4.68)	0.02*	2.80 (1.16-6.79)
Female				
C	118 (71.95)	154 (72.64)		
T	46 (28.05)	58 (27.36)	0.85	1.05 (0.66-1.65)
CC	44 (54.32)	53 (50)		
CT	30 (37.04)	48 (45.28)	0.40	0.77 (0.42-1.41)
TT	7 (8.64)	5 (4.72)	0.27	1.93 (0.59-6.31)
Male				
C	87 (63.04)	93 (71.54)		
T	51 (36.96)	37 (28.46)	0.14	1.47 (0.88-2.47)
CC	29 (42.03)	31 (47.69)		
CT	29 (42.03)	31 (47.69)	1.00	1.0 (0.49-2.05)
TT	11 (15.94)	3 (4.62)	0.04*	3.92 (0.99-15.48)
-7C/A				
Allele				
C	265 (88.33)	282 (82.46)		
A	35 (11.67)	60 (17.54)	0.03*	0.62 (0.40-0.97)
Genotype				
CC	117 (77.61)	115 (67.25)		
CA	31 (20.90)	52 (30.41)	0.04*	0.59 (0.35-0.98)
AA	2 (1.49)	4 (2.34)	0.68	0.49 (0.09-2.74)
Female				
C	152 (89.41)	153 (82.26)		
A	18 (10.59)	33 (17.74)	0.05*	0.55 (0.30-1.02)
CC	68 (80)	62 (66.67)		
CA	16 (18.82)	29 (31.18)	0.05*	0.50 (0.25-1.01)
AA	1 (1.18)	2 (2.15)	0.61	0.46 (0.40-5.15)
Male				
C	112 (86.15)	129 (82.69)		
A	18 (13.85)	27 (17.31)	0.42	0.77 (0.40-1.47)
CC	48 (73.85)	53 (67.95)		
CA	16 (24.61)	23 (29.49)	0.49	0.77 (0.36-1.62)
AA	1 (1.54)	2 (2.56)	1.00	0.55 (0.50-6.28)

*: p≤0.05; n: individual number; OR: Odds ratio; p: Significant value.

As a result of the ANOVA test for TLR3 c.1377C/T, we observed that platelet, sediment, d-dimer, LDH and alanine aminotransferase (ALT) parameters were statistically significant in CT and TT genotypes (p< 0.05) (Table 3). Tukey test was used to investigate the differences of clinical parameters between the genotypes. Platelet, sediment, d-dimer, LDH and ALT levels were statistically significant in individuals with CT and TT genotypes according to the Tukey test results. The findings obtained in this study showed that platelet level was statistically significant different in individuals with CT genotype. In addition, we observed that sediment (p= 0.02) and d-dimer (p= 0.04) were statistically significant in CC, CA and AA genotypes for TLR3 -7A/C. Individuals with CC, CA and AA genotypes showed statistically significant difference regarding sediment and d-dimer (Table 3).

In this study, a decision tree based on the CHAID tree-based classification algorithm was created to determine the severity of the patients with COVID-19 (Figure 2). We found that sediment, d-dimer, leukocyte and activated partial thromboplastin time (aptt) variables were important predictors in determining the severity of patients (Adj p= 0.00). The findings showed that 76.4% of the patients were non-severe and 23.6% were severe patients according to the root node (Node 0), which is at the top of the classification tree diagram. Sediment showed a more significant effect on the severity of patients than the other variables found in the classification tree (Adj p=0.000; Chi-square=18.359; df1=1) (Figure 2).

Table 3. Association between genotype and clinical parameters of COVID-19 patients

TLR3 c.1377C/T Genotype	Platelet (mean ±std. error)	Sediment (mean ±std. error)	D-dimer (mean ±std. error)	LDH (mean ±std. error)	ALT (mean ±std. error)
CC	206.59 ± 9.38b	31.92 ± 3.11a	638.86 ± 149.07a	269 ± 14.18a	19.82 ± 1.73b
CT	244.92 ± 12.51a	24.40 ± 3.38b	269.27 ± 60.74b	221.65 ± 12.56b	24.41 ± 2.54a
TT	223.94 ± 11.87b	20.07 ± 2.56b	223.33 ± 57.86b	239.06 ± 16.25b	29 ± 4.26a
Sig.	0.03*	0.04*	0.04*	0.04*	0.04*
TLR3 -7C/A Genotype					
CC	217.22 ± 7.4	31.25 ± 2.41a	553.47 ± 105.12a	253.70 ± 10.67	23.03 ± 1.76
CA	234.09 ± 14.9	19.55 ± 2.99c	152.68 ± 21.26c	235.60 ± 14.25	23.90 ± 3.04
AA	222 ± 0.00	27.05 ± 0.05b	431 ± 0.00b	246 ± 0.00	23 ± 0.00
Sig.	0.53	0.02*	0.04*	0.64	0.96

*: $p \leq 0.05$; [a, b, c] Mean values with different symbols in the same column differ from each other ($p<0.05$). ALT: Alanine aminotransferase; LDH: Lactate dehidrogenase; Sig.: Significant value; std. error: Standard error

Also, descriptive statistics for d-dimer, aptt and leukocyte were shown in the classification tree (Figure 2). Statistically insignificant clinical parameters were removed from the classification tree.

Discussion

In the last 20 years, there have been pandemics worldwide that have been exposed to important coronavirus strains outbreaks. These outbreaks have been most often caused by severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV). The SARS-CoV-2 has caused the COVID-19 outbreak and continues to be effective worldwide. Today, genetic polymorphisms are efficient in pathways that demonstrate a crucial role in the binding of microbiological agents to the host cell, host disease resistance, disease susceptibility and severity. There are few studies in the literature on COVID-19 and gene polymorphisms. Genetic variations in the TLR pathway contribute to resistance or susceptibility to various infections. Thus, in this study, we examined the effects of TLR3 c.1377C/T (rs3775290) and TLR3 -7C/A (rs3775296) polymorphisms on COVID-19 infection.

TLR3, TLR7, TLR8 and TLR9 recognize viral pathogen-associated particles, and these molecules are called antiviral TLRs. Such TLRs have a major role in reducing viral infection, causing a decrease in disease severity. Any mutation or SNPs in the TLR pathway may cause impairment in signal transduction, and this impairment may cause recurrence of viral infections [20]. In this study, there was no statistically significant difference in TLR3 c.1377C/T T allele frequencies between the case and control group (p= 0.18). We found that T allele frequency was 27.49% (Table 1). The T allele frequency of healthy control ranges from 19% to 38% in different populations. T allele frequency was 33.7% in Chinese Han population [21]; 35.66% in Eastern Indian population [22]; 25.5% in Egyptian population [23]. We found a significant difference in TT genotype between COVID-19 cases and controls (p= 0.02; Table 1). We also found that individuals with TT genotype had approximately three times greater risk than individuals with CC genotype for COVID-19 (OR= 2.80; Table 2). In a recent study, this polymorphism was investigated in COVID-19 disease. Consistent with our results, the TT genotype was found to be statistically significant between patient and control group [24].TLR3 c.1377C/T polymorphism has been associated with another viral disease as our present study result. One of them, an association analysis by Huang (2015), showed that the TT genotype of TLR3 c.1377C/T polymorphism was related to decreased risk for chronic hepatitis B, HBV-related liver cirrhosis, and HBV-related hepatocellular carcinoma diseases [25]. In another study, frequency of polymorphic genotype TLR3 c.1377C/T TT was not significantly different between hepatitis C virus (HCV) infection-positive patient and control group, whereas TLR-3 c.1377 T allele was found to be associated with advanced hepatic fibrosis stage [23]. Engin et al. found that the homozygous mutant genotype (TT) frequency of TLR3 c.1377C/T in patients with CCHF was significantly higher than that of the controls [19]. A study among patients with DENV-CHIKV co-infection and CHIKV mono-infection revealed that patients with the TLR3 rs3775290 TT genotype exhibited a significant susceptibility to co-infection [22]. Mosaad et al. (2019) found that a significant higher frequency was found for the CT genotype of TLR3 rs3775290 in chronic HCV infection [26].

In humans, the TLR3 promoter region maintains promoter integrity and promoter-specific virus responsive elements. It has been suggested that promoter polymorphisms, such as TLR3 -7C/A, may cause transcriptional regulation of TLR3 and alter gene expression in response to inflammatory cytokines [27]. TLR3s have the ability to initiate a signaling cascade that activates type 1 interferons and inflammatory cytokines [28]. Thus, they trigger the initiation of the immune response against both DNA and RNA viruses. When the human body is exposed to a viral infection, interferons are induced by the TLR3/TRIF pathway within a few hours. TLR3 identifies dsRNA and viral infection causes dsRNA generation either as a replication intermediate for ssRNA viruses or as a by-product of symmetrical transcription in DNA viruses. Since dsRNA is a universal viral pathogen-associated molecule, TLR3 may have an effective role in

antiviral immunity against both DNA and RNA viruses [29]. In our study, we have also investigated TLR3 -7C/A promoter polymorphism in COVID-19 infection. There was a significant difference TLR3 -7C/A A allele frequency between case and control group (p= 0.03; Table 2) We have found that mutant A allele frequency was 17.54% in the control group (Table 2). A allele frequency ranged from 0.00% to 27% in different population. A allele frequency was 26.7% in Iran population [30]; 10% in Egyptian population [22]; 21.5% in Chinese population [31]; 0.00% in Cyprus population [32]. We found that CA genotype frequency was significantly different between the case and control (p= 0.04; Table 2). We also found that individuals with CA genotype have approximately 0.6 times greater protective effects than individuals with the CC genotype for COVID-19. However, there was any significant difference in allele and genotype frequencies between severe and non-severe. In a study of CCHF disease, there was no significant difference in distribution of TLR3 -7C/A genotype and allele frequencies [19]. In another study, frequency of polymorphic genotypes in TLR3 -7C/A were not significantly different between studied HCV-positive patients and controls. Consistent with the findings obtained in the present study, Deeba et al. (2019) reported that the distribution of polymorphic TLR3 -7C/A A allele showed a statistical significance in natural killer cells [31]. There was also a significant difference in the frequency distribution of TLR3 -7C/A CA heterozygous genotypes and mutant A alleles in the human T-lymphotropic virus type 1 (HTLV-1) disease like our result. Habibabdi et al. (2020) reported that these observations might indicate a protective factor to prevent HTLV-1 infection for the Iranian population [30]. Similar to the findings obtained in Habibabdi et al.'s (2020) study, we observed CA genotype and mutant A allele might have a protective role for COVID-19 infection (OR= 0.59 and OR= 0.62, respectively; Table 2).

Patients with COVID-19 presented with higher coagulatory potential [5]. Liver biochemical parameters like aspartate aminotransferase (AST), ALT and LDH were strongly correlated with COVID-19 mortality. Non-survivors had higher levels of AST, ALT and LDH [33]. In this study, we have also analyzed the relationship between allele-genotype distribution and clinical parameters that affects disease severity. We found that patients with COVID-19 with CC genotype had the highest sediment, d-dimer, LDH level regarding two polymorphisms. Patients with CT genotype had the highest platelet level, whereas patients with TT genotype had the highest ALT level for TLR3 c.1377C/T (Table 3). In addition, individuals with CA genotype had the highest platelet and ALT level for TLR3-7C/A (Table 3).

Gender differences may exist in patients with COVID-19 of severe type. Male patients may have more complicated clinical conditions and worse in-hospital outcomes than women [34]. We found that male patients with TT genotype showed a statistically significant difference for TLR3 c.1377C/T. Also, females with A allele and CA genotype differed statistically significant for TLR3-7C/A.

In conclusion, TLR3 c.1377 TT genotype frequency in patients with COVID-19 was higher than the healthy controls. TLR3 c.1377 TT genotype, especially in males, might increase susceptibility to COVID-19 disease. TLR3 -7C/A A allele and TLR3 -7C/A CA genotype may be protective factors for COVID-19. In addition, clinical parameters like sediment and d-dimer are among the most important factors that may affect COVID-19 severity for TLR3 c.1377C/T and -7C/A polymorphisms.

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Conflict of interest

The authors state that did not have a conflict of interests

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