Tohoku J. Exp. Med., 2023, **261**, 179-185

Impact of Inflammation-Related Genes on COVID-19: Prospective Study at Turkish Cohort

Ahmet Cevdet Ceylan,[1,2] Büşranur Çavdarlı,[2] Gülay Güleç Ceylan,[1,2] Vehap Topçu,[2] S. Betül Arslan Satılmış,[2] Şerife Gökbulut Bektaş,[3] Ayşe K. Kalem,[4,5] Bircan Kayaaslan,[4,5] Fatma Eser,[4,5] Emra Asfuroğlu Kalkan,[6] Osman İnan,[6] İmran Hasanoğlu,[4,5] Selcen Yüksel,[7] İhsan Ateş,[8] Seval İzdeş,[3,9] Rahmet Güner[4,5] and C. Nur Semerci Gündüz[1,2]

[1]Department of Medical Genetics, Faculty of Medicine, Ankara Yıldırım Beyazıt University, Ankara, Turkey
[2]Department of Medical Genetics, Ankara City Hospital, Ankara, Turkey
[3]Department of Anesthesiology and Reanimation-Critical Care, Ankara City Hospital, Ankara, Turkey
[4]Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Ankara Yıldırım Beyazıt University, Ankara, Turkey
[5]Department of Infectious Diseases and Clinical Microbiology, Ankara City Hospital, Ankara, Turkey
[6]Department of Internal Medicine, Ankara City Hospital, Ankara, Turkey
[7]Department of Biostatistics, Ankara Yıldırım Beyazıt University, Ankara, Turkey
[8]Department of Internal Medicine, Ankara City Hospital, Health Science University, Ankara, Turkey
[9]Department of Anesthesiology and Reanimation-Critical Care, Faculty of Medicine, Ankara Yıldırım Beyazıt University, Ankara, Turkey

The pandemic coronavirus disease 2019 (COVID-19) has caused a high mortality rate and poses a significant threat to the population. The disease may progress with mild symptoms or may cause the need for intensive care, depending on many factors. In this study, it was aimed to determine if there is a tendency due to genetic factors in COVID-19 patients. Ninety-four of 188 patients with mild clinical and 94 with severe clinical symptoms were included in the study. The targeted panel including coagulopathy (*F2, F5*), viral invasion (*ACE2*), and inflammation (*CXCL8, IFNAR2, IFNL4, IL10, IL2, IL6, IRF7, TLR3, TLR7, TNF)* related genes was performed sequenced by the next generation sequencing (NGS). The variants found were classified and univariate analyses were performed to select candidate variables for logistic model. Risk factors and variants were compared. It was revealed that the presence of 2 or more risk factors caused the disease to progress severely ($p < 0.001$). Heterozygous *IRF7*:c.1357−23dup variant had a 2.5 times higher risk for mild disease compared to severe disease. Other variants were found to be more significant in mild disease. Since polymorphic variants were not evaluated in the literature, the findings of our study could not be compared with the literature. However, as variants that may be effective in the severity of infections may differ according to ethnicity. This study has the feature of being a guide for subsequent studies to be carried out especially in Turkish population. Clinical course of the COVID-19 is likely to depend on a variety of risk factors, including age, sex, clinical status, immunology and genetic factors.

Keywords: COVID-19; *IRF7*; new variants; polymorphic variants; risks of severe disease
Tohoku J. Exp. Med., 2023 November, **261** (3), 179-185.
doi: 10.1620/tjem.2023.J071

Introduction

Coronaviruses (CoVs) are transmitted to humans through mammalian hosts and cause respiratory system, gastrointestinal system and central nervous system diseases. The new coronavirus started an epidemic in the Wuhan province of China, causing a pandemic that also affected our country at the end of 2019. This coronavirus that

Received June 16, 2023; revised and accepted August 9, 2023; J-STAGE Advance online publication August 25, 2023
Correspondence: C. Nur Semerci Gündüz, M.D., Department of Medical Genetics, Ankara City Hospital, University District 1604. Street No.9, Bilkent, Çankaya, Ankara 06800, Turkey.
e-mail: nsemerci1@yahoo.com

caused the pandemic was named as SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), the disease as coronavirus disease 2019 (COVID-19) (Chakravarty 2021). Since it is able to spread quickly and cause deaths, studies on pathogenesis, treatment and vaccination of the virus have been started rapidly in many centers worldwide, as well as in our country. The severity of the disease ranges from asymptomatic to fatal for the individuals affected by SARS-CoV-2 infection (Guo et al. 2020). It has been known that genetic factors play an substantial role in the emergence of many infectious diseases observed in humans as well as microorganisms (Pairo-Castineira et al. 2021). In some cases, severe mutations in a gene may cause susceptibility to infections, whereas coexistence of mutations in more than one gene may lead to the development of infection or susceptibility to infection (Anastassopoulou et al. 2020; Wang et al. 2020). Advances in genetics have provided understanding of the onset, progression and prognosis of the disease. Thus, it is possible to see which genetic variants create a response against the infectious agent. It has been observed that there is a clinical variability among patients with COVID-19 disease, which has affected more than 500 million people and caused the death of more than 6 million people so far.

In the course of the disease, patients are divided into two groups: those who are generally asymptomatic or recover with mild clinical findings, and those who are required intensive care due to respiratory or multisystem failure (Anastassopoulou et al. 2020). The reason for this clinical variability among patients shows the possibility that differences in human genetic structure may be effective, as well as mutations in the virus (Chakravarty 2021). It has been suggested that *ACE2* receptor gene polymorphisms may be effective in the intracellular entry of the virus by changing the virus affinity to this receptor (Hou et al. 2020; Pairo-Castineira et al. 2021; Flemming 2021). Genes involved in the immune system and coagulopathy pathway may also be effective. In this study, it was aimed to determine if there is a tendency to infectious diseases due to genetic factors in COVID-19 patients.

Methods

Patients

Patients over 18 years old who were evaluated in the Ankara City Hospital Infectious Diseases and Clinical Microbiology Clinic, General Hospital Anaesthesia Intensive Care Service, and Internal Medicine Intensive Care Service were included in the study. Real-time PCR results of all the patients were positive for COVID-19 disease. The patients were grouped as nonsevere and severe/critical according to the World Health Organization interim guidance (Guner et al. 2021). Ninety-four out of 188 patients had mild clinic (nonsevere), 94 had severe clinic. Risk factors of the patients such as hypertension, diabetes mellitus, chronic renal failure, coronary artery disease and chronic lung disease were not taken into account at the patient selection criteria. Ages and sex of the patients were among the inclusion criteria. Demographic findings are shown in Table 1.

Genetic analysis

Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA of patients were extracted by QIAsymphony® automated DNA isolation system (Qiagen Inc., Mississauga, ON, Canada).

A targeted next-generation gene panel was designed based on the literature data. The genes in the panel were related with coagulopathy (*F2, F5*), viral invasion (*ACE2*), and inflammation (*CXCL8, IFNAR2, IFNL4, IL10, IL2, IL6, IRF7, TLR3, TLR7* and *TNF*).

The fragmented DNA was barcoded with unique molecular indices to track the original DNA molecule and provide a highly sensitive detection. Then, targeted genes were amplified with single primer extension technology and bead clean-up step was performed to discard unwanted fragments. The concentration optimization of libraries was performed with Qiaseq Quant Assay Kit (QIAGEN, Hilden, Germany) and all libraries were diluted to 4 nM. Libraries with different sample indexes were combined in equimolar amounts in the final pool. Then, the final pool was sequenced in Miseq System, Illumina (Illumina Inc., San Diego, CA, USA) according to the manufacturer's guide. The secondary analysis of FASTQ files were performed on Qiagen Clinical Insight-Analyse Universal with panel-specific pipeline. The VCF files were clinically interpreted using Qiagen Clinical Insight-Interpret (QIAGEN). Firstly, the pathogenic/likely pathogenic variants were classified according to the ACMG 2015 criteria (Richards et al. 2015). Statistical analysis was performed according to the mildly and severely affected patient groups.

Statistical analysis

First, univariate analyses were performed to select candidate variables for logistic model. $\alpha = 0.25$ was taken while selecting candidate variables. To purify age effect on univariate model results, age variable was taken as covariate for all univariate analyses. Odds ratio (OR) and 95% confidence interval (CI) of the estimates were reported. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA).

Results

A total of 188 patients followed up with the diagnosis of COVID-19, 94 with mild clinics and 94 with severe clinics, were included in our study. The mean age of mild and severe patient groups was 47.35 ± 10.84 (mean ± SD) and 54.59 ± 8.87, respectively. Of these individuals, 122 were male, 66 were female (Table 1). The effect of sex on the severity of the disease was not significant ($p = 0.760$). In addition, risk factors were evaluated. Hypertension was not

Table 1. Demographic and clinical characteristics of the patients.

	Number of mild clinic	Number of severe clinic	p value
Sex (male)	62 (66.0%)	60 (63.8%)	0.760
Sex (female)	32 (34.0%)	34 (36.2%)	
Risk Factors (Co-morbidities)			
HT	27 (28.7%)	38 (40.4%)	0.092
DM	18 (19.1%)	32 (34.0%)	0.021
CRF	0	11 (11.7%)	0.001
CAD	6 (6.4%)	17 (18.1%)	0.014
CLD	3 (3.2%)	11 (11.8%)	0.025
Risk factor (less than < 2)	26 (27.7%)	32 (34.4%)	0.318
Risk factor (more than ≥ 2)	18 (19.1%)	45 (47.9%)	< 0.001

HT, hypertension; DM, diabetes mellitus; CRF, chronic renal failure; CAD, coronary artery disease; CLD, chronic lung disease.

found to be significantly different between the groups ($p = 0.092$). However, diabetes mellitus, chronic kidney disease, coronary artery disease and chronic lung disease were statistically significantly higher in the severe group ($p = 0.021$, $p = 0.001$, $p = 0.014$, $p = 0.025$, respectively). Although the presence of less than 2 risk factors did not have a significant effect on the severity of the disease, it was revealed that the presence of 2 or more risk factors caused the disease to progress severely ($p < 0.001$).

We sequenced 13 genes with the NGS method. As a result of the evaluation, 103 variants were detected. A statistically significant difference was detected for 29 variants at the genes in the panel list. The incidence of 29 variants associated with mild and severe COVID-19 infection groups were shown in the Table 2. Statistical analyses in which each variable was evaluated independently are also included in Table 2.

In our study, heterozygous *IRF*7:c.1357−23dup variant had a 2.5 times higher risk for mild disease compared to severe disease ($p = 0.019$). Heterozygous *IRF*7:c.847+28T>C variant had a 2 times higher risk for mild disease than severe disease ($p = 0.031$). Homozygous *IRF7*:c.847+28T>C variant had a 4.4 times higher risk for mild disease than severe disease ($p < 0.001$).

Nine variants in the *IFNAR2* gene were statistically significant. Heterozygous *IFNAR2*:c.841−33C>T variant had a 3.3 times higher risk for severe disease than mild disease ($p = 0.040$). c.394+250A>G ($p = 0.030$), c.541−50A>G ($p = 0.001$), c.841−220G>A ($p = 0.021$), c.841−290C>T ($p < 0.001$), c.841−4del ($p < 0.001$), c.841−5del ($p < 0.001$), c.97+138T>C ($p = 0.001$), c.98−43T>C ($p < 0.001$) variants were found statistically more frequent in the mildly affected patient group compared to the severely affected group.

Heterozygous *IL10*:c.225+56A>G variant had a 3.3 times higher risk for mild disease than severe disease ($p = 0.032$). Heterozygous *IL10*:c.378+140A>T variant had a 13.2 times higher risk for mild disease than severe disease ($p = 0.001$). Heterozygous *IL10*:c.378+19T>C variant had a 6.2 times higher risk for mild disease than severe disease ($p = 0.015$). If there was homozygosity for this variant, the odd ratio would be 4.7 ($p = 0.035$).

Heterozygous *CXCL*8:c.200+125dup variant had a 20.8 times higher risk for mild disease than severe disease ($p < 0.001$). If there was homozygosity for this variant, the odd ratio would be 3.3 ($p = 0.001$). Heterozygous *CXCL*8:c.284+161C>T variant had a 6.4 times higher risk for mild disease than severe disease ($p < 0.001$). If there was homozygosity for this variant, the odd ratio would be 7.9 ($p = 0.001$). Heterozygous *CXCL8*:c.284+129del variant had a 3.4 times higher risk for mild disease than severe disease ($p = 0.001$).

Heterozygous *IL*2:c.351+45del variant had a 15.2 times higher risk for mild disease than severe disease ($p < 0.001$).

Heterozygous *TLR3*:c.634−135G>A variant had a 15.6 times higher risk for mild disease than severe disease ($p < 0.001$). If there was homozygosity for this variant, the odd ratio would be 6.8 ($p = 0.006$). Heterozygous *TLR*3:c.633+136G>T variant had a 11.1 times higher risk for mild disease than severe disease ($p = 0.027$).

Heterozygous *TNF*:c.186+123G>A variant had a 8.8 times higher risk of mild disease than severe disease ($p = 0.001$).

Heterozygous *IL*6:c.210+180A>G variant had a 142.857 times higher risk of mild disease than severe disease ($p < 0.001$). If there was homozygosity for this variant, the odd ratio would be 90.9 ($p < 0.001$). Heterozygous *IL6*:c.211−188C>A variant had a 62.5 times higher risk of mild disease than severe disease ($p < 0.001$). If there was homozygosity for this variant, the odd ratio would be 142.9 ($p < 0.001$). Heterozygous *IL6*:c.211−93C>T variant had a 5.5 times higher risk of mild disease than severe disease ($p = 0.004$).

Heterozygous *F*5:c.4095C>T variant had a 4.8 times higher risk of mild disease than severe disease ($p = 0.001$). There were no significant variants in F2.

Homozygous *ACE*2:c.2115−268A>G variant had a 17.5 times higher risk of mild disease than severe disease ($p < 0.001$). Homozygous *AC*E2:c.1297+68insCTTAT variant had a 4.2 times higher risk of mild disease than severe dis-

Table 2. Univariate logistic regression analysis of the variants.

Gene Name	Variant	Type	Number of mild clinic	Number of severe clinic	B	Standard error of B	p	Odds Ratio (OR)	95%CI for OR	
									Upper	Lower
IRF7	c.1357−23dup	Het	26	14	−0.932	0.398	0.019	2.538	5.525	1.166
	c.847+28T>C	Het	41	28	−0.714	0.331	0.031	2.041	3.906	1.067
	c.847+43C>T	Het	39	14	−1.476	0.380	< 0.001	4.367	9.174	2.079
IFNAR2	c.394+250A>G	Het	6	1	−2.423	1.118	0.030	11.236	100.000	1.259
	c.541−50A>G	Het	37	26	−1.587	0.483	0.001	4.902	12.658	1.898
	c.541−50A>G	Hom	45	40	−1.351	0.462	0.003	3.861	9.524	1.563
	c.841−220G>A	Het	34	20	−0.834	0.361	0.021	2.304	4.673	1.134
	c.841−290C>T	Het	9	1	−3.959	1.113	< 0.001	52.632	500.000	5.917
	c.841−290C>T	Hom	50	5	−3.560	0.549	< 0.001	35.714	100.000	12.048
	c.841−33C>T	Het	33	44	1.197	0.583	0.040	3.309	10.379	1.055
	c.841−4del	Het	82	12	−4.019	0.480	< 0.001	55.556	142.857	21.739
	c.841−5del	Het	36	10	−1.555	0.414	< 0.001	4.739	10.638	2.101
	c.97+138T>C	Het	29	11	−1.444	0.419	0.001	4.237	9.615	1.862
	c.98−43T>C	Het	24	1	−4.334	1.056	< 0.001	76.923	500.000	9.615
	c.98−43T>C	Hom	37	5	−3.298	0.566	< 0.001	27.027	83.333	8.929
IL10	c.225+56A>G	Het	44	36	−1.187	0.555	0.032	3.279	9.709	1.105
	c.225+56A>G	Hom	42	41	−1.148	0.552	0.037	3.155	9.259	1.070
	c.378+140A>T	Het	18	2	−2.576	0.780	0.001	13.158	62.500	2.849
	c.378+19T>C	Het	38	30	−1.827	0.749	0.015	6.211	27.027	1.433
	c.378+19T>C	Hom	51	53	−1.539	0.731	0.035	4.651	19.608	1.111
CXCL8	c.200+125dup	Het	19	2	−3.036	0.793	< 0.001	20.833	100.000	4.405
	c.200+125dup	Hom	38	27	−1.194	0.359	0.001	3.300	6.667	1.631
	c.284+129del	Hom	73	50	−1.223	0.356	0.001	3.401	6.849	1.689
	c.284+161C>T	Het	33	10	−1.850	0.428	< 0.001	6.369	14.706	2.747
	c.284+161C>T	Hom	15	4	−2.059	0.616	0.001	7.813	26.316	2.347
IL2	c.351+45del	Het	21	2	−2.715	0.771	< 0.001	15.152	66.667	3.333
TLR3	c.633+136G>T	Het	9	1	−2.408	1.088	0.027	11.111	90.909	1.318
	c.634−135G>A	Het	22	2	−2.748	0.770	< 0.001	15.625	71.429	3.448
	c.634−135G>A	Hom	13	3	−1.909	0.696	0.006	6.757	26.316	1.724
TNF	c.186+123G>A	Het	22	3	−2.174	0.652	0.001	8.772	31.250	2.451
IL6	c.210+180A>G	Het	33	5	−4.980	0.746	< 0.001	142.857	500.000	33.333
	c.210+180A>G	Hom	54	11	−4.490	0.634	< 0.001	90.909	333.333	25.641
	c.211−188C>A	Het	23	3	−4.129	0.724	< 0.001	62.500	250.000	14.925
	c.211−188C>A	Hom	49	2	−5.013	0.815	< 0.001	142.857	1,000.000	30.303
	c.211−93C>T	Het	17	4	−1.712	0.599	0.004	5.525	17.857	1.712
F5	c.4095C>T	Het	31	7	−1.567	0.468	0.001	4.785	12.048	1.916
ACE2	c.2115−268A>G	Hom	21	2	−2.858	0.776	< 0.001	17.544	76.923	3.817
	c.1297+68insCTTAT	Hom	55	34	−1.441	0.363	< 0.001	4.219	8.621	2.075
	c.584−71A>G	Het	14	5	−2.042	0.604	0.001	7.692	25.000	2.364
	c.584−71A>G	Hom	51	22	−1.883	0.370	< 0.001	6.579	13.514	3.185

CI, confidence interval; Het, heterozygous; Hom, homozygous.

ease ($p < 0.001$). Homozygous ACE2:c.584−71A>G variant had 6.6 times higher risk of mild disease than severe disease ($p < 0.001$). If there was heterozygous for this variant, the odd ratio would be 7.7.

Discussion

Many different reasons including viral agent-related factors and host-related factors are held responsible for the

clinical heterogeneity of COVID-19 (Pairo-Castineira et al. 2021). Determination of the factors causing different clinical courses among the asymptomatic and severely affected patients could improve understanding of the pathogenesis of the disease. SARS-CoV-2 can infect people of any age, but those over 60 years of age and those with underlying comorbidities (such as smoking habits, diabetes, hypertension, obesity, cardiovascular disease, chronic lung and kidney diseases, immune suppression, cancer, and organ transplantation) are more likely to develop serious or fatal conditions. In addition, sex, lifestyle choices, and viral exposure levels can determine the susceptibility of an individual to infections and critical diseases (Ou et al. 2020; Yin et al. 2021). In our study, no significant difference was found between the severely and mildly affected patient groups in terms of age. In addition, in accordance with the literature, as well as two or more accompanying comorbidities, each of the comorbidity was observed more frequently in the severely affected patient group compared to the mildly one.

Although there is a positive correlation between disease severity and the accompanying risk factors, young or healthy individuals are reported to have severe or life-threatening disease, too. Many researchers have shown that clinical diversity observed in the SARS-CoV-2 infection are result from genetic factors affecting disease severity, susceptibility and resistance (Gupta et al. 2022).

In our study, we have screened genes implicated in different pathways for sequence variants that may have modifying effect in the pathogenesis of COVID-19 in a panel including *ACE2, CXCL8, F2, F5, IFNAR2, IFNL4, IL10, IL2, IL6, IRF7, TLR3, TLR7* and *TNF* genes.

Cytokine storm, autoimmune features, and dysfunctions of myeloid cells are known to be significant contributing factors in severe COVID-19. It has been suggested that different genes in the inflammatory pathway may cause a serious disease. In our study, the effect of cytokine pathway proteins was also investigated. We sequenced the *CXCL8, IFNAR2, IFNL4, IL10, IL2, IL6, IRF7, TLR3, TLR7* and *TNF* genes in inflammatory pathway. Many variants in these genes were found in both of the groups and most of these variants were found significantly higher in the mildly affected patient group. Although, it was previously reported that patients with immunodeficiency had a more severe disease, different underlying conditions and comorbidities could be suggested for the severe course of the disease in some patients who had no immunodeficiency (Manik and Singh 2022). It is noteworthy that most of these variants are located in the intronic regions of the genes. The significant excess of variants in these genes in the mild group raises the idea of whether intronic variants of these immune pathway genes may have a protective effect from severe disease by increasing the activity of the immune system (Manik and Singh 2022). This issue needs to be investigated in more patients.

Interleukin-8 (IL8) synthesized by *CXCL8* is a member of the CXC chemokine family. IL8 is highly expressed at the tracheal aspirates of infants with respiratory syncytial virus (RSV) bronchiolitis, and the level of IL8 was correlated with disease severity (Melero et al. 2022; Sagulkoo et al. 2022). In relation with this, *CXCL8* may be considered to be involved in the pathogenesis of COVID-19, but in our study, no variant of *CXCL8* had a significant association in severely affected patient group.

SARS-CoV-2 infection activates both the innate and adaptive immune system in the alveolar tissue, inducing cytokine release syndrome. Cytokine release syndrome causes high levels of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-α and IFN), which have important roles for mortality. The type I IFN pathway plays a crucial role in mediating innate immune responses to viral infections. This cytokine family is comprised of 13 IFN-α subtypes, IFN-β, IFN-ω, IFN-κ, and IFN-ϵ, all of them signal through the heterodimeric IFN I receptor, composed of IFN-α/β receptor 1 (IFNAR1) and IFNAR2 (Beck and Aksentijevich 2020). By analysing patients with severe COVID-19, these two studies provide evidence that type I IFNs are actually protective against COVID-19; however, through either gene mutations or autoantibodies, they lead to severe disease. We observed *IFNAR2*:c.841−33C>T variant had a 3.309 times higher risk for severe disease than wild type. *IFNAR2*:c.841−33C>T is located at intron 8 of the *IFNAR2* gene. Although it has no effect on protein structure, it may have an effect on protein function.

In another study of 987 patients with COVID-19 pneumonia, it was reported that 101 patients had autoantibodies against a variety of type I interferons (IFNs), including IFN-ω and/or IFN-α. These autoantibodies were absent in patients with mild or asymptomatic disease, and noted in only a few healthy controls (Beer et al. 2022).

Since the underlying mechanisms of thromboembolic events, which are known to be one of the major mortality and morbidity reasons for COVID-19, include complex processes and interactions, it has been demonstrated in different studies that variants in *F2* and *F5* predispose to thrombosis (Klok et al. 2020). Inherited thrombophilia has been suggested to be a risk factor for severe COVID-19. Investigations on the genetic profiles of thrombophilia-related genes are important, for both in treating and avoiding COVID-19-related mortality (Badulescu et al. 2022). In our study, not only known variants but also all *F2* and *F5* genes were sequenced and no significant differences were detected. A pilot study for the association between COVID-19 and thrombophilia recommended that a large number of patients should be studied to find a significant result (de la Morena-Barrio et al. 2021).

ACE2 was also reported to have an important role in the pathogenesis of the COVID-19 (El-Arif et al. 2021). It is expressed mostly in the alveolar epithelial cells. Polymorphisms in the protein encoding regions of *ACE2* can affect the binding affinity of the viral spike protein to host cells, as well as membrane fusion efficiency, modulat-

ing the host susceptibility to COVID-19. *ACE2* gene can have rarely synonymous and nonsynonymous mutations, and some of them could modify the susceptibility to human coronavirus infections (Badawi et al. 2022). It has been suggested that some polymorphisms (S19P, I21V, E23K, K26R, T27A, N64K, T92I, Q102P, and H378R) located in the coding region of the *ACE2* and *ACE2* peptidase domain (PD), which binds SARS-CoV-2, may increase the risk of virus infection (Suryamohan et al. 2021). In a study from Turkey, *ACE1* insertion/deletion polymorphisms and *ACE2* PD domain variants were evaluated in patients with COVID-19 pneumonia and they were found to have no impact on severe COVID-19 infection (Bastug et al. 2022). In another study from Turkey, the changes in the *ACE2* gene were evaluated retrospectively, and 2 nonsynonymous variants were observed. However, the relationship between these variants and COVID-19 disease could not be established (Duman et al. 2022). These two nonsynonymous variants (p.Lys26Arg and p.Asn720Asp) in the *ACE2* gene were not detected in our study. In our study, the entire *ACE2* gene, also consisting the PD domain, were sequenced. There was no significant difference between the severe and mild patient groups. The *ACE2* gene is important in binding the spike protein, but we revealed that changes on the gene had no effect on the course of the disease.

It is critical to assess variations across all genes in an unbiased way, rather than preselecting genes representing as a priori hypothesis. While this candidate-gene approach was widely employed in the past, it has largely failed. In new cohorts, reported candidate-gene studies are not replicable. In our study, we designed a prospective study by carefully selecting and classifying the cohort. Data from other studies were analyzed, but we studied targeted gene panels in selected cohorts, demonstrating that reported variants and hypotheses may not be valid.

As a result, in order to confirm that the 29 variants detected in our study are effective in the clinical severity of the disease, studies with larger numbers of patients are required.

Since polymorphic variants were not evaluated in the literature, the findings of our study could not be compared with the findings in the literature. However, as variants that may be effective in the severity of infections may differ according to ethnicity, this study has the feature of being a guide for subsequent studies to be carried out especially in Turkish population.

In summary, there is currently no convincing evidence that individuals with monogenic immune disorders are at increased risk for severe COVID-19 outcomes. The hypothesis remains viable, but more compelling evidence will be needed to substantiate it. Therefore, clinical course of the COVID-19 infection is likely to depend on a variety of risk factors, including age, sex, clinical status, immunology and genetic factors.

Acknowledgments

We thank all participants for their participation in the current study. A prospective study was planned after obtaining the approval of the Ankara Yıldırım Beyazıt University, School of Medicine, Ethics Committee, No.28 (approval date 13.05.2020). The study was conducted in concordance with the Helsinki declaration and written in accordance with the STROBE statement. This research was supported by the Ankara Yıldırım Beyazıt University, Scientific Research Project Number TSG-2020-2106.

Conflict of Interest

The authors declare no conflict of interest.

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