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Polymorphisms of ACE (I/D) and ACE2 receptor gene (Rs2106809, Rs2285666) are not related to the clinical course of COVID-19: A case study

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Coronavirus disease 2019 (COVID-19) is an infectious disease, and the reason behind the currently ongoing pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Angiotensin-converting enzyme (ACE2) has been recognized as the specific receptor of the SARS-CoV-2 virus. Although the possible effect of ACE2 gene polymorphism remains unknown, human ACE2 receptor expression influences SARS-CoV-2 susceptibility and COVID-19 disease outcome. In this study, we aimed to investigate the relationship between ACE gene I/D polymorphism, ACE2 receptor gene polymorphism, and COVID-19 severity. ACE gene insertion/deletion (I/D) polymorphism and ACE2 receptor gene rs2106809 and rs2285666 polymorphisms were determined using polymerase chain reaction (PCR) and PCR-based restriction fragment length polymorphism methods, respectively, in 155 COVID-19 patients who were divided into three groups (mild, moderate, and severe) according to clinical symptoms. However, the distribution of genotype and allele frequencies of ACE gene I/D, ACE2 receptor gene rs2106809, and rs2285666 polymorphisms were not statistically significant in all groups. In conclusion, in the study population, ACE gene I/D, ACE2 receptor gene rs2106809, and rs2285666 polymorphisms were not associated with the severity of COVID-19 infection. Although ACE2 receptor gene expression may affect the susceptibility to COVID-19, there is no existing evidence that the ACE or ACE2 gene polymorphisms are directly associated with COVID-19 severity. Interindividual differences in COVID-19 severity might be related to epigenetic mechanisms of ACE2 receptor gene expression or variations in other genes suggested to play a critical role in COVID-19 pathogenesis such as pro-inflammatory cytokines and coagulation indicators.

KEYWORDS
ACE, ACE2, COVID-19, rs2106809, rs2285666

1 | INTRODUCTION

Coronaviruses are a large family of single-stranded RNA viruses, which cause illnesses ranging from a mild cold to more severe diseases such as severe acute respiratory syndrome (SARS-CoV) and Middle East Respiratory syndrome (MERS-CoV).[1] Coronavirus disease 2019 (COVID-19) was first reported in Wuhan, China, and subsequently spread worldwide. As of February 2021, there have been more than a hundred million cases of COVID-19 worldwide and nearly two and a half million deaths.[2]

The entry of SARS-CoV-2, the agent that causes COVID-19, into the cell occurs by binding viral spike proteins to angiotensin-converting enzyme 2 (ACE2) receptors of the host membrane. It was suggested that increased susceptibility to COVID-19 infection is associated with the expression of the target ACE2 receptor in the epithelium exposed to the virus.[3] Low infection and complication level is present among children. Serum ACE levels in children are higher than in adults and ACE2 receptor gene expression in the nasal epithelium, which is the first point of contact for SARS-CoV-2, was age-dependent, lowest in younger children, and increasing with age into adulthood.[4] It was also suggested that the lower risk among children is due to the lower expression of the ACE2 receptor.[5] Epidemiological data show that a majority of pediatric COVID-19 cases showed mild to moderate clinical features, and only a few developed severe or critical diseases (0.6% and 0.3%, respectively).[6] Of a total of 44 672 cases, only 1% is under 10 years old and in China.[7] Also, there is lower ACE2 receptor expression in children, highlighting the importance of ACE and ACE2 receptor expression level in COVID-19 severity.

A counter-regulatory relationship between ACE2 and ACE located on opposite axes in the renin-angiotensin system (RAS) has been reported. ACE plays an important role in converting angiotensin I to angiotensin II and ACE2 is a negative regulator of the RAS and counterbalances the function of ACE. The lungs are the primary organs for ACE receptor expression and generating circulating angiotensin II. RAS plays a role in the pathogenesis of pulmonary hypertension and fibrosis, which are common chronic lung diseases. Recent studies show that RAS also plays an important role in acute lung diseases, particularly acute respiratory distress syndrome (ARDS).[8]

A common 287 base pair insertion/deletion (I/D) polymorphism has been reported in intron 16 of the ACE gene and is known to be associated with serum levels of circulating ACE. Serum ACE concentrations were reported to be significantly higher in subjects with the D/D genotype compared to the I/D and I/I genotypes.[9] Considering the opposite effect between ACE and ACE2, decreased ACE2 receptor gene expression is strongly related to an increase in ACE expression. So it could be hypothesized that having a D allele for ACE I/D polymorphism affects the clinical course of the COVID-19 by decreasing the ACE2 receptor level.

Besides this, it has been shown that patients with chronic obstructive pulmonary disease (COPD) have increased gene expression of ACE2 receptor in bronchial epithelial cells in the lower respiratory tract, and also smoking has been shown to increase both the expression and activity of ACE2 in the airways. Although upregulation of ACE2 is beneficial in protecting the host against acute lung injury, this makes individuals more susceptible to coronavirus infections that use this receptor to enter epithelial cells.[10,11]

In the changes in the ACE2 receptor gene expression level, genetic variations play a very important role as well as environmental factors. Of the many polymorphisms identified in the ACE2 receptor gene, rs2106809 and rs2285666 are particularly remarkable. A bioinformatics tool called Human Splicing Finder (HSF) (v.3.0) predicts that the rs2106809 polymorphism, intronic single-nucleotide polymorphism (SNP) found in the ACE2 receptor gene, might create an intronic-exonic splicing enhancer site (ESE). It was suggested that the splicing efficiency of the ACE2 receptor gene may be influenced by the creation of these enhancer motifs.[12] It was also found that ACE2 rs2106809 CC or CT genotype carriers had higher circulating ACE2 receptor levels compared with TT genotype carriers.[13]

The G8790A (rs2285666) polymorphism is at the fourth base of the third intron and situated in the intron adjoined to the exon, suggesting, this locus could alter messenger RNA (mRNA) alternate splicing and affect ACE2 receptor gene expression. It has also been reported that this polymorphism shows a strong linkage disequilibrium with the other SNPs (rs1978124 intron 1 and rs714205 intron 16) in the ACE2 receptor gene.[14]

The relationship between ACE2 gene expression and COVID-19 has been shown in the literature, however, most of the studies conducted to investigate the role of ACE receptor gene polymorphisms are in-silico analyzes or epidemiological studies. Also, to the best of our knowledge, there is no study investigating the relationship between ACE I/D and ACE2 receptor gene rs2106809 and rs2285666 polymorphism and COVID-19 severity. In this study, we aimed to investigate whether the course of the disease (mild, moderate, and severe) is predictable by determining the genotypes of ACE I/D and ACE2 receptor gene polymorphism.

2 | MATERIALS AND METHODS

One hundred fifty-five patients with COVID-19 were included in this study. A confirmed case with COVID-19 was defined as a positive result to real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay for nasal and pharyngeal swab specimens.[15] Written informed consent was obtained from all the patients that participated in this study, and the local ethics committee of Bulent Ecevit University, Faculty of Medicine approved the study. Patients with COVID-19 were grouped according to the severity of the disease. Clinical characteristics of patients are shown in Table 1. COVID-19 severity is classified as follows; Mild type: Outpatients, the clinical symptoms are mild, with no abnormal radiological findings, Moderate type: Hospitalized for persistent fever or pneumonia or mild respiratory distress or present pneumonia on chest computed tomography, Severe type: The disease is classified as severe if one of the following conditions is met:

1. Respiratory distress, respiratory rate ≥ 30/min.
2. Oxygen saturation on room air at rest ≤ 93%.
3. The partial pressure of oxygen in arterial blood/FiO_2 ≤ 300 mm Hg.
4. Respiratory failure occurs and mechanical ventilation is required.
5. Another organ dysfunction is present, requiring intensive care unit monitoring and treatment.

2.1 | DNA isolation and genotyping

Genomic DNA was extracted from 200 μl of peripheral blood by a Macherey–Nagel DNA isolation Kit (Cat No: 740.951.250) following manufacturer instructions.

TABLE 1 Demographic characteristics and signs and symptoms of patients with COVID-19

		Severity		
	Total n (%)	Mild n (%)	Moderate n (%)	Severe n (%)
Age	52.25 ± 17.52	44.60 ± 15.52	54.57 ± 16.62	66.54 ± 12.68
Sex				
Female	77 (49.7)	49 (62.8%)	14 (33.3%)	14 (40.0%)
Male	78 (50.3)	29 (37.2%)	28 (66.7%)	21 (60.0%)
Signs and symptoms				
Fever or chills	55 (35.5)	15 (19.2)	23 (54.8)	17 (48.6)
Cough	55 (35.5)	24 (30.8)	17 (40.5)	14 (40.0)
Fatigue	46 (29.7)	29 (37.2)	8 (19.0)	9 (25.7)
Diarrhea	25 (16.1)	16 (20.5)	4 (9.5)	5 (14.3)
Sore throat	9 (5.8)	17 (21.8)	5 (11.9)	8 (22.9)
Muscle soreness	28 (18.1)	18 (23.1)	4 (9.5)	6 (17.1)
Nausea or vomiting	17 (11.0)	6 (7.7)	4 (9.5)	7 (20.0)
respiratory distress	39 (25.3)	1(1.3)	14 (33.3)	24 (68.6)
Persistent pain or pressure in the chest	9 (5.8)	1 (1.3)	1 (2.4)	7 (20.0)
Joint pain	26 (16.8)	15 (19.2)	4 (9.5)	7 (20.0)
Loss of sense of smell and taste	18 (11.6)	10 (12.8)	2 (4.8)	6 (17.1)
Headache	33 (21.3)	21 (26.9)	5 (11.9)	7 (20.0)

Abbreviation: COVID-2019, coronavirus disease 2019.

PCR was used to detect the I/D polymorphism of the *ACE* gene. A PCR-based restriction fragment-length polymorphism method was used to genotype *ACE2* rs2106809 and rs2285666 polymorphisms. The PCR was performed in a 25 μl volume for each polymorphism that is containing 10× PCR buffer, 3.0 mM MgCl2, 0.25 mMdNTPs, 1.5 units of Taq polymerase (Promega), and 0.3 μM each primer (5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′ and 5′-GAT GTG GCC ATC ACA TTC GTC AGA-3′ for *ACE* I/D polymorphism and F; 5′-GAA AGC CAG ATG CTT TAA CAA G-3′ and R; 5′-TTTTTCCATATCTCTATCTGATCG-3′ for *ACE2* rs2106809 polymorphism and F; 5′-CATGTGGTCAAAAGGA TATC-3′ R; 5′-AAAGTAAGGTTGGCAGACAT-3′ for *ACE2* rs2285666 polymorphism.

For *ACE* I/D polymorphism, the PCR products were run at 2% agarose gel. Allele (I/D) types and genotype for each sample were determined based on the PCR product sizes. The major allele indicates the 490 bp fragment that embraces the 287 bp Alu sequence, known as an insertion, whereas the 190 bp fragment represents the minor allele with the deletion of the Alu sequence. Heterozygosity specifies the combination of both major and minor fragments, that is, 490/190 bp.

For the *ACE2* rs2106809 polymorphism, the PCR products of 207 base pairs (bp) were digested with restriction enzyme TaqI resulting in two products of 183 bp and 24 bp in the presence of the A allele.

For *ACE2* rs2285666 polymorphism, the PCR products were incubated with 5 U of AluI restriction enzyme at 37°C overnight. After digestion, fragments of 281 and 185 bp identify the T allele and a 466 bp band identifies the C allele.

All digestion products were electrophoresed on 3% agarose gel and visualized by staining with ethidium bromide and evaluated using the gel documentation system (Syngene, Genegenius Bio Imaging System).

2.2 | Statistical analysis

Genotype and allele frequency of the polymorphism was calculated in cases according to the severity of COVID-19. The χ^2 test was used to compare the genotype frequency of the *ACE* and *ACE2* receptor gene polymorphisms in patients who have mild, moderate, or severe clinical symptoms. As the *ACE2* receptor gene is located on the X chromosome and as males are hemizygous for *ACE2*, males and females were analyzed separately. A p value less than 0.05 was considered statistically significant. The software used for the calculations was SPSS version 18 (SPSS Inc.).

3 | RESULTS

The present study included 155 patients with COVID-19, of which 78 were in the mild, 42 were in the moderate and 35 were in the severe group. Seventy-seven subjects were women

and 78 were men who enrolled in the study. The mean (±SD) age was 52.25 ± 17.52 in patients, and 44.60 ± 15.52, 54.57 ± 16.62, and 66.54 ± 12.68 for mild, moderate, and severe groups, respectively. The severity of the disease increased with age ($p < 0.001$). Also, it was found that gender differences affect COVID-19 severity and men tended to be affected more seriously than women ($p = 0.003$). The percentage of males was 60% in the severe groups and 40% for females. The clinical signs and symptoms of the patients with COVID-19 are given in Table 1.

The distribution of each genotype for ACE I/D, ACE2 rs2106809, and rs2285666 in the COVID-19 patients according to the severity of the disease are shown in Table 2. There was no statistically significant association between groups according to genotype ($p = 0.191$) and allele frequencies ($p = 0.392$) for ACE I/D gene polymorphism.

As ACE2 is on the X chromosome, data for each sex were analyzed separately in Tables 2. It was also found that the distribution of genotype and allele frequencies of ACE2 rs2106809 and rs2285666 gene polymorphisms were similar in all groups according to the severity of disease for both genders ($p > 0.05$). Haplotype frequency and association analysis were also performed (Table 3). We identified one haplotype for two polymorphisms (rs2106809 and rs2285666) in the ACE2 receptor gene. However, no statistical difference was noted ($p = 0.519$).

Also, genotypic and allelic distribution of ACE I/D and ACE2 rs2106809 and rs2285666 polymorphisms were analyzed according to clinical symptoms and no significant relationship was found.

4 | DISCUSSION

ACE2 has been established as the functional host receptor for SARS-CoV-2, responsible for the current devastating worldwide pandemic of COVID-19.[16] COVID-19 displays symptoms ranging from a mild cold to cardio-respiratory failure that may lead to death.[17,18] Both ACE and ACE2 receptor genes have numerous genetic variations including functional polymorphism of I/D for ACE and rs2106809 and rs2285666 polymorphisms for the ACE2 receptor gene. The D allele shows higher ACE activity and polymorphisms in the ACE2 receptor gene affect circulating ACE2 receptor levels.[12,13] Considering the role of ACE in COVID-19 pathogenesis and the variation in disease severity, ACE I/D, and ACE2 receptor gene variants have attracted the attention of researchers. However, studies have been limited to in silico analyzes and epidemiological studies. To the best of our knowledge, our work is the first study including wet lab analysis for investigating ACE I/D, ACE2 rs2106809, rs2285666 polymorphisms. Unfortunately, no association was found between these polymorphisms and the severity of symptoms of COVID-19.

Delanghe et al.[19] designed multiple regression analysis models to compare the prevalence and mortality of the COVID-19 infection from a number of European countries and frequency data of the geographical variation of I/D polymorphism in the ACE gene. Differently, the authors reported a negative correlation between the frequency of the D allele of the ACE I/D polymorphism and the prevalence and mortality rates of COVID-19 in 33 countries. It should be noted that this study does not involve wet lab analysis and the results conflicted with eastern Asian

TABLE 2 Allele and genotype distribution of ACE I/D polymorphism and genotype frequency of ACE2 rs2106809 and rs2285666 polymorphisms in COVID-19 patients according to the severity of the disease

SNP	Gender	Genotype/Allele	Severity Mild n (%)	Moderate n (%)	Severe n (%)	χ^2	p
ACE I/D Polymorphism	Female and Male (all subjects)	II	17 (21.8)	4 (9.8)	6 (17.1)	6.11	0.191
		ID	37 (47.4)	27 (65.9)	15 (42.9)		
		DD	24 (30.8)	10 (24.4)	14 (40.0)		
		I	64 (50.0)	32 (60.4)	24 (49.0)	1.87	0.392
		D	64 (50.0)	21 (39.6)	25 (51.0)		
rs2106809	Female	AA	22 (44.9)	6 (42.9)	9 (64.3)	2.72	0.606
		AG	22 (44.9)	6 (42.9)	3 (21.4)		
		GG	5 (10.2)	2 (14.3)	2 (14.3)		
	Male	A	22 (75.9)	19 (67.9)	18 (85.7)	2.07	0.354
		G	7 (24.1)	9 (32.1)	3 (14.3)		
rs2285666	Female	CC	23 (46.9)	7 (50.0)	7 (50.0)	0.76	0.942
		CT	22 (44.9)	5 (35.7)	6 (42.9)		
		TT	4 (8.2)	2 (14.3)	1 (7.1)		
	Male	C	22 (75.9)	18 (64.3)	17 (81.0)	1.87	0.391
		T	7 (24.1)	10 (35.7)	4 (19.0)		

Abbreviations: ACE, angiotensin-converting enzyme; COVID-2019, coronavirus disease 2019; SNP, single-nucleotide polymorphism.

TABLE 3 Haplotype frequency of *ACE2* rs2106809 and rs2285666 polymorphisms in COVID-19 patients according to the severity of the disease

Haplotype rs2106809/rs2285666	Severity			χ^2	p
	Mild n (%)	Moderate n (%)	Severe n (%)		
AC	85 (66.9)	35 (63.6)	36 (73.5)	5.19	0.519
AT	3 (2.4)	1 (1.8)	3 (6.1)		
GC	5 (3.9)	1 (1.8)	1 (2.0)		
GT	34 (26.8)	18 (32.7)	9 (18.4)		

Abbreviation: COVID-2019, coronavirus disease 2019.

populations' data. It was suggested that there is a converse association between *ACE* D allele frequency and prevalence of COVID-19. Considering that the D allele frequency is lower in Asian populations than in European populations, the mortality rate and prevalence of COVID-19 in the Asia population is expected to be higher. However, the prevalence and mortality of COVID-19 in Europe are higher than in Asia.[19,20] In contrast to Delanghee et al.,[19] Pati et al.[21] suggested a significant positive correlation of the D allele of *ACE* polymorphism with SARS-CoV-2 infection and mortality rate in their epidemiological investigation.

Hatami et al.[22] conducted a meta-analysis study and showed the recovery rate significantly increased with the I/D allele frequency ratio. However, this study provides an ecological perspective but does not provide a direct clinical relevance between the COVID-19 and *ACE* I/D polymorphism.[22]

In two different populations, genetic variants in the *ACE2* receptor gene were analyzed by whole-exome sequencing in hospitalized COVID-19 patients. Different from our study, they investigated the relationship of COVID-19 with coding-region variants in the *ACE2* receptor gene. These studies also provided no strong evidence that *ACE2* receptor gene variants are a consistent association with COVID-19 severity. The *ACE2* receptor gene sequencing showed no coding sequence variants that could explain an increased risk of developing COVID-19. Similarly, in our study, no relationship was found between *ACE-2* receptor gene intron variants and COVID-19 severity.[23,24]

Our results showed that the *ACE* I/D, *ACE2* rs2106809, rs2285666 polymorphisms have no role in the severity of COVID-19. Novelli et al.[23] also reported that *ACE2* receptor gene coding-region variants have no effects on COVID-19 severity. Taking the fact that ACE2 receptor gene expression may affect the susceptibility of individuals to infection into consideration, we speculate that genetic variations in the noncoding regions of the *ACE2* receptor gene or in other noncoding DNAs that control the expression levels of ACE genes may have a potential role in the severity of the disease.

A growing number of recent findings point out that epigenetic mechanisms such as DNA methylation and histone modifications, play key roles to control gene expression. A recent analysis of public genomic and transcriptomic data outlined the role of histone methylation to regulate *ACE2* receptor gene transcription.[25] Further regulation occurs at the mRNA level. From putative microRNA-binding sites identified in vitro, Lambert et al.[26] demonstrated that miR-421 downregulates the *ACE2* receptor gene. Besides undergoing posttranslational modifications by glycosylation and phosphorylation, the *ACE2* receptor gene is also posttranslationally regulated.[27] Therefore, variations in other genes related to

epigenetic mechanisms of ACE2 receptor gene expression may have a potential role in the severity of COVID-19.

Second, mortality or severity of disease in patients with COVID-19 might be linked to excessive production of pro-inflammatory cytokines leading to ARDS aggravation and widespread tissue damage, resulting in multiorgan failure and death, not linked to increased ACE2 receptor expression. Wang et al.[28] showed that most of the severely ill patients had viral shedding in a variety of tissues for 20–40 days after onset of disease, while the majority of mildly ill patients had viral shedding restricted to the respiratory tract and had no detectable virus RNA 10 days after onset. Similarly, Liu et al.[29] reported that mild cases were found to have an early viral clearance. These results may indicate that the nasopharyngeal SARS-CoV-2 RNA load is higher in the severe group, or they may be related to the immune response of the host. In the current literature, impairment of SARS-CoV-2 clearance is related due to genetic and viral features, enhanced levels of interferons, neutrophil extracellular traps and pyroptosis, and possibly other unknown mechanisms were reported.[30] Besides this, the patients from the severe group showed elevated levels of inflammatory cytokines (such as IL-2, IL-2R, RIL-6, IL-8, and IL-10) and significant enhancement in coagulation parameters (such as D-dimer, prothrombin time, and fibrinogen), and increases in myocardial injury indicators. Therefore, inflammatory release, coagulation dysfunction, and myocardial injury correlate with disease severity and rise throughout the course of the disease.[31,32] Thus, the severity of COVID-19 may be related to variations in genes encoding pro-inflammatory cytokines or other genes that are suggested to be associated with the occurrence of severe COVID-19.

In conclusion, our study does not support the hypothesis that *ACE* I/D, *ACE2* rs2106809, and rs2285666 polymorphisms are related to COVID-19 severity. Considering that little is known about the genetic basis of the difference of COVID-19 severity and the analysis of genetic polymorphisms reveals important information, the possible relation between *ACE2* rs2106809, rs2285666 polymorphisms, and COVID-19 severity needs to be investigated. However, to explain the personal variations in COVID-19 pathogenesis, further studies are needed to investigate variations in other genes related to the epigenetic mechanisms of ACE2 receptor gene expression and variation in genes encoding pro-inflammatory cytokines and coagulation indicators.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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