



Association investigations between ACE1 and ACE2 polymorphisms and severity of COVID-19 disease

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Abstract

Due to the unique affinity of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to the angiotensin-converting enzyme 2 (ACE2) receptor in patients, the foremost recent evidence indicated that ACE1 and ACE2 polymorphisms could affect the susceptibility of individuals to SARS-CoV-2 infection and also the disease outcome. Here, we aimed to assess the possible association between two polymorphisms and the severity of disease in patients. In the present study, 146 patients with COVID-19 who were admitted to the Mazandaran University of Medical Sciences hospitals between March 2020 and July 2020 were enrolled in this case–control study. The patients were divided into four groups based on clinical symptoms and severity of the diseases (mild, moderate, severe, and critical). After DNA extraction, the ACE gene I/D polymorphism (rs4646994) and ACE2 gene polymorphism (rs2285666) were genotyped using Gap-PCR and PCR–RFLP techniques, respectively. Then, five samples from each obtained genotype were confirmed by Sanger sequencing technique. Data were analyzed with SAS software version 9.1 using appropriate statistical procedures. The ACE gene I/D polymorphism (rs4646994) genotypes were classified into three types: I/I, I/D, and D/D. Our finding indicated that the prevalence of ACE1 D/D genotype was significantly higher in severe and critical COVID-19 patients ($P = 0.0016$). Additionally, the analysis revealed a remarkable association between rs4646994 SNP and the HB and ESRI levels in patients ($P < 0.05$). Although the ACE2 rs2285666 SNP was not related to the severity of disease, this variant was significantly associated with ALT, ESRI, and P. These results provide preliminary evidence of a genetic association between the ACE-D/D genotype and the D allele of ACE1 genotype and the disease severity. Therefore, our findings might be useful for identifying the susceptible population groups for COVID-19 therapy.

Keywords COVID-19 · Angiotensin-converting enzyme · rs4646994 · rs2285666

Introduction

Due to the SARS-CoV-2 virus, a novel virus from the Coronaviridae family, the world has been struggling with the epidemic of the COVID-19 (World Health Organization 2020). COVID-19 has been impacting an outsized number

of individuals worldwide. Evidence indicated the COVID-19 case mortality rate had varied among nations. So far, around 7.2 million cases have been reported in Iran in keeping with data reported by WHO with 140,000 confirmed deaths.

The clinical spectrum of the COVID-19 virus appears very diverse, starting from mild flu to severe pneumonia and even acute respiratory distress syndrome (ARDS) (Xia et al. 2020). Other complications include septic shock, metabolic acidosis, coagulopathy, acute cardiac and arrhythmic damage, secondary infections and multiple organ damage, neurological and ocular manifestations, and renal and hepatic dysfunction (Chang et al. 2020; Pambuccian 2020). It has been hypothesized that viral infection has a severe inflammatory response and leads to severe lung damage that may require ICU admission, mechanical ventilation, and increase the risk of multiple organ failure and death (Jose and Manuel 2020).

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The renin–angiotensin–aldosterone (RAAS) system appears to play a crucial role in the COVID-19 pathogenesis (Ingraham et al. 2020). Angiotensin-converting enzyme (ACE or ACE1) catalyzes the synthesis of angiotensin-II (Ang-II) from Ang-I, and ACE2 hydrolyzes Ang-II to Ang-1-7. Ang-II binds to the AT1 receptor, causing vasoconstriction, fibrosis, inflammation, thrombosis, and other responses. Ang-1-7, on the other hand, binds to the AT2 receptor, dilating blood vessels and reducing fibrosis, inflammation, and thrombosis. Thus, ACE and ACE2 play a key and opposite role in the balance that leads to the risk of hypertension and cardiovascular disease. In the lung, ACE2 produces a protective response by reducing edema, permeability, and lung damage (Kuba et al. 2005). Previous studies revealed that the high ACE activity increases the risk of pulmonary and cardiovascular disease by increasing Ang-II/AT1R axis activity.

The human ACE gene is located on chromosome 17 with 21 kb long, and contains 26 exons and 25 introns (Riordan 2003). The genetic variants in the two ACE1 and ACE2 genes are associated with various diseases such as the risk of hypertension, heart disease, kidney failure, and lung disease. Reports revealed associations of COVID-19 severity with genetic variants in RAAS-related genes including ACE1, ACE2. From a systematic review, ACE1 rs4646994 and ACE2 rs2285666 were noted as common polymorphisms of RAAS-related genes. Also, meta-analyses studies uncovered that both ACE1 rs4646994 and ACE2 rs2285666 were significantly associated with the severity of COVID-19 (Saengsiwaritt et al. 2022). The functional polymorphism observed in this gene is insertion/deletion (rs4646994 I/D SNP) associated with increased activity and concentration of the enzyme. The two alleles D and I of the ACE gene are different in size due to the absence of a 287 bp sequence in intron 16 of this gene and three genotypes DD, DI, and II are created. Individuals with the D/D genotype indicated the highest blood ACE levels, and this rise in expression increases the risk of cardiovascular and respiratory diseases among people with the homozygous elimination genotype. This polymorphism is associated with acute respiratory distress syndrome (ARDS) as well as the progression of pneumonia (SARS) in SARS (Marshall et al. 2002; Matsuda et al. 2012).

The human ACE2 gene is located on the X chromosome and has been linked to several single nucleotide polymorphisms (SNPs) that have been reviewed as important risk factors for hypertension and heart failure, including G to A change in nucleotide +4 intron 3 (SNP rs2285666). In fact, the location of the ACE2 gene on the X chromosome is considered a disadvantage for male carriers who have alleles associated with low ACE2 expression, and this may explain the higher prevalence of severe COVID-19 in men. SARS-CoV reduces cardiac ACE2 expression and this may

explain inflammation and myocardial damage and adverse cardiac outcomes in patients with SARS (Oudit et al. 2009). rs2285666 genetic variant is one of tagged-SNP that could alter mRNA alternate splicing and affect ACE2 receptor gene expression. Also, the SNP is found to be associated with lower plasma ACE2 level (Chen and Yu 2018). 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 (Çelik et al. 2021).

To date, it is clear that ACE/ACE2 imbalance plays an eminent role in the pathogenesis of COVID-19. Therefore, different types of genetic variants in these genes may increase the risk of disease in susceptible individuals by altering gene expression and protein function. Some researchers have also reported that regional differences in allele abundance may explain differences in incidence and mortality rate of the disease. Although some studies proposed a role for rs4646994 and rs2285666 SNPs in COVID-19 severity, the evidence has not been conclusive among different nations as some findings have not been replicated. Therefore, our study aimed to identify different allelic forms in ACE and ACE2 genes associated with COVID-19 among Iranian patients to better understand the pathogenic mechanism of SARS-CoV-2 virus and how the immune system responds to the virus to explain the wide range of disease severity in individuals.

Materials and methods

Subjects

In the present study, 146 patients with COVID-19 infection admitted to Mazandaran University of Medical Sciences hospitals with the diagnosis of COVID-19 during late March, April and May 2020 (first peak), June and July 2020 (second peak) were examined. After obtaining consent from the study participants, all information related to age, sex, history of chronic diseases, early symptoms, CT scan, laboratory tests, medications used, and the course of the disease were recorded in a questionnaire.

The severity of the disease is defined into four groups by the WHO including mild, moderate, severe, and critical. The manifestation of “Mild” stage included usually fever less than 38°, sore throat with or without dry cough, chills, headache, loss of appetite, loss of taste or smell, nausea, vomiting, anorexia, diarrhea, weakness, and fatigue. At this stage, vital signs (pulse, pressure, and number of breaths) are stable and oxygen saturation level (SPO₂) is greater than or equal to 93%. In the “Moderate” stage, gastrointestinal and neurological symptoms (severe headache) are observed. Criteria for entering this stage are including (1) shortness

of breath, feeling of pain, and pressure in the chest with or without fever equal to or greater than 38° (2) SPO₂ between 90% and 93%. In the “severe” stage, there is a range of more severe clinical symptoms. Criteria for entering this stage are including (1) rapid progression of respiratory symptoms (2) tachypnea (RR > 30) and shortness of breath (3) SPO₂ less than 90 and (4) increased involvement of more than 50% of the lungs in CT-scan. In critical stage, the patient needs special care. The criterion for entering this stage is the presence of at least one of the following symptoms: (1) patient with symptoms of respiratory failure despite non-invasive oxygen therapy (2) patient with shock symptoms (3) patient in need or under mechanical ventilation and (4) patient with multiple organ failure.

DNA analysis

DNA extraction

Blood samples were collected in EDTA-containing tubes and DNA was isolated using the commercial DNA extraction kit of Aria Gene Farzam Madiar (AGFM) Company according to the protocol of the DNA extraction manufacturer. Then, the quantity and quality of the extracted DNA were determined using two methods of spectrophotometry and agarose gel electrophoresis.

Amplification of gene fragments

Specific primers were designed using Primer premier software to amplify fragments of the ACE and ACE2 genes (Table 1), and using the NCBI website, a sequence of primers designed with the entire human genome will be blasted and featured. The desired fragments for both two genes are then amplified using Amplicon Mastermix in Biorad thermocycler and agarose gel electrophoresis is used to determine the quantity and quality of the amplified fragments.

Genotyping

The I/D polymorphism (rs4646994) located in intron 16 of the ACE gene was determined by Gap-polymerase chain reaction (PCR) followed by agarose gel electrophoresis to visualize the I and D alleles (Alvarez et al. 1998).

For the ACE2 rs2285666 A/G SNP the PCR products were digested with the *AluI* restriction enzyme and followed by electrophoresis on 2% agarose gels. To perform polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), 5 µL of the PCR product of ACE2 gene was digested with 0.3 µL of restriction enzymes at 37 °C for 14 h. Digested products were segregated by agarose gel electrophoresis, then visualized by Gel Doc instrument.

Sequencing of different genotypes

We used the direct sequencing technique to confirm the obtained results and also to determine the exact length of the obtained parts. We examine five samples of each genotype (wild and mutant forms) using ABI Genetic Analyzer 3130XL. After obtaining the results, evaluation of reads was done by bioinformatics software such as BioEdit.

Statistical analysis

In this experimental and laboratory study, due to the limitations in sample sizes and selection of samples with complete information, 146 patients were evaluated with complete information. Genotypes and clinical data collected in Excel software were entered and then analyzed using SAS statistical software version 9.1 by appropriate statistical tests such as chi-square and logistic regression. The chi-square test (χ^2) was used to verify the equilibrium of the genotypic frequencies with the Hardy–Weinberg equilibrium (HWE). The effects of observed genotypes on the clinical values were evaluated with the GLM procedure. Continuous and categorical variables were expressed as mean \pm standard deviation and as percentages, respectively. Significant level in all tests is considered 0.05.

Results

ACE I/D polymorphism

After isolating DNA from blood samples, the ACE I/D polymorphism was genotyped by Gap-PCR. PCR products of ~490 and ~200 bp characterize as I and D alleles, respectively (Fig. 1).

Table 1 Primer sequences and specific information for each locus

Gene	Sequence (5'-3')	TM ^a (°C)	Reference
ACE I/D (rs4646994)	F-TGTAAGCCACTGCTGGAGAG R-CCCATGCCATAACAGGTCTTC	60	NC_000017.11
ACE2 (rs2285666)	F-TGGCTGCCTCACTGTCCTATG R-AGGTTGGCAGACATCAGGTCAT	62	NG_012575.2

^aTM annealing temp

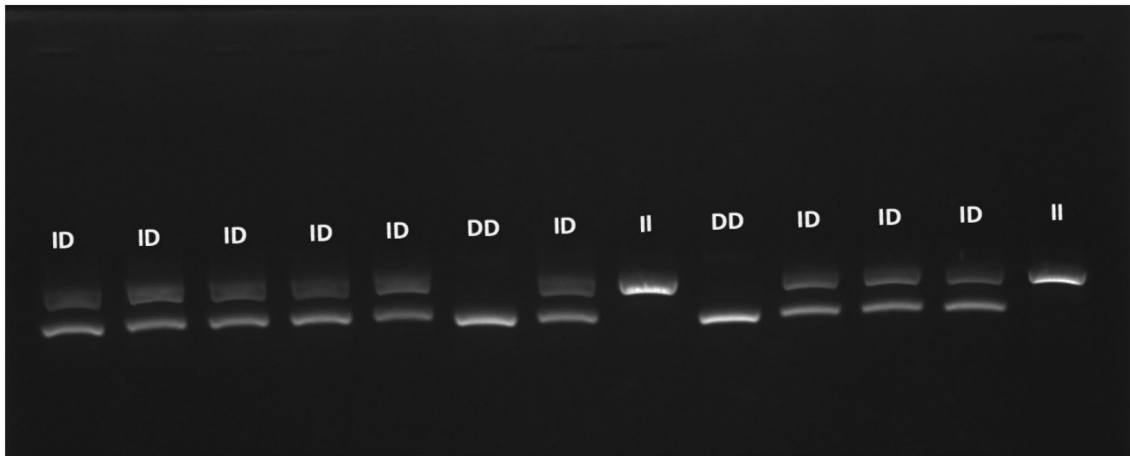


Fig. 1 Agarose gel electrophoresis of some samples for the ACE I/D SNP

ACE2 rs2285666 SNP

For ACE2 rs2285666 polymorphism, PCR–RFLP was applied. After digestion of the PCR products with restriction enzyme *AluI*, fragments of 499 and 277 bp identify A allele (AA genotype) and a 776 bp band identifies G (GG genotype) (Fig. 2). For confirmation of genotyping, five samples were randomly evaluated by Sanger sequencing. The sequencing results confirmed the results of PCR–RFLP technique (Fig. 2).

The present study included 146 COVID-19 patients of which 102 were in the mild and moderate and 44 are in the severe and critical group. 76 subjects were women, and 70 were men enrolled in the study. Additionally, our analysis has indicated that gender, location, and blood groups were insignificant between two severity groups ($P > 0.05$) (Table 2).

Table 3 shows the distribution of Allelic and genotypic frequencies for ACE I/D (rs4646994) and ACE2 rs2285666 SNPs in the COVID-19 patients. The analysis revealed a significant association between groups according to genotype ($P = 0.0016$) for ACE I/D gene polymorphism. It was found that the distribution of genotype of ACE2 rs2285666 gene polymorphisms was similar in two groups according to the severity of disease. There was no statistically significant association between groups and genotypic frequencies ($P = 0.8472$).

Association analysis was also performed between observed genotypes and clinical values using GLM procedure (Table 4). The analysis revealed a remarkable association between rs4646994 SNP and the HB and ESRI levels in patients ($P < 0.05$). Although the ACE2 rs2285666 SNP was not related to the disease severity, the SNP was significantly associated with ALT, ESRI, and P (Table 4).

Discussion

Since the outbreak of COVID-19, several researchers have noted the importance of ACE and ACE2 gene polymorphisms in COVID-19 outcomes. Finding the genetic risk factors for assessing the individual's sensitivity to COVID-19 could be important. Although several studies reported various factors including male sex, age, obesity, diabetes and ethnicity are reported to be associated with an increased risk of COVID-19 infection (Hastie et al. 2020), no significant difference in age, gender, location, and blood groups was observed in our findings.

The COVID outbreak has evoked a wave of interest focused on potential SNPs correlated with the severity of the disease. So far, several polymorphisms (Di Maria et al. 2020) with potential to affect SARS-CoV-2 susceptibility were noted by researchers, but undoubtedly confirmed and widely replicated genetic determinants of SARS-CoV-2 infection and COVID-19 severity remain unknown and vary among different populations. The genome-wide association (GWAS) method was applied to the detection of genetic susceptibility to COVID-19, suggesting promising candidate genes such as CCR9 and CXCR6, and SLC6A20, which interacts with ACE2 (Group 2020). Considering the role of ACE gene in the renin–angiotensin (RA) system and consequently, in the pathogenesis of COVID-19, there have been many trials verifying the hypothetical effect of genetic variants in ACE1 and ACE2 genes.

In this regard, the ACE gene I/D (insertion or deletion) SNP (rs4340, rs4646994), is probably the most studied polymorphism. In the present study, we found a significant association between ACE gene I/D SNP and COVID-19 severity which is consistent with former reports (Bellone and Calvisi 2020; Delanghe et al. 2020a;

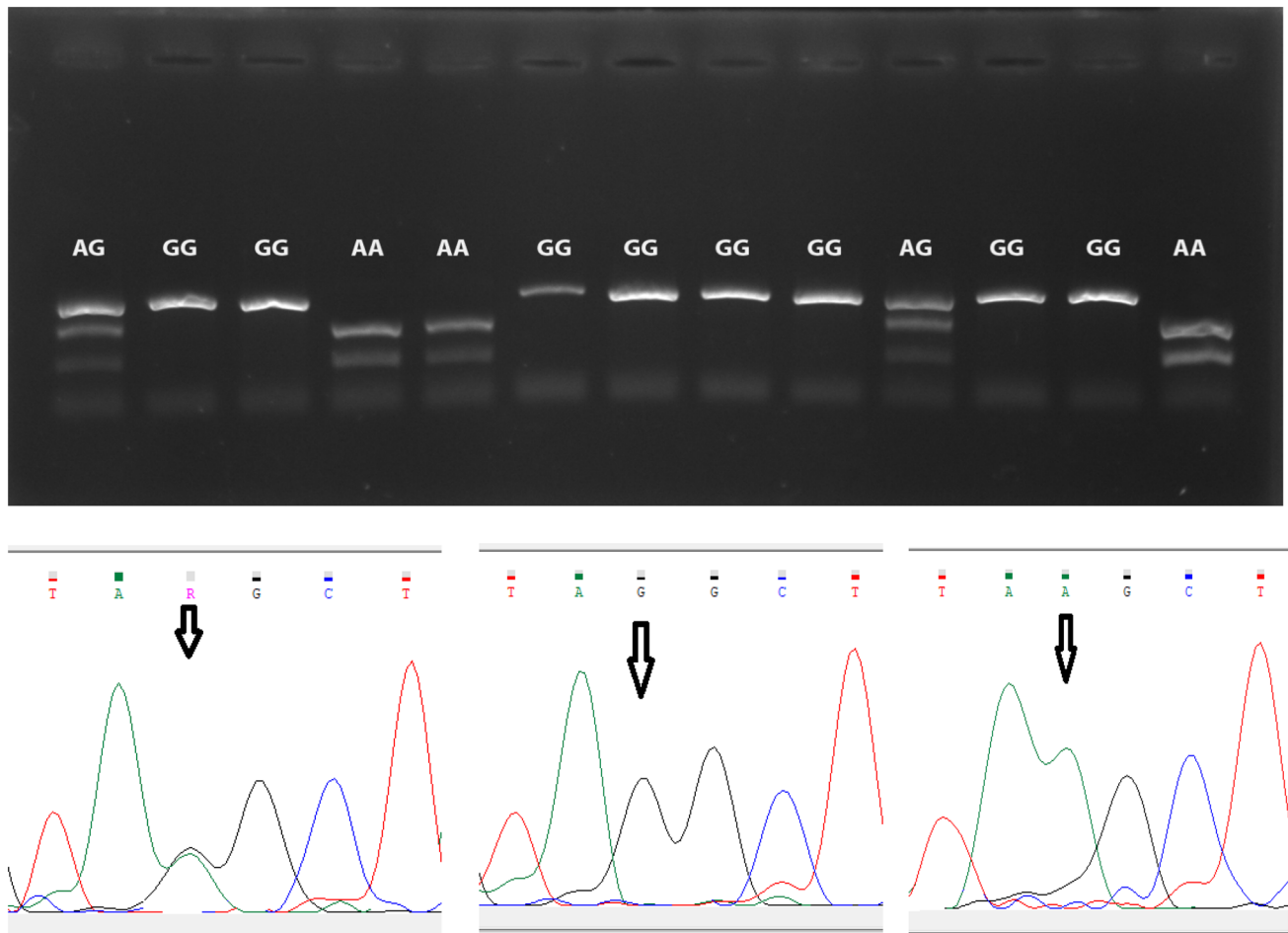


Fig. 2 Agarose gel electrophoresis of some samples for the ACE2 rs2285666 SNP. Capillary electrophoresis of three ACE2 genotypes (AA, AG, GG), showing the SNP (arrow)

Table 2 Main clinical values between two studied groups.

Parameters	Mild and moderate	Severe and critical	Odds ratio	P value
Gender (female/male)	55/47	21/23	0.936	0.8545
Location (city/village)	56/34	28/10	1.700	0.2149
Blood group RH (+/-)	48/4	26/3	0.722	0.6847

Saadat 2020; Yamamoto et al. 2020). Conclusion based on which allele (D or I) is as deleterious, is controversial among different studies. Our finding indicated that ACE1 D/D genotype was significantly higher in severe and critical COVID-19 patients ($P = 0.0016$) which is in agreement with the results described by studies performed

Table 3 Allelic and genotypic frequencies of ACE and ACE2 loci and χ^2 test for HWE

Severity	SNP							P value	
	rs4646994			P value	rs2285666				P value
	II	ID	DD		AA	AG	GG		
	N (%)	N (%)	N (%)		N (%)	N (%)	N (%)		
Mild and moderate	15 (14.71)	44 (43.14)	43 (42.16)	0.0016	22 (21.57)	22 (21.57)	58 (56.86)	0.8472	
Severe and critical	4 (9.09)	33 (75.0)	7 (15.91)		11 (25.00)	8 (18.18)	25 (56.82)		
Total	19 (13.01)	77 (52.74)	50 (34.25)		33 (22.6)	30 (20.55)	83 (56.85)		

Significant p-Values (A p-value less than 0.05) were shown as bold

Table 4 Association analysis between observed genotypes and clinical values using GLM procedure

Parameters	rs646994				rs2285666				P value
	II	ID	DD	P value	AA	AG	GG	P value	
ALB	3.44±0.16	3.67±0.08	3.68±0.10	0.4480	3.52±0.12	3.75±0.13	3.65±0.08	0.4683	
ALP	300.71±39.34	245.08±18.15	239.11±23.10	0.3802	271.92±28.83	232.0±29.46	247.57±17.86	0.6157	
ALT	52.57±10.96	35.28±4.73	43.63±5.91	0.2663	57.43±7.28	35.82±7.15	34.64±4.64	0.0275	
AST	56.11±13.60	44.26±5.87	54.93±7.44	0.4623	52.29±9.21	58.76±9.21	44.0±5.87	0.3768	
BS	163.04±23.83	153.44±11.68	148.42±15.03	0.8738	172.89±18.12	154.35±19.47	145.15±11.17	0.4303	
Ca	8.08±0.22	8.61±0.11	8.61±0.14	0.0903	8.47±0.18	8.86±0.18	8.43±0.11	0.1270	
CPK	222.49±71.27	159.86±32.71	189.86±45.84	0.6853	207.40±55.36	166.55±51.05	169.51±33.47	0.8224	
CR	1.30±0.42	1.74±0.21	2.10±0.26	0.2521	1.88±0.33	2.10±0.33	1.66±0.20	0.5094	
CRP	59.40±9.32	47.67±4.90	42.34±6.04	0.3111	50.51±7.72	48.80±7.72	46.01±4.68	0.8687	
Tc	37.25±0.16	37.19±0.08	37.18±0.11	0.9405	37.20±0.12	36.99±0.13	37.27±0.08	0.2111	
Troponin	16.01±5.37	12.35±3.14	5.36±4.47	0.2735	9.81±4.28	8.53±6.25	12.64±3.12	0.7805	
ESR	72.25±6.36	48.73±3.23	42.40±4.28	0.0007	62.39±5.51	44.41±5.51	47.91±3.20	0.0426	
FBS	149.49±32.21	171.87±15.36	191.97±21.71	0.5300	192.27±28.69	162.35±24.89	174.37±15.21	0.7309	
Ferritin	338.52±108.14	415.66±56.97	491.20±70.41	0.4656	338.20±86.32	404.81±100.8	469.95±52.19	0.4149	
HB	9.40±0.58	11.21±0.27	10.87±0.36	0.0233	10.52±0.42	11.35±0.45	10.84±0.28	0.4110	
INR	1.07±0.03	1.05±0.01	1.04±0.02	0.6998	1.02±0.03	1.02±0.02	1.06±0.01	0.3379	
K	4.32±0.17	4.21±0.08	4.44±0.10	0.2513	4.38±0.12	4.18±0.14	4.32±0.08	0.5650	
L	22.07±3.21	19.50±1.70	19.70±2.11	0.7738	15.37±2.56	21.02±2.56	21.34±1.61	0.1332	
LDH	624.70±71.73	563.65±38.57	600.71±52.69	0.7052	599.50±58.89	509.81±61.51	606.51±37.96	0.3953	
Mg	2.04±0.16	2.01±0.08	2.07±0.11	0.9263	2.04±0.13	2.05±0.14	2.03±0.08	0.9856	
Na	136.0±1.28	135.0±0.63	135.70±0.78	0.6906	135.08±0.96	136.09±1.02	135.24±0.60	0.7314	
P	71.44±3.27	74.07±1.73	71.43±2.10	0.5698	80.0±2.55	71.15±2.55	70.61±1.58	0.0073	
PR	81.81±3.86	90.79±1.94	90.32±2.67	0.1150	87.50±2.94	90.23±3.24	89.83±2.02	0.7697	
RR	21.51±1.16	20.18±0.57	20.85±0.79	0.5464	21.20±0.83	20.98±0.97	20.10±0.58	0.5030	
PT	12.30±0.29	12.23±0.14	12.39±0.15	0.7710	12.13±0.20	12.10±0.21	12.45±0.12	0.2502	
PTT	31.7±2.28	32.31±1.14	34.24±1.17	0.4152	33.38±1.67	33.75±1.74	32.74±1.02	0.8640	
Systolic	116.23±5.47	116.26±2.74	115.92±3.72	0.9972	110.27±3.90	124.25±4.44	115.94±2.68	0.0666	
Diastolic	72.35±3.26	69.93±1.63	69.91±2.21	0.7918	68.28±2.39	69.66±2.71	71.43±1.64	0.5399	
SPO ₂ A	64.19±9.63	60.61±5.54	68.96±7.64	0.6803	54.59±8.78	76.03±8.0	61.48±5.24	0.1749	
First PO ₂	92.90±3.51	87.15±1.64	91.12±2.30	0.1958	88.15±2.55	92.38±2.81	88.26±1.69	0.4222	
Fifth PO ₂	95.42±1.83	92.69±0.93	93.47±1.31	0.4138	92.52±1.42	94.29±1.69	93.37±0.94	0.7233	
Discharge PO ₂	95.66±1.61	94.83±0.88	93.96±1.23	0.6958	93.49±1.33	95.37±1.66	95.05±0.83	0.5617	
WBC	7723.91±1137.82	9359.47±561.02	8194.88±690.99	0.2715	8975.64±856.19	8130.55±898.06	8874.46±543.24	0.7422	
PLT	245.858.8±29,097.4	246.858.1±14,037.6	225,141.2±17,554.7	0.6101	221,045.3±21,101.6	259,751.2±22,930.3	239,738.3±13,561.2	0.4636	

Significant *p*-Values (A *p*-value less than 0.05) were shown as bold

ALB albumin; ALP alkaline phosphatase; ALT alanine transaminase; AST aspartate aminotransferase; BS blood glucose; Ca calcium; CPK creatine phosphokinase; CR creatinine; CRP C-reactive protein; Tc temperature cycle; Troponin troponin; ESR erythrocyte sedimentation rate; FBS fast blood sugar; Ferritin ferritin; HB hemoglobin; INR international normalized ratio (PT test); K potassium; L lymphocyte; LDH lactate dehydrogenase; Mg magnesium; Na sodium; P pulse rate; PR prothrombin time; PTT partial thromboplastin time; Systolic systolic blood pressure (the pressure in the heart beats); Diastolic diastolic blood pressure (the pressure in the heart beats between beats); SPO₂ saturation of peripheral oxygen; FirstPO₂ partial pressure of oxygen at the time of hospitalization; FifthPO₂ partial pressure of oxygen during hospitalization; DischargePO₂ partial pressure of oxygen after hospital discharge; WBC white blood cell count; PLT platelet

on Asian populations (Pourbagheri-Sigaroodi et al. 2020; Yamamoto et al. 2020; Verma et al. 2021), reflecting the ethnicity-specific allelic effect. A linear regression analysis indicated an association between D/D genotype and COVID-19 mortality in 25 European nations (Bellone and Calvisi 2020). Other researchers have noted opposite results and have recognized I allele as a predictive marker of COVID-19 severity (Delanghe et al. 2020a, b; Hubacek et al. 2021). Additionally, a few studies have reported no association between this SNP and the disease severity (Çelik et al. 2021). However, the probable reasons for this difference among the abovementioned studies are due to ethnicity, sample size, and the protocol performed.

It has been stated that several ACE2 variants are correlated to various diseases such as hypertension, whose incidence depends on the balance in the renin–angiotensin–aldosterone pathway (Lu et al. 2012; Hidalgo-Tenorio et al. 2017; Pan et al. 2018). Some of these variants influenced the response to ACE inhibitors (Fan et al. 2007; Chen et al. 2016). These variants may be particularly undesirable in men who have only one copy of the X-linked ACE2 gene. The rs2285666 polymorphism of the ACE2 gene is located at the splicing site, which can affect the processing of total ACE2 RNA into mRNA and, ultimately, the amount of protein. In this regard, a study reported higher levels of circulating ACE2 in male than in female (Salah and Mehta 2021). Furthermore, rs2285666 (A allele) is related with a lower probability of cardiovascular death in female (Vangjeli et al. 2011; Malard et al. 2013; Pan et al. 2018). Our finding shows no association between rs2285666 SNP and the severity of the disease which is in agreement with previous studies (Gómez et al. 2020).

Another study verified the serum ACE2 levels in A-carriers of rs2285666 was significantly higher compared to G-homozygotes reflecting the effect of rs2285666 on serum ACE2 levels (Wu et al. 2017). Therefore, it is hypothesized that these functional variants of ACE2 can affect the severity of pathogenicity. Moreover, pathogenic variants of ACE2 are very rare on a population scale, and survival is not possible without the receptors for this gene. In addition, according to the databases associated with human genome variants, there is no missense change in the encoded sequence of the ACE2 gene. At a frequency of minor alleles less than 0.01, only four missense SNPs have been investigated, and rs2285666 is the variant that can affect splicing (Gómez et al. 2020).

Recently, rs190509934 genetic variant was identified through a genome-wide association study as a low-frequency variant (minor allele frequency 0.2–2%) near ACE2 gene that downregulates ACE2 expression and affect the risk of SARS-CoV-2 infection, providing evidence that ACE2 expression levels influence COVID-19 risk (Horowitz et al. 2022). We exploited GWAS meta-analysis data from the COVID-19 Host Genetics Initiative (Initiative 2020) for

two genetic variants were associated with severe COVID-19 at the genome-wide significance level ($P \leq 5 \times 10^{-8}$). The results indicated that both rs190509934 and rs2285666 SNPs at the ACE2 gene showing suggestive association with the severity of disease (Supplementary Table 1).

Furthermore, to evaluate the association between SNPs and gene expression level to better understanding the mechanism of disease susceptibility, the expression quantitative trait locus (eQTL) analysis in different tissues was performed using the GTEx database (<http://www.gtexportal.org/home/>). The eQTL analysis from GTEx indicated that rs2285666 variant (chrX_15592225_C_T_b38) was associated with ACE2 gene expression in the different tissues, including nerve tibial (NERVET), brain nucleus accumbens (BRNNCC), brain hypothalamus (BRNHPT), brain frontal cortex (BRNCTXB), brain cortex (BRNCTXA), and brain caudate (BRNCDT). The relative expression of ACE2 gene was significantly lower in subjects with the CC genotype of rs2285666 compared with those carrying the CT or TT genotype (Fig. 3).

As presented, the association of the rs4646994 and rs2285666 polymorphisms with COVID-19 severity is inconsistent among former studies around the world, and possible reason is due to ethnic variations between nations as the above-mentioned variants indicate some population-based differences (Srivastava et al. 2020; Sarangarajan et al. 2021). I/D ACE polymorphism revealed significant geographical and ethnic variability like an increase at the I allele frequency in Asian and African population compared to that in Caucasians (Hubacek et al. 2021). In our study, we worked on patient subjects, individuals who suffering from COVID-19 disease, and assessed association between obtained genotypes and COVID-19 severity. Regardless of variety at methodology, sample size, ethnicity among former researches, we have obtained similar results. Therefore, we may encounter contradictory findings but this should not undervalue the importance of ACE and ACE2 genes variants in COVID-19 severity.

Additionally, our analysis revealed a remarkable association between rs4646994 SNP and the HB and ESRI levels in patients ($P < 0.05$). Although the ACE2 rs2285666 SNP was not related to the severity of the disease, the SNP was significantly associated with ALT, ESRI, and P. Erythrocyte sedimentation rate (ESR) is used as a marker for indicating inflammation, and mostly shows changes in a variety of plasma proteins (Tan et al. 2020). In the present study, ESR showed a significant association with the rs4646994 and rs2285666 SNPs, which could play an eminent role in assessing the severity of COVID-19. Although there are few studies available evaluating ESR as a single predictor of the prognosis and mortality in COVID-19 patients (Kaya et al. 2021), However, these results remain controversial because some other studies have reported no

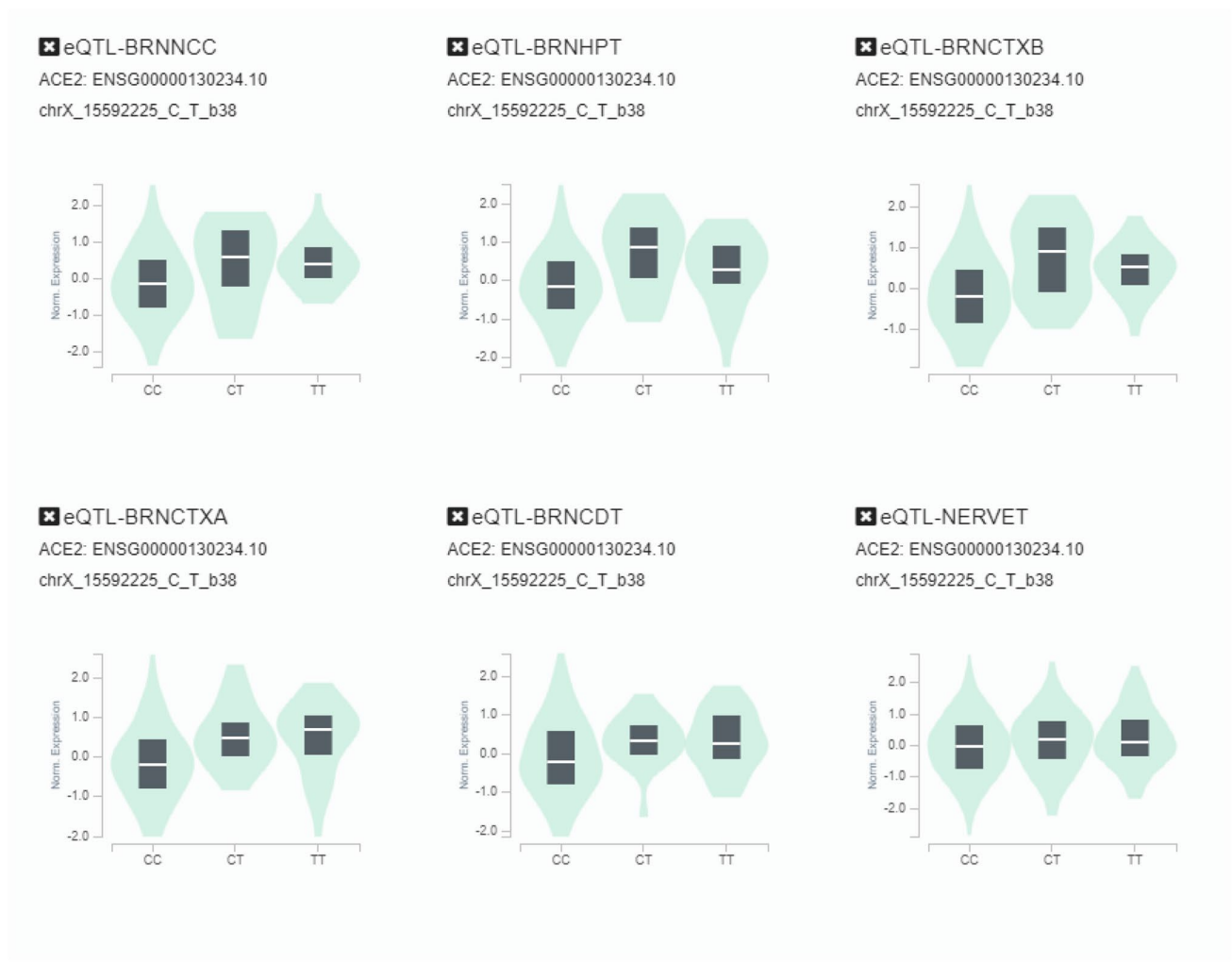


Fig. 3 eQTL violin plot of rs2285666 variant. The genotype of rs2285666 was associated with gene expression of ACE2 in different tissues on GTEx database

significant difference in the serum levels of ESR between the two severe and no-severe groups (Zeng et al. 2020).

Conclusion

The results of our study suggest that ACE D/D homozygote patients may be at an increased risk of symptomatic COVID-19. Thus, ACE I/D SNP may be a biomarker to predict the COVID-19 severity and may influence on its treatment strategies to offer a population-based therapeutic development. The ACE2 rs2285666 variant was not associated with severity of the disease. A significant association between rs4646994 SNP and the HB and ESRI levels in patients was observed ($P < 0.05$). ACE2 rs2285666 SNP was also significantly associated with ALT, ESRI, and P.

Limitation

Our study has several limitations, mainly the reduced sample size of the patients. In addition, we could not assess individuals exposed to the virus and show resistant to infection; thus, they have not any disease symptoms. These individuals are important for the detection of genetic variants related to disease susceptibility.

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Author contributions All the authors contributed to this work by performing the genetic and statistical analysis. MN wrote the manuscripts. All the authors read and approved the submission of this paper.

Declarations

Conflict of interest The study was approved by Ethics Committees of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1400.9183), and written informed consent was obtained from all subjects. The authors declare that there is no conflict of interest.

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