Differential impact of the angiotensin-converting enzyme-2 (ACE2 rs4343 G>A) and miR-196a2 rs11614913 C>T gene alterations in COVID-19 disease severity and mortality

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Abstract. The recent coronavirus outbreak from Wuhan China in late 2019 caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) resulted in a global pandemic of coronavirus-19 disease (COVID-19). Understating the underlying mechanism of the pathogenesis of coronavirus infection is important not only because it will help in accurate diagnosis and treatment of the infection but also in the production of effective vaccines. The infection begins when SARS-CoV-2 enters the cells through binding of its envelope glycoprotein to angiotensin-converting enzyme2 (ACE2). Gene variations of ACE2 and microRNA (miR)-196 are associated with viral infection and other diseases. The present study investigated the association of the ACE2 rs4343 G>A and miR-196a2 rs11614913 C>T gene polymorphisms with severity and mortality of COVID-19 using amplification refractory mutation system PCR in 117 COVID-19 patients and 103 healthy controls from three regions of Saudi Arabia. The results showed that ACE2 rs4343 GA genotype was associated with severity of COVID-19 (OR=2.10, P-value 0.0028) and ACE2 rs4343 GA was associated with increased mortality with OR=3.44, P-value 0.0028. A strong correlation between the ACE2 rs4343 G>A genotype distribution among COVID-19 patients was reported with respect to their comorbid conditions including sex (P<0.023), coronary artery disease (P<0.0001), oxygen saturation <60 mm Hg (P<0.0009) and antiviral therapy (0.003). The results also showed that the CT genotype and T allele of the miR-196a2 rs11614913 C>T were associated with decreased risk to COVID-19 with OR=0.76, P=0.006 and OR=0.54, P=0.005, respectively. These results need to be validated with future molecular genetic studies in a larger sample size and different populations.

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Introduction

A recent and ongoing pandemic that originated from Wuhan China, caused by a new β coronavirus termed severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) results in a disease termed coronavirus-19 disease (COVID-19) (1). COVID-19 presents with varied clinical features ranging from asymptomatic course to acute respiratory distress syndrome associated with high morbidity and mortality (2,3). The majority of COVID-19 patients (~80%) recover by their own in due course of time, but the rest suffer from moderate to severe disease (4). To date, ~29 million people have been infected with COVID-19 resulting in more than 5.4 million

mortalities (https://www.worldome-69 ters.info/coronavirus, accessed on Jan, 02, 2022).

COVID-19 has been associated with age, blood group type and ACE-2 gene polymorphism (5-7). The severity of the disease has also been linked with some comorbidities including hypertension, obesity and diabetes (8,9). Angiotensin converting enzyme (ACE) converts angiotensin (Ang) I to Ang II and breaks down bradykinin which serves a role in the control of blood pressure (10). ACE2 converts Ang II into Ang (1-7), which is a vasodilatory peptide (11). The ACE2 gene is found on chromosome Xp22 (12). The ACE and ACE2 share 42% amino acid similarity as the ACE2 originates through duplication of genes (12). The ACE2 is a glycoprotein and consists of 805 amino acids (12). The N-terminal of ACE2 (catalytic domain) is a signal peptide region containing an HEXXH zinc binding metalloprotease motif (12,13). The C-terminus of the ACE2 is the functional transmembrane domain (12).

ACE2 is expressed in the respiratory system, renal system, lungs, heart, blood vessels, testes, gastrointestinal tract and central nervous system (12). ACE and ACE2 gene variations are associated with different diseases such as hypertension, cardiovascular disease (CVD) and diabetes mellitus (14-16). The ACE2 counterbalances the ACE to regulate the level of circulating Ang II (15). Ang II is the main effector of the classic RAS (15). RAS dysfunctions are associated with pulmonary injury and acute respiratory distress syndrome caused by a number of factors such as viral infections (17). Dysregulation of ACE2 expression is associated with CVD in experimental models (15). In humans, the levels of ACE2 are elevated in atherosclerosis and heart failure (15). The SARS-CoV-2 uses the spike glycoprotein on its envelope to bind the ACE2 and enter the host cells (18). It has been reported that the binding between the spike glycoprotein of the novel coronavirus (2019-nCoV) is stronger than the binding between the ACE2 with the spike glycoprotein of the SARS virus (19). It is suggested that the ACE2 levels correlate with SARS-CoV-2 infection susceptibility (13). Our recent work found a strong association between ACE2 DD genotype and COVID-19 mortality and also reported that two genotypes ACE2-CC and CT are associated with COVID-19 severity (20).

microRNAs (miRNAs) are short non-coding RNA molecules with 18-23 nucleotides and are involved in the regulation of the expression of their target genes (21). They serve important roles in differentiation, apoptosis, inflammation, diabetes, cardiovascular disease and also in diagnosis and prognosis of various diseases (22). The genome wide association studies uncovered the association of different miRNA loci with different diseases (23-26).

It has been reported that miR-196b inhibits the hepatitis C virus (HCV) replication (27) and is gradually upregulated following COVID-19 infection (28). In a report from Turkey, miR-1962a rs3217927 SNP was found to be a very effective prognostic marker for multiple myeloma (29) but to the best of the authors' knowledge the role of miR-1962a rs3217927 SNP in COVID-19 has not been reported. The present study investigated the association of ACE2 rs4343 G>A and miR-196a2 rs11614913 C>T gene variations with the COVID-19 disease severity and mortality in a patient population from the Asir and Tabuk regions of Saudi Arabia.

Materials and methods

Study population. The present collaborative and population-based case-control study involved 117 COVID-19 patients and 200 healthy controls. The blood specimens from 117 reverse transcription (RT) PCR confirmed positive COVID-19 patients were collected from different hospitals in Saudi Arabia (Bisha, Abha and Tabuk; Table I). The patient group included 85 males and 32 females with a male to female ratio of 2.66 and their ages ranged between 32 and 69 years. The recruitment time for the patients was between January 15, 2021 and August 31, 2021. The ethical approvals were obtained from three local institutional ethics committees of College of Medicine, University of Bisha (Ref. no. UBCOM/H-06-BH-087(05/25), University of Tabuk (Decision no. KAEK2020/4/4) and College of Medicine, King Khalid University, Abha (Ref. no. H-06-B-091) in accordance with local guidelines which complied in essence with the principles of the Helsinki Declaration. Written informed consent was obtained before the collection of blood samples from the patients.

Data collection. A structured and bilingual (Arabic and English) questionnaire was given to all study subjects before enrolling for the present study. The subjects were interviewed for details of epidemiological/demographic data, history of co-morbid conditions such as cardiovascular diseases, type 2 diabetes mellitus (T2DM), history of addiction particularly smoking and family history of any other significant diseases.

Sample collection from COVID-19 patients. A lavender top (LT) tube containing EDTA was used for the collection of 3 ml of peripheral blood from all the COVID-19 patients. The blood specimens were immediately stored at -20˚C until further analyses.

Sample collection from control subjects. Written consent was obtained from healthy and age matched controls and the purpose of their participation was explained to them using a structured bilingual questionnaire. The sample collection was timed in such a way that it coincided with the routine blood draws of such subjects who reported to the hospital for their routine health checkups. This group comprised of RTPCR confirmed negative individuals who attended hospital for general health checkups. As a matter of policy, RTPCR was conducted on all those individuals who wanted to see a physician in the outpatient departments during first wave of COVID-19 pandemic. 3 ml peripheral blood samples were collected in LT tubes containing EDTA and were immediately stored at -20˚C until further analyses.

Genomic DNA extraction. A commercial kit from Qiagen GmbH (DNeasy) was used for DNA extraction according to the instructions provided by the manufacturer. The extracted DNA from patients and control group was dissolved in nuclease-free water and was stored at 4˚C further analyses. NanoDrop (Thermo Fisher Scientific, Inc.) was used to establish the quality and integrity of extracted DNA samples. The ratio of optical density at 260 nm (OD_{260}) and 280 nm (OD_{280}) was used to verify the purity of the DNA samples. The OD_{260}/OD_{280} ratios ranged from 1.83-1.99, thus confirming good quality DNA.

Table I. Baseline characteristics of the COVID-19 patients.

Patient characteristics	n=117	%
Age (years)		
>40	97	82.90
≤40	20	17.09
Sex		
Male	85	72.64
Female	32	27.36
CKD		
Yes	11	9.40
No	106	90.60
T2DM		
Yes	47	40.17
No	70	59.83
Oxygen saturation		
<60	47	40.17
>80	70	59.83
Hypertension		
Yes	37	31.62
No	80	68.37
CAD		
Yes	17	14.53
No	100	85.47
Duration in hospital (days)		
>30	57	48.71
<30	60	51.29
CRP		
<10 mg/l	13	2.56
≥10 mg/l	104	97.44
ALT		
<36 U/l	72	61.53
>36 U/l	45	38.57
AST		
<40 U/l	69	58.97
>40 U/l	48	41.3
Steroids therapy		
Yes	77	65.81
No	40	34.19
Antiviral therapy		
Yes	79	67.52
No	38	32.48
Survival		
Yes	43	36.75
No	74	63.24

CKD, chronic kidney disease; T2DM, type 2 diabetes mellitus; CAD, coronary artery disease; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate transaminase.

Genotyping of ACE2-rs4343 G>A and miR-196a2 rs11614913 C>T. ACE2 rs4343 G>A and miR-196a2 rs11614913 C>T genotyping was performed by using amplification refractory mutation system (ARMS-PCR) on T100 Thermocycler from Bio-Rad Laboratories, Inc. Primer3 software (version 4, https://primer3.ut.ee/) was used to design ARMS PCR primers and the details are given in Table II.

Preparation of PCR cocktail. A 25 μl ARMS-PCR cocktail, containing 50 ng DNA was prepared by adding 0.25 μl solution containing 25 pmol of Fo, Ro, FI and RI primers respectively. 10 μl PCR master mix (DreamTaq Green, Thermo Fisher Scientific, Inc.) was added and the final volume of 25 μl was made by using nuclease-free double distilled water.

Thermocycling conditions. The thermocycling conditions included a hot start at 95˚C for 8 min, followed by 40 amplification cycles at 95˚C for 35 sec, 60˚C for miR-196a2 rs11614913 C>T and 58˚C for *ACE2* rs4343 (2350A>G) for 40 sec and 72˚C for 45 sec. This was followed by an elongation step at 72˚C for 10 min and storage at 4˚C.

Gel electrophoresis for ACE2 rs4343 G>A. The PCR products of *ACE2* rs4343 (2350A>G) genotyping were separated by electrophoresis on 2% agarose and visualized on a UV transilluminator. GelPilot 100 bp Plus ladder (100) from Qiagen (cat. no. 239046) was used as a marker. Primers Fo and Ro flank the exon of the *ACE2* rs4343 (2350A>G) gene and gave a band corresponding to 268 bp that acted as a control for quality and quantity of DNA. Primers FI and Ro that amplified T allele gave a band corresponding to 190 bp and primers Fo and R1 gave a band corresponding to 125 bp from the allele G as depicted in Fig. 1.

Gel electrophoresis for miR-196a2 rs11614913 C>T. The ARMS-PCR products for *miR-196a2* were analyzed by electrophoresis on 2% agarose gel and visualized on a UV transilluminator. Primers Fo and Ro flanked the exon of the miR-196a2 rs11614913 C>T gene and gave a band corresponding to 297 bp that acted as a control for quality and quantity of DNA. Primers F1 and Ro amplified T allele and generated a band corresponding to 199 bp and primers Fo and R1 gave a band corresponding to 153 bp from the C allele as depicted in Fig. 2.

Healthy controls

For ACE2 rs4343 G>A gene polymorphism. The age matched and healthy control group comprised 103 subjects out of whom 70 (67%) were males and 33 (33%) were females. The age distribution of the control group showed that 75 (72%) patients were >40 years and 28 (27%) were ≤40 years old.

For miR-196a2 rs11614913 C>T gene polymorphism. The miR-196a2 rs11614913 was studied in 200 age matched healthy controls comprising 130 (65%) males and 70 (35%) females. The age distribution of the control group showed that 146 (73%) were >40 years and 54 (27%) were ≤40 years old.

Statistical analysis. Deviations from Hardy-Weinberg disequilibrium (HWD) were calculated by Chi-square (χ^2) goodness-of-fit test. Group differences were compared using Student's two-sample t-test and one-way analysis of

Table II. ARMS primer details.

Direction	Sequence	Product size	Annealing temperature
ARMS primers for *ACE2* rs4343 (2350A>G)			
*ACE*rs4343 FO	5'-CTGAAATTCTCTGAGCTCCCCT-3'	268 bp	58˚C
*ACE*rs4343 RO	5'-GAAAATGAAGGGACCCAAGTGC-3'		
*ACE*rs4343 FIA	5'-CTGACGAATGTGATGGCCCCA-3'	190 bp	
*ACE*rs4343 RIG	5'-CATAACAGGTCTTCATATTTCCGGTAC-3'	125 bp	
ARMS primers for miR-196a2 rs11614913 C>T			
miR-196a2 FO	5'-ACCCCCTTCCCTTCTCCTCCAGATAGAT-3'	297 bp	61˚C
miR-196a2 RO	5'-AAAGCAGGGTTCTCCAGACTTGTTCTGC-3'		
miR-196a2 FI (T allele):	5'-AGTTTTGAACTCGGCAACAAGAAACGGT-3'	199 bp	
miR-196a2 RI (C allele)	5'-GACGAAAACCGACTGATGTAACTCCGG-3'	153 bp	

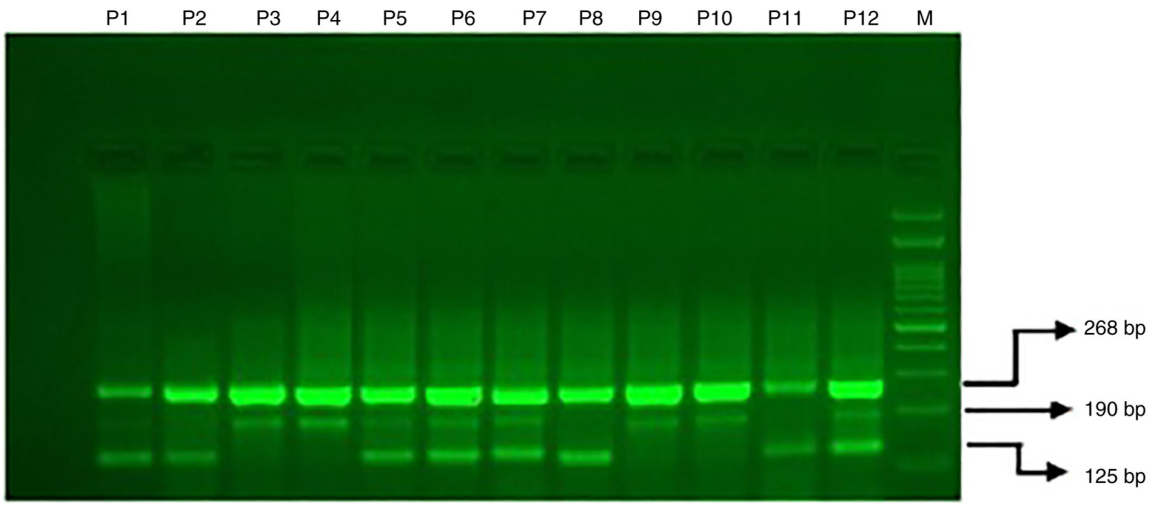

Figure 1. ACE2 rs4343 (2350A>G) genotyping utilizing amplification refractory mutation system (ARMS-PCR) in COVID-19 patients. M, 100 bp DNA ladder; P1, P2, P5, P6, P7 and P12, heterozygous; P3, P4, P9 and P10, homozygous GG-(190 bp); P2, P8 and P11, homozygous TT-(125 bp).

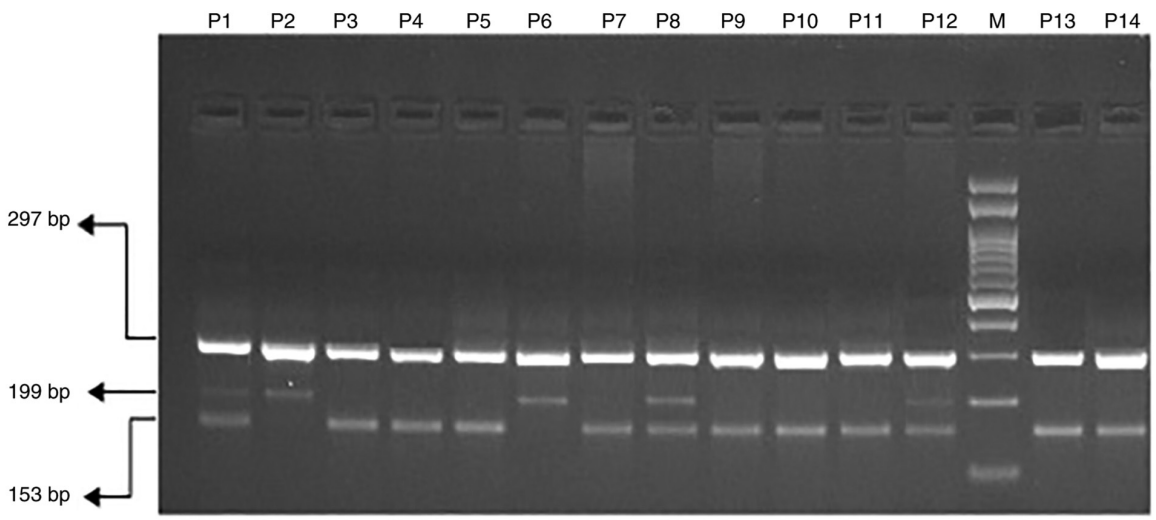

Figure 2. MicroRNA-196a2 rs11614913 C>T genotyping utilizing amplification refractory mutation system (ARMS-PCR) in COVID-19 patients. M, 100 bp DNA ladder; P1, P8 and P12, heterozygous; P3, P4, P5, P7, P9, P10, P11, P13 and P14, homozygous CC-(153 bp); P2 and P6, homozygous TT-(199 bp).

Table III. Association of ACE2 rs4343 G>A gene variation in COVID-19 cases and controls.

Subjects	n=	GG %	GA %	AA %	G	A	Degree of freedom	χ^2	P-value
Cases	117	57 (48.71)	53 (45.29)	7 (5.98)	0.71	0.29	2	6.10	0.047
Controls	103	65 (63.10)	30 (29.12)	8 (7.76)	0.78	0.22			

COVID-19, coronavirus-19 disease.

variance (ANOVA) with Tukey's post hoc test for continuous variables and χ^2 for categorical variables. Differences in the ACE-rs4646994 I/D, and ACE2 rs4240157 C>T allele and genotype frequencies between groups were evaluated using χ^2 test. The associations between ACE2-rs4646994 I/D, ACE2 rs4240157 C>T genotypes, miR-196a2 rs11614913 C>T and risk of Covid-19 patients were estimated by computing the odds ratios (ORs) and risk ratios (RRs) with 95% confidence intervals (CIs). Allele frequencies among patients as well as controls were evaluated by using the χ^2 Hardy-Weinberg equilibrium (HWE) test. All statistical analyses were performed with SPSS 16.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Demographic characteristics and baseline features. The demographic features and the baseline characteristics for 117 COVID-19 patients are given in Table I. Of the patients, 97 (82.90%) were >40 years of age and 20 (17.10%) patients were ≤40 years old. From the patients, 85 (72.64%) were male and 32 (27.36%) were female. Regarding the co-morbidities, 47 (40.17%) were T2DM patients, 37 (31.62%) had hypertension and 11(9.40%) had chronic kidney disease. A total of 47 (40.17%) patients had low oxygen saturation (<60 mm Hg) at the time of admission and 57 (48.71%) patients stayed >30 days in hospital. In the COVID-19 patient group, 79 (67.52%) patients received antiviral therapy whereas 77 (65.81%) received steroid therapy. Out of 117 COVID-19 patients, 43 (36.75%) patients succumbed and 74 (63.24%) survived and were discharged from the hospital. As can be seen in Table I, out of 117 COVID-19 patients, 45 (38.57%) had elevated levels of alanine aminotransferase, 104 (97.44%) had high levels of C-reactive protein and 48 (41.3%) had high levels of aspartate transaminase (AST).

Association of ACE2 rs4343 G>A SNP between COVID-19 patients and controls. The present study found the frequency of ACE2 rs4343 G>A in compliance to the Hard-Weinberg equation (HWE) in all the study subjects and randomly chose only 10% samples from control group to analyze genotyping results, ensuring an accuracy rate of more than 99%. The GG, GA and AA genotype frequencies were 48.71, 45.29 and 5.98% in COVID-19 patients respectively, whereas in healthy controls GG, GA and AA genotype frequencies were 63.10, 29.12 and 7.76% respectively (Table III). The difference in the distribution of ACE2 rs2323G>A genotypes in COVID-19 patients and healthy controls was significant (P<0.047). The frequency of G allele (fG) was also found to be significantly higher in COVID-19 patients as compared with the control group (0.71 vs. 0.29; Table III).

Association between ACE2 rs4343G>A genotypes and COVID-19 severity. Table IV summarizes the data on the association between ACE2 rs4343G>A genotypes and risk to COVID-19. These data were obtained by using a multivariate analysis model based on logistic regression such as odds ratio (OR) and risk ratio (RR) with 95% confidence intervals (CI). The results indicated that the COVID-19 disease severity correlated significantly with ACE2 genotypes (GG vs. GA) in the codominant model with OR 2.10 CI=1.13-3.56, RR=1.47 (1.05-2.05) and P<0.016. A strong association was also observed between ACE2 GG vs. ACE2 (GA+AA) genotype in dominant inheritance model that leads to increased COVID-19 severity with OR=1.80, 95% CI=1.04-3.08, RR=1.37 (1.01-1.85) and P<0.032 as depicted in Table IV. The A allele was not associated with COVID-19 severity with an OR 1.39, 95% CI=0.90-2.15, RR=1.20 (0.93-1.54) and P-value=0.131 on making allelic comparisons. No significance was observed between different alleles and COVID-19 severity in over dominant inheritance model. The results indicated a potential dominant effect of ACE2-AA genotype but not A allele on COVID-19 severity in the patients from Asir and Tabuk regions of KSA. The results also showed that in case of overdominant inheritance model, the *ACE2 rs4343*-GG+AA vs. GA genotype of the *ACE2 rs4343 G>A* was not associated with susceptibility to COVID-19 with OR=1.89 (1.29-1.90) and P=0.170.

Association of ACE2 rs4343 G>A genotypes with gender and comorbid conditions and COVID-19 severity. Table V summarizes the statistical comparisons (P-values) of ACE2 rs4343 G>A genotypes with comorbid conditions of COVID-19 patients and disease severity. A multivariate analysis based on logistic regression such as OD and RR with 95% CI was used to analyze these results. The results showed that the there was a significant correlation between the ACE2 rs4343 G>A genotypes with respect to the sex of the COVID-19 patients (P<0.023) and COVID-19 patients having coronary artery disease (P<0.0001). Similarly, a significant correlation was also reported between ACE2 rs4343 G>A genotypes and COVID-19 patients with oxygen saturation <60 mm Hg (P<0.0009). The duration of hospital stays of COVID-19 patients also correlated with ACE2 rs4343 G>A genotype distribution (P<0.496) but a non-significant association was observed. As can be seen in Table V, a non-significant correlation was also observed between antiviral therapy and ACE2 rs4343 G>A genotypes.

Table IV. Association of the ACE2 rs4343 G>A polymorphism with the COVID-19.

Genotypes	Healthy controls (n=103)		Covid-19 cases (n=117)		OR (95% CI)	Risk Ratio (RR)	P-value
Codominant	n	%	n	%			
ACE2-GG	65	63.10	57	48.71	1 (ref.)	1 (ref.)	
ACE2-GA	30	29.12	53	45.29	2.10 (1.13-3.56)	1.47 (1.05-2.05)	0.016
ACE2-AA	08	7.76	07	5.98	1.01 (0.34-2.92)	1.01 (0.60-1.64)	0.99
Dominant							
ACE2-GG	65	63.10	57	48.71	1 (ref.)	1 (ref.)	
ACE2-(GA+AA)	38	29.12	60	45.29	1.80 (1.04-3.08)	1.37 (1.01-1.85)	0.032
Recessive							
ACE2-(GG+GA)	95	92.23	110	94.01	1 (ref.)	1 (ref.)	
ACE2-AA	08	7.76	07	5.98	0.75 (0.26-2.16)	0.86 (0.52-1.42)	0.60
Allele							
ACE2-G	160	81.63	167	71.36	1 (ref.)	1 (ref.)	
ACE2-A	46	23.46	67	28.3	1.39 (0.90-2.15)	1.20 (0.93-1.54)	0.131
Overdominant							
ACE2-G/G-A/A	73	26.16	64	21.47	1 (ref.)	1 (ref.)	
ACE2-G/A	206	73.83	234	78.52	1.29 (0.88-1.90)	1.13 (0.94-1.37)	0.17

COVID-19, coronavirus-19 disease.

Correlation of ACE2 rs4343 G>A genotypes with mortality of COVID-19 patients. In a co-dominant model, ACE2-DD genotype heterozygosity showed a strong association with increased COVID-19 mortality with OR 3.44, 95% CI=1.53-7.72 and P=0.0028 as depicted in Table VI. However, ACE2-AA genotype (GG vs. AA) was not associated with COVID-19 mortality with OR 0.51 95% CI=0.056-4.62 and P=0.55 as depicted in Table VI. In dominant inheritance model, ACE2-GA+AA genotype (GG vs. GA+AA) was strongly associated with increased COVID-19 mortality with OR 2.87, 95% CI=1.30 to 6.31 and P<0.008. However, in recessive inheritance model, ACE2-genotype (AA vs. GG+GA) was not associated with COVID-19 mortality with OR 3.7, 95% CI=0.43 to 31.86 and P<0.23. The A allele too did not show any association with COVID-19 mortality, with an OR 1.60, 95% CI=0.90-2.86 and P=0.108, on allelic comparisons. In overdominant inheritance model, ACE2-genotype (GA vs. GG+AA) was strongly associated with increased COVID-19 mortality with OR 1.89, 95% CI=1.004 to 3.58 and P<0.040.

Comparison of miR-196 rs11614913 C> SNPs between COVID-19 patients and controls. As the miR-196 rs11614913 C>T frequency was in agreement with HWE in all study subjects, only 10% samples were chosen randomly to analyze the results from the control group.

As is evident in Table VII, the CC, CT and TT genotype frequencies in COVID-19 patients were 76.92, 18.80 and 4.27% respectively whereas in healthy controls CC, CT and TT genotype frequencies were 60, 32 and 8% respectively. The distribution of miR-196a2 rs11614913 C>T genotypes between COVID-19 patients and healthy controls was significant (P<0.008). Moreover, the frequency of C allele (fC) was found to be higher among COVID-19 patients than in control group (0.86 vs. 0.76; Table VII).

Potential association of miR-196a2 rs11614913 C>T genotypes with COVID-19. A multivariate analysis based on logistic regression such as OD and RR with 95% CI was used to determine the association between miR-196a rs11614913 C>T genotypes and risk to COVID-19 and the data are summarized in Table VIII. The results showed that the CT genotype of the miR-196a2 rs11614913 was associated with decreased susceptibility to COVID-19 with OR=0.452 (0.26-0.79), RR=0.76 (0.64-0.91) and P=0.006. The T allele of the miR-196a2 rs11614913 was also associated with decreased susceptibility to COVID-19 with OR=0.54 (0.35-84), RR=0.81 (0.71-0.92) and P=0.005 (Table VIII). The results showed that in case of the overdominant model, the miR-196-CC+TT vs. CT genotype of the miR-196a2 rs11614913 was associated with decreased susceptibility to COVID-19 with OR=0.49 (0.28-0.85), RR=0.79 (0.67-0.93) and P=0.0016.

Association of miR-196a2 rs11614913 C>T genotypes with gender, comorbid conditions and COVID-19 severity. A multivariate analysis was used to elucidate the association of miR-196a2 rs11614913 C>T genotypes with sex, comorbid conditions and COVID-19 severity and the results are summarized in Table IX. The results indicated that there was a significant difference (P=0.006) in rs11614913 genotype distribution between patients >40 years old and patients ≤40 years old (Table IX). The results also showed that there

Table V. Association of ACE2 rs4343 G>A polymorphism with COVID-19 patient characteristics.

Patient characteristics	n=117	GG	GA	AA	Degree of freedom	χ^2	P-value	
Age (years)							0.44	NS
>40	97	46	44	07	1.64	2		
≤40	20	11	09	00				
Sex							0.023	SG
Male	85	42	41	02	7.47	2		
Female	32	15	12	5				
T2DM							0.61	NS
Yes	47	23	20	04	0.97	2		
No	70	34	33	03				
CKD							0.47	NS
Yes	11	07	04	00	1.5	2		
No	106	50	49	07				
Hypertension							0.11	NS
Yes	37	14	21	02	4.37	2		
No	80	43	32	05				
CAD								
Yes	17	06	05	06	30.41	2	0.0001	SG
No	100	51	48	01				
Oxygen saturation							0.0009	SG
Yes	47	15	29	03	9.24	2		
No	70	42	24	04				
Duration in hospital (days)							0.490	NS
>30	57	25	29	03	1.4	2		
<30	60	32	24	04				
ALT							0.82	NS
<36 U/l	45	23	20	02	0.39	2		
>36 U/l	72	34	33	05				
CRP								
<10 mg/l	13	51	47	06	0.09	2	0.956	NS
≥10 mg/l	104	06	06	01				
AST							0.014	SG
<40 U/l	48	30	18	0	9.14	2		
>40 U/l	69	27	35	07				
Antiviral therapy							0.003	SG
Yes	79	30	43	06	3.87	2		
No	38	27	10	01				
Steroids therapy							0.400	NS
Yes	77	35	36	06	1.82	2		
No	40	22	17	01				
Survival							0.004	SG
Yes	43	14	28	1	11.6	2		
No	74	43	25	6				

COVID-19, coronavirus-19 disease; T2DM, type 2 diabetes mellitus; CKD, chronic kidney disease; CAD, coronary artery disease; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate transaminase; NS, non-significant; SG, significant.

was a significant difference (P=0.035) in rs11614913 genotype distribution between male and female patients (Table IX). The results showed that there were significant differences in patients with hypertension and coronary artery disease

Table VI. Statistical comparisons (P-values) of ACE2 rs4343 G>A genotypes with mortality of COVID-19 patients.

Model	Genotype	Survival	Mortality	OR (95% CI)	P-value	
Codominant		n=74	n=43			
	GG	43	14	1 (ref.)		
	GA	25	28	3.44 (1.53-7.72)	0.0028	SG
	AA	06	01	0.51 (0.056-4.62)	0.55	NS
Dominant						
	GG	43	14	1 (ref.)		
	GA+AA	31	29	2.87 (1.30-6.31)	0.008	SG
Recessive						
	AA	06	01	1 (ref.)		
	GG+GA	68	42	3.7 (0.43-31.86)	0.23	NS
Allele						
	G	111	56	1 (ref.)		
	A	37	30	1.60 (0.90-2.86)	0.108	NS
Overdominant						
	GG+AA	49	15	1 (ref.)		
	GA	148	86	1.89 (1.004-3.58)	0.040	SG

Table VII. Association of miR-196a2 rs11614913 C>T gene variation in COVID-19 cases and controls.

Subjects	n	CC	CT	TT	Degree of freedom	χ^2	C	T	P-value
Cases	117	90 (76.92%)	22 (18.80%)	05 (4.27%)	2	9.48	0.86	0.14	0.008
Controls	200	120 (60%)	64 (32%)	16 (8%)			0.76	0.24	

COVID-19, coronavirus-19 disease.

(CAD) compared with patients without hypertension and CAD (P=0.044 and 0.035, respectively; Table IX). Results also indicated that there was a significant difference (P=0.01) in patients with oxygen saturation <60 and those with oxygen saturation >80. Furthermore, the results showed that there was a significant difference (P=0.01) in rs11614913 genotype distribution between the patients who survived from the COVID-19 and the patients who succumbed (Table IX).

Discussion

The diverse clinical manifestations of the SARS-CoV-2 infection vary from no symptoms to severe disease (ICU admission) and mortality in COVID-19 patients. The results of the present study indicated that there was a significant difference in the ACE2 rs4343 G>A genotype distribution between the patient and the control groups (P<0.05; Table III). Results also showed that the GA genotype of the rs4343 G>A was associated with increased susceptibility to COVID-19 (9) (Table IV). rs4343 G>A influences the activity and the levels of ACE and increases susceptibility to hypertension, T2DM, obesity, renal disease, CVD and autoimmune diseases (30). The results of the present study are consistent with a recent study that reported the association of the G allele with the SARS-CoV-2 severity in the presence or absence of metabolic and other comorbidities (30). Furthermore, it has been suggested that GG genotype of the rs4343 SNP is associated with increased circulating ACE levels and its activity (31,32). The increased activity and levels of the ACE2 are reported to increase the susceptibility to COVID-19 (33). The results of the present study seem to be in agreement with these studies (31-33) as rs4343 GA genotype increases the activity and levels of ACE2 (30) which may increase the susceptibility to COVID-19 (9) (Table IV). The results also showed that there was a significant difference in ACE2 rs4343 G>A SNPs between male and female patients (P<0.023; Table V). This result is in agreement with earlier studies that report higher expression of ACE2 in males compared with females and the increased expression of ACE2 is reported to promote the entry of SARS-CoV-2 (9,13). It is suggested that the reduced expression of ACE2 in females renders them less sensitive to severe adverse effects of COVID-19 (13). The results of the present study also indicated that there were significant differences (P<0.05) in the rs4343 G>A genotype distribution between the patients with CAD and reduced oxygen saturation and patients without CAD and with normal oxygen saturation (Table V). This result is in agreement with a study that reports the association of the ACE2 rs4343 G>A with dyslipidemia and severity of COVID-19 (30).

Table VIII. Allele and genotype distribution of *miR*-196a2 rs11614913 C>T polymorphism in the COVID-19 patients and control groups.

Genotypes	Healthy controls	Covid-19 cases	OR (95% CI)	Risk Ratio (RR)	P-value
Codominant	(N=200)	(N=117)			
miR-196a2-CC	120	90	1 (ref.)	1 (ref.)	
miR-196a2-CT	64	22	0.452 (0.26-0.79)	0.76 (0.64-0.91)	0.006
miR-196a2-TT	16	05	0.41 (0.14-1.17)	0.75 (0.57-0.97)	0.09
Dominant					
miR-196-CC	120	90	1 (ref.)	1 (ref.)	
miR-196-CT+TT)	80	27	0.45 (0.26-0.75)	0.76 (0.65-0.89)	0.001
Recessive					
miR-196-(CC+CT)	184	112	1 (ref.)	1 (ref.)	
miR-196-TT	16	05	0.51 (0.18-1.43)	0.81 (0.63-1.05)	0.20
Allele					
miR-196-C allele	304	202	1 (ref.)	1 (ref.)	
miR-196-T allele	96	35	0.54 (0.35-84)	0.81 (0.71-0.92)	0.005
Over dominant					
miR-196-CC+TT	136	95	1 (ref.)	1 (ref.)	
miR-196-CT	64	22	0.49 (0.28-0.85)	0.79 (0.67-0.93)	0.0016

COVID-19, coronavirus-19 disease.

Moreover, the results of the present study indicated that there was a significant difference (P<0.05) of rs4343 G>A genotype distribution with elevated patient AST levels (Table V). This result is in agreement with an earlier study that reports the association of SARS-COV-2 with liver dysfunction (34). As ACE2 is expressed in the hepatic tissues (9), it is possible that the rs4343 G>A SNP modulates the SARS-COV-2 infection and increases the liver damage but this need further validation. The results of the present study also indicated that there was no significant difference (P>0.05) in rs4343 G>A genotype distribution between diabetic and non-diabetic subjects (Table V). This result was rather unexpected and was inconsistent with a study that reported diabetes to increase the susceptibility to coronavirus infection since ACE2 is highly expressed in T2DM patients (9). This inconsistency may be because the sample size used in the present study was relatively small (n=117). In addition, the results showed that the genotype distribution of rs4343 G>A was significantly different (P<0.05) between patients who needed antiviral therapy and those who did not (Table V). This result is in agreement with Íñiguez *et al* (30), who report that the G allele of the rs4343 increases the severity of COVID-19. The results of the present study also showed that there was a significant difference (P<0.05) in ACE2 rs4343 G>A genotype distribution between patients who survived and those who did not (Tables V and VI), i.e. the G allele of the rs4343 increased the severity and mortality of COVID-19. Again, this result is in agreement with Íñiguez *et al* (30), who demonstrate that the ACE2 rs4343 G allele increases the severity of COVID-19.

miRNAs serve important and diverse roles in cellular physiology and pathology including immunity, development, apoptosis and types of cancer (35-38). miRNA gene variations are associated with various metabolic diseases (25,26,39-41) and have been demonstrated to influence the susceptibility to viral infections and the clinical course of the viral disease (42,43). miR-196 is found in the regions of homeobox clusters within the vertebrates genome (44) and is located in the 3'-untranslated region of the miR-196a2 precursor. Polymorphism of miR-196 rs11614913 not only influences the transcription level of mature miR-196a, but also has a biological effect on target gene production (42).

The results of the present study indicated that the miR-196a rs11614913 C>T genotype distribution was significantly different (P<0.05) between patients and controls (Table VII). The results also showed that the CT genotype and the T allele of the miR-196a rs11614913 C>T were associated with the decreased risk to COVID-19 (Table VIII). It is reported that miR-196 is among interferon-induced miRNAs and that miR-196 directly targets the CORE and NS5A coding region of genomic RNA of the HCV and thereby suppresses the replication of the virus by ≤80% (45). In addition, it has been demonstrated that miR196 inhibits the expression of the HCV (46) by repressing the expression of the Bach-1 protein (46). Bach-1 is an inhibitor of the anti-oxidative and anti-inflammatory heme oxygenase 1 (HMOX1) (46,47). miR-196 mimics significantly repress the expression of the protein Bach1 and upregulate the gene expression of HMOX1 and thereby inhibit the HCV expression (46). In an experiment conducted in lung tissues of hamster, it was shown that miR-196a is among five miRNAs that commonly bind to SARS-CoV, MERS-CoV and SARS-CoV-2 viruses (48). It is reported that miR-196a is gradually upregulated after SARS-CoV-2 infection (48). The present study hypothesized that rs11614913 affected the immune response against SARS-CoV-2 and that the T allele and CT genotype carriers became less susceptible to the SARS-CoV-2 infection (Table VIII). The results are in partial agreement with the result of Tian *et al* (47), who report that miR-196a-2 C>T (rs11614913) is probably

Table IX. Allele and genotype distribution of miR-196a rs11614913 C>T polymorphism in the COVID-19 patients.

Patient characteristics	n=117	CC	CT	TT	Degree of freedom	χ^2	P-value
Age (years)							0.006
>40	97	82	30	05	02	10.18	
≤40	20	12	06	02			
Sex							0.035
Male	85	63	20	02	02	6.69	
Female	32	27	02	03			
T2DM							0.16
Yes	47	36	10	00	02	3.6	
No	70	54	12	05			
CKD							0.017
Yes	11	08	06	02	02	8.0	
No	106	82	16	03			
Hypertension							0.044
Yes	37	28	05	04	02	28	
No	80	62	17	01		62	
CAD							0.035
Yes	17	15	00	02	02	6.68	
No	100	75	22	03			
Oxygen saturation							0.035
Yes	47	33	14	00	02	8.86	
No	70	57	08	05			
Duration in hospital (days)							0.83
>30	57	44	10	03	02	0.35	
<30	60	46	12	02			
ALT							0.82
<36 U/l	45	72	54	13	05	02	
>36 U/l	72	45	36	09	00		
CRP							
<0.8 mg/dl	27	27	00	00	02	10.53	0.005
>0.8 mg/dl	90	63	22	05			
AST							0.25
<40 U/l	69	55	10	04	02	2.75	
>40 U/l	48	35	12	01			
Antiviral therapy							0.42
Yes	79	58	17	04	02	1.7	
No	38	32	05	01			
Steroids therapy							0.733
Yes	77	58	16	03	02	0.62	
No	40	32	06	02			
Survival							0.010
Yes	43	31	07	05	02	9.0	
No	74	59	15	00			

COVID-19, coronavirus-19 disease; T2DM, type 2 diabetes mellitus; CKD, chronic kidney disease; CAD, coronary artery disease; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate transaminase.

associated with reduced susceptibility of HBV and HCV-related HCC, particularly in the Chinese population.

The results of the present study further showed that the carriers of the CT genotype and the T allele of the miR-196a

rs11614913 in >40-year-old patients were at reduced risk to SARS-CoV-2 infection (Table IX). It also observed that the males who are carriers of the CT genotype and T allele of the miR-196 rs11614913 were less susceptible to the SARS-CoV-2 infection compared with females (Table IX). The results also showed that miR-196 rs11614913 significantly (P>0.05) increased the risk to SARS-CoV-2 infection in patients with hypertension and CAD (Table IX). This result is consistent with a study that indicated the association of miR-196 rs11614913 with CAD (41). The results of the present study also suggested that 69% of the patients who succumbed were miR-196 rs11614913 CC genotype carriers (Table IX) suggesting that CC genotype contributed to disease severity and mortality. Limitations of the present study included the relatively small sample size. Further studies with larger sample size and on different ethnic populations are recommended.

Taken together, the present study examined the association of the ACE2 rs4343 G>A and miR-196a rs11614913 C>T with the severity and mortality of SARS-CoV-2 infection in a study population from Asir and Tabuk regions of Saudi Arabia. The results clearly indicated that the GA genotype of the ACE2 rs4343 was associated with increased severity and mortality of COVID-19. The results also showed that the CT genotype and T allele of the miR-196a rs11614913 C>T were associated with decreased susceptibility of COVID-19. More studies in different ethnic populations and bigger sample sizes are necessary to further investigate the roles of genetic alterations of ACE2 and miR-196a in the molecular pathogenesis of SARS-CoV-2 and COVID-19.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon a reasonable request.

Authors' contributions

All the authors were involved in the conception and planning of the study. MMM, RM, MAAA, RF, MMSA and MA designed the study. MAAA, BAA, AAB, MMSA and AMA were involved in the recruitment of patients. BAA, AAB and AMA collected the patient data and analyzed the clinical outcomes of patients with COVID-19. MHA, RM and IE performed the experiments. MMM, RM and IE wrote the initial draft. which was revised and edited by all the authors. MMM and MAAA were involved in the acquisition of grants and project administration. RM, MMM and IE confirm the authenticity of all raw data. All the authors read and approved the final version of the manuscript for publication and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The ethical approvals were obtained from three local institutional ethics committees of College of Medicine, University of Bisha (Ref. no. UBCOM/H-06-BH-087(05/25), University of Tabuk (Decision No: KAEK2020/4/4) and College of Medicine, King Khalid University, Abha (Ref. no. H-06-B-091) in accordance with local guidelines which complied in essence with the principles of the Helsinki Declaration. Written informed consent was obtained before the collection of blood samples from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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