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Research Article

Cancer patients with Angiotensin-converting enzyme (*ACE*) gene polymorphism and COVID-19 phenotypic expression predisposed to SARS-CoV-2 infection

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Abstract
Pathogenesis of COVID-19 has been linked to the Angiotensin system. Angiotensin-converting enzyme (ACE2) has been recognized as the specific receptor of the SARS-CoV-2 virus, serves as a cellular receptor for SARS-CoV-2, suggesting that a person's vulnerability to infection may be controlled by how much of the ACE2 gene is expressed. It is also possible that the severity of COVID-19 is related to the equilibrium between ACE1 and ACE2 activity, which has been linked to the etiology of respiratory disorders. This study aimed to investigate the association of ACE1 I/D polymorphism with the severity of Covid-19. The study looked at 113 people-(50 healthy controls, 63 people with Covid). Results for the ACE2 rs4240157 T > C polymorphism were obtained. Logistic regression was used to evaluate the distribution frequencies of variables across the study groups. The ACE1-CC*CT genotype (p = 0.049) and male gender (p0.001) were related to severe COVID-19. COVID-19 severity was found to be associated with the ACE2–CT genotype through multiple logistic regression under the co-dominant inheritance model: CC*CT Allele, 95% CI (0.0104 to 0.2954), Significance level, (0.0007) Odd Ratio (0.0556); CC*TT Allele, 95% CI (0.1854 to 6.1927), Significance level, (0.9386) Odd Ratio (1.0714); and CT*TT (19.2857). This was assuming the ACE2–CC*CT genotype was connected with covid-19 severity. However, the ACE2 polymorphism did not affect the development of illness. In conclusion, male gender, malignancy, and the ACE1 genotype were linked to a negative result of COVID-19. Our results indicated that ACE1-C/T might affect COVID-19 severity; however, this association was hypertensive status-specific. However, this finding needs to be confirmed in additional large samples.

Keywords: ACE, COVID-19, ACE1, PCR, Gene polymorphisms, TETRA-ARMS

INTRODUCTION

The coronavirus illness 2019 was caused by the severe acute respiratory syndrome coronavirus 2, often known as SARS-CoV-2. This virus is a member of the family Coronaviridae (COVID-19). Fever, coughing, and shortness of breath are the most often experienced symptoms (Wiersinga *et al*.,2020). Despite extensive investigation, it has not yet been determined what causes some people to become sick while others who are exposed to the same settings do not become infected (e.g. medical history or exposure to SARS-CoV-2). The progression of the disease can range from mild symptoms to death, and the overall fatality rate is approximately two percent (as of the 25th of January 2021), while the in-hospital mortality rate can range anywhere from twenty-eight to seventy-two percent (Karagiannidis *et al*.,2020). Furthermore, it is unknown why the course of the disease can vary so drastically between subjects with similar preconditions.

There is an outbreak of the new SARS-CoV-2 virus, which caused Coronavirus Disease 2019, in 2020 and beyond (COVID-19). At the end of 2019, it was found in the Chinese province of Hubei for the first time and the WHO declared COVID-19 a worldwide pandemic in March 2020 (Mondragon, (2020).

Host cells are infected with SARS-CoV-2 via an interaction between its spike protein and the entrance receptor ACE2 (Hoffmann *et al*.,2020). Thus, a large number of research projects focusing on ACE2 have been carried out, including treatments (Du *et al*.,2009). An androgen-induced increase in TMPRSS2 activity is required for the viral invasion process to begin, since this enzyme is required to priming viral spike proteins (Ko *et al*.,2015). SARS-CoV-2 binding to ACE2 and TMPRSS2 processing of the S protein may result in the downregulation and depletion of ACE2 (Tang *et al*., 2021). This suggests that the multi-organ failure seen in COVID-19 may have a common cause: widespread expression of ACE2 across the body. ACE1 regulates ACE2 expression and function by regulating angiotensin II (Ang II) levels, as will be addressed more below. Reduced ACE2 expression may prevent viral infections because SARS-COV-2 downregulates ACE2 expression. However, it lessens the positive effects of ACE2 in the lungs and other organs. Endothelial and neurological protection are among the many benefits of the protective RAAS, including anti-inflammatory, anticoagulant, and antifibrotic properties. At the beginning of COVID-19, the imbalance of the ACE1/ACE2 arms and the disturbance of the RAAS homeostasis appear to be the most significant.

The ACE and ACE2 activities in COVID-19 may also play an important role in the thrombo-inflammatory process. One of the main functions of ACE is to break down Angiotensin (Ang) I into Ang II, which is then converted into Ang 1-7 by ACE 2. Angiotensin II's unopposed actions on ACE2 cause vasoconstriction, endothelial damage, endovascular thrombosis, and increased blood volume in the absence of ACE2's receptor (McFadyen *et al*.; Verdecchia *et al*, 2020). A 287-base pair (bp) Alu repeat sequence in intron 16 of the ACE gene has been documented in the literature as either inserted (allele I) or deleted (allele D). Serum ACE levels and these polymorphisms have been linked, with D/D homozygotes having 65% more and I/D heterozygotes having 31% more ACE than I/I homozygotes, according to research (Rigat *et al*.,1990). The frequency of ACE D/D polymorphism has also been linked to both the prevalence and death rates of COVID-19 (Zheng & Cao; Gemmati & Tisato 2020). The current pilot research aimed to evaluate the role of the ACE I/D polymorphism in COVID-19 that was complicated by a pulmonary embolism.

MATERIALS AND METHODS

Study subjects
The practical side of the present study was done during the period from November 2021 to March 2022.Fifty healthy and sixty-three patients were selected for the study. Patients hospitalized at the Medical center in Mergan Medical City, Iraq, diagnosed with Covid -19 were compared to 50 healthy controls and 63 patients with the disease. The current study comprised 19 males and 44 females, ranging in age from 20 to 80, who were diagnosed with covid-19 using serological and molecular assays. Patients' blood and serum samples were properly analyzed.

Ethical approval
The study was approved by an independent ethics committee, and it followed the Declaration of Helsinki's guidelines for conducting ethical research. Prior to sample collection, the patient's verbal and analytical consent were obtained. Document 5907 (containing the number and the date in 8/11/2021) attests to the local ethics committee's approval of the study protocol, subject information and permission form.

Healthy control group
Fifty healthy individuals (Aged 20-80 years) from Babylon Iraqi communities who have been proven laboratory, clinically, and genetically that they do not have covid-19, were included.

Blood samples collection
The blood samples totalling around five millilitres of patients in this trial were collected. EDTA-containing tubes were used to collect around two millilitres of blood for genetic testing. Two samples of blood were taken from each subject and the first was put in gel tubes for 30 minutes; the second was centrifuged for 15 minutes and the serum was recovered and stored in the freezer (-20 ^{o}C).

Control Samples collection
Fifty healthy individuals with a similar age distribution as the patients were selected to take their venous blood samples for the study.

Table 1. Sequence of primers for ACE2 gene

Gene		Sequence	bp.
ACE2 rs4240157 T > C	F0	GCTGAGTTCTCAAAATAATGCCATAGAT	386
	R0	GCATTTCTTTCCAATCATTAAGAGTTCA	
	FI-T	GCCTCAGAACATTACAGAATCAACCT	244
	RI-C	GAGGGTTGGTAAATAGTGTTCAGTGG	194

Isolation of genomic DNA

EDTA tubes were used to collect human genomic DNA for molecular analysis, and proteinase K is advised to purify DNA from frozen blood samples. The Geneaid and Promega kits Geneaid- Ltd were used.

Estimation of DNA concentration and purity

Patients' and controls' whole blood samples were used to extract genomic DNA using the protocol for DNA separation from patient and control subjects' whole blood samples. Blood samples were analyzed using the gSYNCTm gDNA Extraction kit from fresh blood to extract DNA and RNA (Geneaid-Ltd).

With the Nano drop, 2.5 l of DNA extracted from the samples was put into the machine to measure the concentration (ng/L) and purity (OD: 260/280nm), which was used to determine the presence of protein in the samples. According to the standard 260/280 ratio for purifying DNA, this ranged from one to two. Following genomic DNA extraction, agarose gel electrophoresis was used to confirm the closeness and uprightness of the separated DNA. Acrylate dissolved in 1x TBE buffer and Safe stain were used to expose the DNA bands (75 min/100 Volt).

Primer's preparation

ACE2 rs4240157 T > C primer given in the accompanying was used to identify diagnostic and virulence genes (Table 1). Ligo /USA provided the primer. All primer pairs were spun down before removing the cap from the primer's tubes. As per the manufacturer's instructions, a specified volume of nuclease-free water was added to each primer, resulting in a primer stock solution with a 100 Pico-mole/microliter concentration. Transferring 10 l of the primer stock solution into an Eppendorf tube containing 90 l of free nuclease water yielded 10 Pico-mole/microliter of free nuclease water that was utilized in PCR amplification.

PCR was used to examine the I\D polymorphism in the ACE gene's 16th intron. Primers were used to perform the PCR (Akbari *et al.*, 2022; Yaeghmaie *et al.*, 2018). A gradient temperature was used for PCR optimization as a first step. This is critical in determining the ideal annealing temperature. It took 20 µl of total reaction volume to make up the PCR reaction mixture for gradient, which included 5 µl of template DNA, 5 l of the master mix, as well as 5 µl of each forward and reverse primer. Table 2 shows the PCR conditions for gradients.

DNA and master mix was mixed with 1.5 forward and reverse primers in 5l of PCR mixture after choosing the clearest band at the optimal annealing temperature for the ACE genes, 57°C. According to the accompanying table, PCR was carried out as in Table 3).

Tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) is a promi-

nent assay for SNP genotyping. However, some reports of published data may question the reliability of this method on some occasions, in addition to a laborious and time-consuming procedure of the optimization step (Mesrian Tanha *et al.*,2015).

The tetra-primer ARMS–PCR uses four primers in a single PCR to determine the genotype. In the beginning of the reaction, two non-allele-specific primers amplify the region that comprises the SNP. They are named outer primers. Then, the outer primers fragment is

produced, serving as a template for the two allele-specific primers (inner primers), which will produce the allele-specific fragments. By placing the outer primers at different distances from the polymorphic nucleotide, the two allele-specific fragments can be distinguished by their different sizes in an agarose gel (Rubio *et al.*, 2008).

Hardy-Weinberg equilibrium

According to the Hardy-Weinberg equilibrium, the amount of genetic diversity in a population will not change from generation to generation if no events are considered to be perturbing. Because genotype and allele frequencies are in equilibrium in a big population with no disruptive causes, the law assumes that genotype and allele frequencies would be stable in a large population where no factors might potentially disturb the equilibrium. The equilibrium predicted by Hardy and Weinberg will be thrown off by various factors, including mutations, natural selection, nonrandom mating, genetic drift, and gene transfer. Mutants, for example, throw off the balance of allele frequency distributions in a population. On the other hand, natural selection and nonrandom mating contribute to variations in gene frequency, which in turn causes the Hardy–Weinberg equilibrium to be upset. This occurs as a result of the fact that the alleles in issue either contribute to or detract from the reproductive viability of the species that bears them. Genetic drift is another factor that can potentially throw off the equilibrium, which occurs when the frequency of alleles either rises or falls at random and frequently occurs in samples that are too small. The Hardy-Weinberg equilibrium can be upset when two different species mate and new alleles are produced as a result of the process known as gene migration. Gene migration occurs whenever two different species mate and introduce new alleles into the population, causing the Hardy–Weinberg equilibrium to be thrown off balance. Because both potentially destructive forces are inherent to the natural world, the Hardy–Weinberg equilibrium only seldom holds up in practice. Consequently, the Hardy–Weinberg equilibrium depicts an idealized state, and the hereditary differences in the world may be measured as departures from it (Graffelman, *et al.,* 2017).

Table 2. Gradient condition for ACE C\T

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	45 sec	
Annealing Zones	55-57-59-61-63-65	30 sec	35
Extension	72	1	
Final extension	72	5	
Storage	4	∞	

Table 3. PCR condition for ACE C\T

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	45 sec	
Annealing	57	30 sec	35
Extension	72	1	
Final extension	72	5	
Storage	4	∞	

RESULTS AND DISCUSSION

The correlation of ACE2 rs4240157 T > C genotypes with COVID-19 severity estimated using multivariate logistic regression is given in Table 4. In the co-dominant inheritance model, ACE2–CT genotype was connected to COVID-19 severity with an CC*CT Allele; CI 95% (0.0104 to 0.2954), Significance level, (0.0007) Odd Ratio (0.0556), while CC*TT Allele; CI 95% (0.1854 to 6.1927), Significance level, (0.9386) Odd Ratio (1.0714), and CT*TT Allele; CI 95% (6.8611 to 54.2101), Significance level, (0.0001) Odd Ratio (19.2857). This genotype was linked with COVID-19 severity assuming the ACE2–CC*CT genotype was linked with covid-19 severity.

An analysis of the rs4240157 T > C polymorphism in the acetylcholinesterase gene and Its effect on COVID-19 disease intensity present data showed that ACE2 rs4240157 T > C genotypes were significantly associated with COVID-19 severity in patients (p 0.007) patients (Table 4 and 5). Recent research has identified ACE2 polymorphisms that may influence disease severity. Of the 10 SNPs studied, 5 polymorphisms (rs6632680-rs4830965-rs1476524-rs4240157-rs2048683) showed an association with higher tissue-specific expression of ACE2, leading to hospitalization; in contrast, the rs1548474 polymorphism showed a correlation with low tissue expression and less severity (Magrone *et al.*, 2020; Marshall *et al.*, 2002).

The rs2106809 polymorphism was thought to regulate ACE2 levels in the blood (Khamlaoui *et al.*,2020; Darbani *et al.*, 2020). A previous study by Xiao *et al* (2020). demonstrated that a specific point mutation in the ACE2 gene (Leu584Ala) promotes SARS-CoV-1 entrance into host cells. Recent research has shown that different amino acid variations can modulate the inter-action between the viral S1 protein and ACE2 receptors and, therefore, the severity of infection (Chen *et al.*,2020). Various ACE2 receptor-expressed amino acid residues were found to be critically important in facilitating or inhibiting viral infection. Thirteen different ACE2 polymorphisms increased ACE2/S1 recognition, making SARS-CoV-2 infection more likely, while eighteen other SNPs decreased interactions between ACE2 and S1, making SARS-CoV-2 infection less likely (Pouladi *et al.*,2021). Increased risk of hypertension was observed in people of Australian ancestry with variants of the ACE 2 gene (rs2074192, rs4240157, and rs4646188 (Patel *et al.*, 2012). The present study found that the ACE2 rs4240157 T > C gene polymorphism significantly differed between COVID-19 patients and controls (p 0.0007). Patients with COVID-19 who carry the ACE2 rs4240157 polymorphism are more likely to require hospitalization, as this variation may increase ACE2 expression in specific tissues, as described by Wooster *et al.*, 2020).

The ACE2 rs4240157 T > C gene polymorphism has been linked to high blood pressure and cardiovascular illness (Pouladi and Abdolahi, 2021). The present study found that the ACE2-CC*TT genotype was linked to a higher risk of dying from COVID-19 ACE2-CC*CT genotype was associated with COVID-19 severity with an odds ratio (OR) of 0.0556 (95% confidence interval [CI]: 0.0104 to 0.2954), p 0.0007, and that the ACE2-CC*TT genotype was associated with COVID-19 severity with an OR of 1.0714 (95% CI: 0.1854 to 6.1927), p 0.9386 (Table 5). It was found that the ACE2-CT*TT genotype linked to a higher risk of dying from COVID-19 ACE2-CT*TT genotype was linked to a higher risk of dying from COVID-19, with an odds ratio (OR) of 19.2857 (95% CI = 6.8611 to 54.2101), p 0.0001 (Table 4). The present data suggest that the ACE C/T gene

Table 4. Genotype frequency of polymorphisms of (Genotype ACE2 rs4240157 T > C) gene associated with Covid-19 patients and control

Genotype	Patients (63)		Healthy (50)		Total (113)	
	No	%	No	%	No	%
CC	13	11.50	12	10.62	25	**22.12**
CT	43	38.05	10	8.85	53	**46.90**
TT	7	6.19	28	24.78	35	**30.97**
TOTAL	**63**	**55.75**	**50**	**44.25**	**113**	**100.00**
Allele Frequency						
C	69	54.76	34	34.00	103	**45.58**
T	57	45.24	66	66.00	123	**54.42**
TOTAL	**126**	**100.00**	**100**	**100.00**	**226**	**100.00**

Table 5. Statistical analysis for Genotype (*ACE2* rs4240157)

Allele	Odd Ratio	Significance level	CI 95%
CC*CT	0.0556	0.0007	0.0104 to 0.2954
CC*TT	1.0714	0.9386	0.1854 to 6.1927
CT*TT	19.2857	0.0001	6.8611 to 54.2101
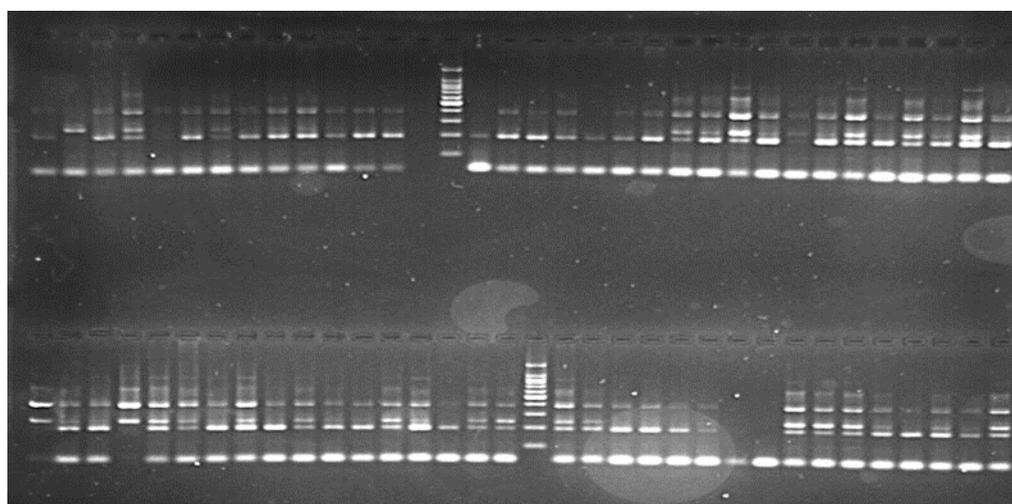
Fig. 1. *Electrophoresis Pattern of ACE2 rs4240157 T > C Polymorphisms. lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.*

polymorphism is closely linked to the clinical severity of COVID-19 disease, despite the limited sample size. Severe COVID-19 disease and death were also independently associated with older age, coronary artery disease, and Cancer. Extreme COVID-19 disease was mitigated in those who had the CC*CT genotype. Since this is the first study of its sort in Saudi Arabia, it is recommended that larger studies be conducted to examine further the association between other ACE2 genotypes and disease severity and clinical outcome in COVID-19 patients. However, none of the SNPs under investigation is widely distributed. This discovery may aid in identifying those who are more and less vulnerable to contracting COVID-19.

Conclusion

In conclusion, male gender, malignancy, and the *ACE1* genotype were linked to a negative result of COVID-19.

Our results indicated that *ACE1-C/T* might affect COVID-19 severity; however, this association was hypertensive status-specific. This finding needs to be confirmed in additional large samples.

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