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Research Article

Single nucleotide polymorphism in Angiotensin-converting enzyme-2 gene as a risk for COVID-19 among Iraqi people

Huda Khadim Hasan*
Ministry of Health and Environment, Hilla city, Iraq
Noor Salman Khadim Al-Khafaji
Biology Department, College of Science, University of Babylon, Hilla city, Iraq

*Corresponding Author email: huda.hassan.scihigh169@student.uobabylon.edu.iq

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Abstract
Angiotensin-converting enzyme-2 (ACE-2) is an essential element in the renin-angiotensin RAS system and plays a key role in coronavirus entrance to the human body and attachment to the cell. Variation in ACE-2 may increase the capability of coronavirus to binding with human tissues and lead to variation in disease severity among patients. For this reason, this study aimed to take some SNPs in different COVID-19 patient cases to show the possible role of Angiotensin-Converting Enzyme-2 (ACE2) polymorphism in people that could have severe infections caused by SARS-CoV-2 by detecting different single nucleotide polymorphisms SNPs on this gene by PCR-sequencing. This cross-sectional study includes 100 diagnosed COVID-19 patients during a period between November and December 2021. The result revealed that three SNPs rs1463669655, rs746202722, and rs201035388 located on ACE-2 (GRCh38.p13) gene did not associate with the severity of COVID-19 disease because all patients have either the wildtype or the heteterotype allele as the following 0 % GG, 100 % AG, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 0 % GG, 100 %AG, and 0 % AA and 100 % AA, 0 % AG, and 0% GG as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % AA, 0 %AG, and 0 % GG and 100 % GG, 0 % GA, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % GG, 0 %GA, and 0 % AA for these three SNPs respectively. The ACE-2 angiotensin-converting enzyme gene was studied because of its active participation in the entry of the Coronavirus into the human body and its binding to alveolar cells and concluded that three SNPs rs1463669655, rs746202722, and rs201035388 located on ACE-2(GRCh38.p13) gene did not associate with the severity of COVID-19 disease.

Keywords: Angiotensin-converting enzyme-2 (ACE-2) gene polymorphism, COVID-19 polymerase chain reaction (PCR), Single nucleotide polymorphisms (SNPs)

INTRODUCTION

COVID-19 is an acute respiratory syndrome caused by coronavirus SARS-CoV-2 that belongs to a subgroup of *Betacorona virus* known as *Sarbeco virus* responsible for causing the ongoing acute respiratory outbreak that swept the world (Devika, 2021). The COVID-19 content spike (S) protein interacts with the host cell's angiotensin-converting enzyme 2 (ACE2) receptor, promoting viral entrance by cleaving ACE-2 and activating the SARS-CoV-2 S protein, which mediates coronavirus entry into the host cells. ACE2 is expressed in target cells of the host, specifically alveolar epithelial type II cells (Hoffmann *et al*., 2020). Harrison , (2015); Wang *et al*., (2022) have shown when the S protein binds to the ACE-2 receptor, it undergoes a conformational change during which the S1 subunit is shed off and the two heptapeptide repeats of the S2 subunit form a six-helix bundle, therefore shortening the distance between viral and host cell membranes and facilitating the virus-cell fusion. It occurs when the S protein fuses with the ACE-II receptor, where it undergoes a configuration change through which the S1 subunit is eliminated and the heptapeptide of the S2 subunit is repeated leading to the formation of a bundle of six helices. The angiotensin-converting enzyme 2 (ACE2) gene and protein exhibit a high degree of genetic polymorphism, including single-nucleotide variation, transcriptional variation, post-transcriptional modifications, and putative protein mutations (Devaux *et al*., 2020a), with single nucleotide

polymorphisms (SNPs) gaining scientific attention. Suryamohan *et al*. (2021) discovered 298 distinct protein-altering variants spanning 256 codons scattered over the 805 amino acid long human ACE2 (hACE2) from different databases. The symptoms and severity of COVID-19 vary ranging from very mild or no symptoms to severe respiratory distress syndrome with multiple organ failure, and even death (Lopera Maya *et al*., 2020). As SARS-CoV-2 primarily depends on ACE2 for fusion and entry, ACE2 polymorphism is considered to be one of the causes of this difference. Thus, it is important to systematically characterize and evaluate angiotensin-converting enzyme 2 (ACE2) polymorphism. Herein, this study aimed to provide the possible role of ACE2 polymorphism in people's susceptibility to SARS-CoV-2 infection and the severity of COVID-19 by detecting different single nucleotide polymorphisms SNPs on this gene.

MATERIALS AND METHODS

Specimens collection

This cross-sectional study includes 100 venous blood spacemen collected from all COVID-19 patients in the amount of 2 ml and transferred to an anticoagulant tube EDTA for DNA extraction. patients aged (15-to 80) years were distributed according to the severity of disease as the following: (46 severe who needed ventilators and lying in the respiratory care unit (RCU) with severe respiratory distress, respiratory rate ≥30 breaths/minute, and pulse oxygen saturation lower than 93% at rest, the case was considered to be in severe illness with PCR positive, 54 non-severe with positive PCR result those who did not need respirators with an oxygen rate higher than 93% (SpO2) ≥93% on resting state who have been hospitalized at the COVID-19 Wards in Merjan Medical City and Al-Imam Al-Sadiq Hospital in Babylon Province during 2 months (November and December 2021). All patients were diagnosed based on previous clinical reports, clinical examinations, and PCR tests. These cases were compared with each other, all of them were asked to fill out a questionnaire and all had no family history of any disease. All patients suffering from Covid-19 pneumonia were included and excluded from other types of respiratory diseases.

Genomic DNA extraction from frozen human blood

Favor prep blood genomic DNA extraction mini kit was used to obtain genomic DNA from frozen human blood following the manufacturer's protocol.

Polymerase chain reaction (PCR)

As previously described, the target DNA was amplified using specific primer pairs in a conventional PCR (Forward: ACE-2 primer 5'-TGGAGGCAAACATCCAATCTCA -3') and (Reverse: primer 5'- CTGTCCTCTCCAGGATGAACTT-3') as shown in Table 1. PCR product (amplicon) is obtained by repeating three consecutive steps for a specific number of cycles, which can then be visualized after agarose gel electrophoresis, as shown in Table 1 which contains information on the thermal cycling conditions.

Primer preparation

TE Buffer (8.0) was used to dissolve the primer pairs used in this study, which contained Tris-HCL (10 Mm) and EDTA-Na2. Initially, the primer stock tube is prepared, and then the working solution is prepared from the primer stock tube

The nuclease-free water was added by the manufacturer's instructions (macrogen/Korea) to obtain a (300 picomole/microliter) concentration of primer stock solution. By diluting the stock solution with nuclease-free water, the working solution was obtained at a concentration of 10 picomole/microliter (Green and Sambrook,2012).

Reaction mixture

Amplification of DNA was carried out in a final volume of 50 µL reaction mixture as mentioned in Table 2.

Agarose gel electrophoresis

Agarose gel electrophoresis is a good way to separate DNA fragments. The amount of agarose in a gel depends on the size of the DNA fragments that need to be separated. It ranges from 0.5% to 2%. (Lee *et al*.,2012). A 1.5 gel was used to get a good picture of small parts of the PCR product (0.2-1kb) with the use of 50°C agarose solution with a 5 µL Simple safe stock solution.

Sequencing of PCR product

Forty microliters of ACE-2 products were sent to Macrogene/ Korea for Sanger sequencing. After trimming each sequence, the result of the trimmed sequence was blasted in the national center for biotechnology information NCBI to check the similarities and differences with the database. Finch TV version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; (http://www.geospiza.com) was used to check the polymorphism of the genes above.

RESULTS AND DISCUSSION

Humane DNA extraction for detecting and identifying specific PCR amplified fragments of ACE-2 which can then be visualized after agarose gel electrophoresis as shown in Figures (1).

Association of ACE-2(GRCh38.p13) SNPs and potential risk of COVID-19

Table 1. Primer sequencing and PCR conditions

Primer for characterization						
Primer	**Sequence 5 to 3**	**product**	**Conditions**		**NO. CY-CLE**	**Reference**
ACE2-F	TGGAGGCAAACATCCAATCTCA	500	Step 1 Step 2 Step 3 Step 4 Step 6 Step 7	95ºC 95ºC 59 ºC 72 ºC 72 ºC	1 35 1	This study
ACE2R	CTGTCCTCTCCAGGATGAACTT					

Table 2. Contents of the Reaction mixture

No.	Contents of the reaction mixture	Volume
1.	Green master mix	25 µL
2.	Upstream primer (10pmol/ µL)	3 µL
3.	Downstream primer (10pmol/ µL)	3 µL
4.	Nuclease free water	14 µL
5.	DNA template	5 µL
Total volume		50 µL

Trimming of Sanger sequencing results for ACA-2 PCR product. Multiple alignments for each were prepared using Finch TV version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; (http://www.geospiza.com) to know the genotype differences between severe and non-severe COVID-19 patients and compared with the NCBI database. Fig. 2 shows SNPs distributions on the ACE-2 (GRCh38.p13) gene.

This study showed that ACE-2 rs1463669655 SNP was 0 % GG, 100 % AG, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 0 % GG, 100 %AG, and 0 % AA, with G-allele and A-allele frequency 46 (0.23) %, 46(0.23) % in severe cases and non-severe were 54 (0.27)% for G-allele frequency and 54(0.27)% for A-allele frequencies and when compared the genotype distribution for ACE-2 rs1463669655 GG and AA the same result was with (P=0.937, OR=1.172, CI=0.02-60.2) and GA result was (p=0.937, OR=0.853, CI=0.01 -43.8). This finding shows that there were no significant differences between ACE-2 rs1463669655 SNP and the COVID-19 disease because all severe and non-severe patients contained the heterozygous genotype and disappearance of the wild genotype and homozygous genotype in all patients as shown in Table 3.

And for ACE-2 rs746202722 SNP was 100 % AA, 0 % AG, and 0% GG as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % AA, 0 %AG, and 0 % GG, with A-allele frequency 92 (0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of G-allele in both two cases and when compared the genotype distribution for ACE-2 rs746202722 (AA/P=0.937, OR=0.853, CI=0.01- 43.8) and AG, GG the same result with (P=0.937, OR=1.172, CI=0.02-60.2). This finding shows that there were no significant differences between ACE-2 rs746202722 SNP and the COVID-19 disease because all severe and non-severe patients contained the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in Table 3.

And when studied ACE-2 rs201035388, SNP was 100 % GG, 0 % GA, and 0% AA as the genotype distribution in severe cases. The genotype distribution in non-severe cases was 100 % GG, 0 %GA, and 0 % AA, with G-allele frequency 92 (0.46)%, 108 (0.54) % in

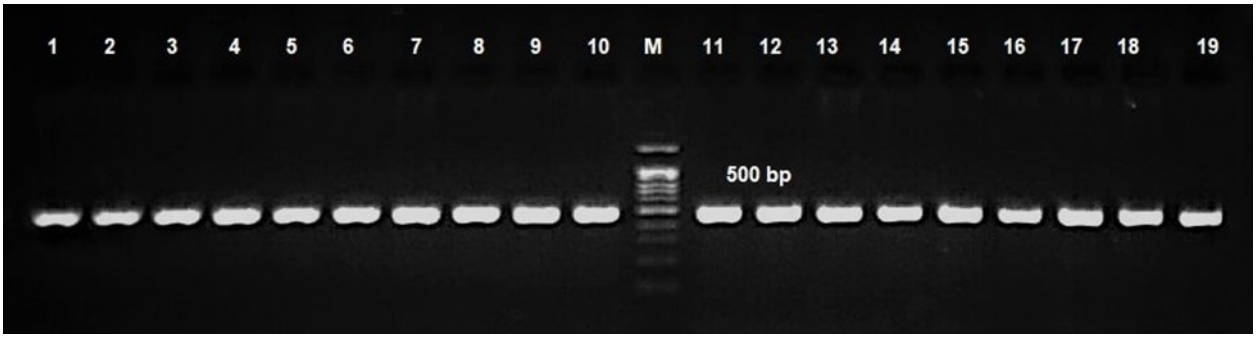

Fig. 1. *1.5 % Agarose gel electrophoresis at 72 volts for 80 minutes of PCR to ACE-2 amplicon (500bp) , 1-19 represented samples , M (DNA marker size (100bp)*

Table 3. Distributions of genotype and allele frequencies in ACE-2 rs1463669655, rs746202722 and rs201035388 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2 rs1463669655		**Severe N=46(%)**	**Non-severe N=54(%)**	**P-Value**	**OR**	**CL=95%**
Wild type GG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Heterozygote GA		52 (100)	58 (100)	0.937	0.853	0.01- 43.8
Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	46 (0.23)	54(0.27)	1.000	1.000	0.57- 1.74
	A	46(0.23)	54(0.27)	1.000	1.000	0.57- 1.74
Genotype ACE-2 rs746202722						
Wild type AA		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote AG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	A	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7
GenotypeACE-2 rs201035388		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG		46(100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote GA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	A	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, CI=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles

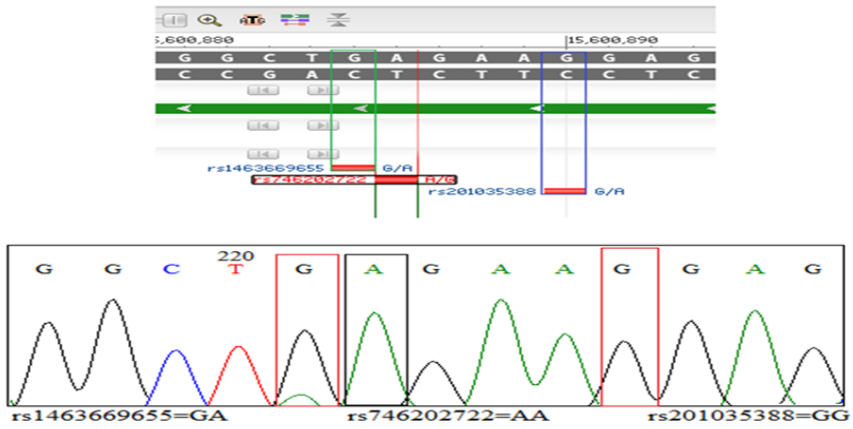

Fig. 2. *Distributions of rs1463669655, rs746202722, rs201035388 SNPs on ACE-2(GRCh38.p13) gene*

severe and non-severe cases, respectively and disappear of A-allele in both two cases. When compared the genotype distribution for ACE-2 rs201035388 (GG/ P=0.937, OR=0.853, CI=0.01- 43.8) and GA, AA the same result with (p=0.937, OR=1.172, CI=0.02-60.2) this finding shows that there were no significant differences between ACE-2 rs201035388 SNP and the COVID-19 disease because all severe and non-severe patients contained the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in Table 3.

A review study by Chen *et al*. (2021) on 23 SNPs present in ACE-2 in different populations showed that they could partially account for the differences in COVID-19 prevalence and mortality rates. And when comparing the affinity of the ACE-2 variant to the S1 protein, their findings were controversial among studies, and the results lack validation by systems biology studies even

though some variants have been believed to enhance the affinity in several reports. Thus, there is an urgent need for *in vitro* validation studies to assess the involvement of population-specific SNPs of ACE-2 and other host factors in susceptibility toward SARS-CoV-2 infection.

Another study by Möhlendick *et al*. (2021) showed that there was a relationship between ACE2 rs2285666 and COVID-19 severity; if A-allele carriers produce more ACE2 than those with GG genotypes, A-allele carriers could be protected by at least partially against the ACE/ACE2. This indicates that the ACE-2 gene polymorphisms may affect the severity of the infection, but the difference in the results of these studies was the difference in the type of SNPs between this study and other studies.

In addition, the study by Cao *et al*. (2020), analyzing 1700 ACE2 variants from China MAP and 1000 Genomes Project databases and comparing the allele frequency differences between different populations, identified a truncating mutation and seven hotspot variants potentially related to differently SARS-CoV-2 infection. Finally, a study by Cafiero *et al*. (2021) on the ACE-2 gene has identified rs2074192 (ACE2), rs1799752 (ACE1), and rs699 (AGT) SNPs could potentially be valuable tools for predicting the clinical outcome of SARS-CoV-2 infected patients. And suggested furthering molecular–epidemiological studies are required to understand the exact mechanisms underlying the clinical variability of COVID-19 disease, even in populations from different ethnic groups, and predict the most severe clinical manifestations, to develop personalized approaches or alternative strategies.

Conclusion

This study concluded that three SNPs rs1463669655, rs746202722, and rs201035388 located on ACE-2GRCh38.p13) gene did not associate with the severity of COVID-19 disease because all patients had either the wildtype or the heteterotype allele and were suggested to further studies about ACE-2-2 polymorphism containing a large population to more insurance.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The approvals were obtained from all the participants and after obtaining the fundamental approval from the official authorities. The information of patient' name, age, sex, date of infection, and chronic disease.

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