International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

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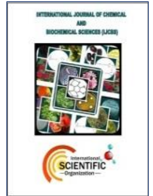

Role of *ACE*, *ACE2* gene variants and serum ACE/ACE2 ratio in COVID-19 severity

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Abstract

As the outbreak of COVID-19 progresses, prognostic markers for identification of high-risk individuals are of urgent medical need. The Angiotensin system is implicated in the pathogenesis of COVID19 as ACE2 is the cellular receptor for SARS-COV-2 virus, and expression of the *ACE2* gene could regulate the individual´s susceptibility to infection. In addition, the balance between ACE and ACE2 activity could play a role in the severity of COVID19. 180 COVID-19 patients were divided into three groups (60 patients as mild, 60 patients as moderate and 60 patients as severe). Enzyme levels of ACE and ACE2 were measured by ELISA. *ACE* I/D (rs4646994) was assayed by PCR and *ACE2 (*rs2285666) gene variant was determined by Real-Time PCR. ACE/ACE2 ratio was significantly lower in mild group versus moderate to severe group (P <0.001). GG (wild) genotype and G allele of ACE2 were more frequent in mild group, AA (variant) genotype and A allele were more frequent in severe group (*P* value <0.001). In the multiple logistic regression, COVID-19 severity was associated with older age (>50y) (OR 10.4, 95% CI 3.8-28.4, P<0.001), Comorbidities (OR 8.2, 95% CI 1.6-42.1, *P*0.012) and higher ACE/ACE2 ratio (OR 8.3 95% CI 3.7-18.6 P<0.001) were independent significant predictors of severity. Haplotype analysis revealed Patients with D allele of *ACE* gene combined with A allele of *ACE2* gene will have nearly double the risk of having severe COVID infection (OR=1.9, CI 1.1-3.5 P=0.024). Old age (>50 years), presence of co-morbidities and high ACE/ACE2 ratio are recognized as pivotal predictors for COVID-19 severity.

Keywords: Angiotensin converting enzyme ACE and ACE2 levels; ACE gene variants; COVID-19 severity.

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1. Introduction

Corona virus disease 2019 (COVID-19), a newly emerged respiratory disease caused by severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), has recently become a pandemic. The mortality rates, as well as the infectious capacity of the virus, ranging from 1% to 5%, have raised a major concern worldwide. Older people with comorbid conditions, such as pulmonary disease, cardiac disease, kidney disease, diabetes, and hypertension, are associated with higher mortality rates [1]. In Egypt, from 3 January 2022 to 8 August 2022, there have been 515,082 confirmed cases of COVID-19 with 24,781 deaths reported to WH [2]. The SARS-CoV-2 virus infects human cells by first binding via its S protein to its target the Angiotensin Converting Enzyme 2 (ACE2) on the surface of cells in the lungs, heart, kidneys and intestine [3]. Renin- Angiotensin Aldosterone system (RAAS) seems to play an important role in the pathogenesis ofCOVID10 [4]. The angiotensin-converting enzyme (ACE) catalyzes the synthesis of Angiotensin-II (Ang-II) from Ang-I, and ACE2 hydrolyzes Ang-II into Ang-1-7. Ang-II binds to the AT1-receptor driving vasoconstriction, fibrosis, inflammation, thrombosis, among other responses; while Ang-1-7 binds to the AT2-receptor with increased vasodilation and reduced fibrosis, inflammation, and thrombosis [5]. The ACE and ACE2 are thus seen as opposite players in the balance that determines the risk of developing hypertension and cardiovascular disease. Therefore, high levels of ACE1 could indicate low levels of ACE2, and vice versa [6]. The *ACE* gene (17q23.3 locus) consists of 26 exons and25 introns, and codes ACE enzyme [7]. *ACE* gene I/D variant consists of the insertion (I)

or deletion (D) of a 286-bp Alu repeat sequence in intron 16 that may affect the expression of *ACE* gene and/or the function of angiotensin I converting enzyme [8]. The presence of D allele is associated with higher ACE enzyme activity and higher production of angiotensin II in comparison to I allele [7]. This increased expression would explain the higher risk for cardiovascular and respiratory disease among individuals who are deletion homozygous. This variant has been related to the outcome in acute respiratory distress syndrome (ARDS) and to the progression of pneumonia in SARS [9]. The *ACE 2* gene is located on Xp22.2 and encoded 805-amino-acid-long protein and belongs to the family of angiotensin-converting enzyme of dipeptidyl-carboxydipeptidases [10]. *ACE2* G8790A (rs2285666) variant is located at the beginning of the intron 3, theoretically affecting gene expression with alternative splicing mechanisms [11], that could affect the processing of ACE2 total RNA to mRNA and eventually, the amount of protein. One study has investigated the effect ofrs2285666 on serum ACE2 levels and found significantly higher levels in A carriers compared to G Homozygote [12]. Early identification of *ACE*1 I/D and *ACE2*G8790A variants as risk factors for COVID-19 may help to provide the appropriate support for critical patients including access to ICU. Our aim is to explore the role of *ACE*1 I/D& *ACE2* G8790A gene variants and serum ACE l/ACE2ratio as risk factors for severity of COVID-19 infection.

2. Materials and Methods

This cross-sectional study was carried out on 180 COVID-19 positive patients attending Internal Medicine isolation hospital, Kasr Al-AinyHospital, Cairo, Egypt, from January 2021 to January 2022. Patients were classified into three groups according to the severity [13]. Group 1: included sixty mild cases, defined by symptomatic cases with lymphopenia or leucopenia with no radiological sign for pneumonia. Group 2: included sixty moderate cases, defined by radiological manifestations for pneumonia with symptoms and or leucopenia or lymphopenia. Group 3: included sixty severe cases, defined as those in need of critical care support, including high-flow oxygen and positive-pressure ventilation. The study protocol conformed to ethical guidelines of the Declaration of Helsinki, 1975 [14] and was approved by the Research Ethics Committee, Faculty of Medicine, Cairo University (MD-299-2020). Policy of data confidentiality was strictly followed. The aim was explained clearly, and informed consent was obtained from all participants before enrolment.

2.1. Patient's Inclusion criteria

We included adult patients, their ages ranged from 20 to 64 years old of both sexes who are PCR Positive for SARS-Cov-2 from nasal swabs or tracheobronchial aspirates. Children were excluded from the study.

2.2. Patients were subjected to the following

History taking included symptoms: cough, fatigue, dyspnea, and fever. Previous medical history: diabetes, hypertension, cardiac disease, and smoking were also taken. Clinical assessment included general examination and local chest examination. CT chest was performed. Laboratory investigations included: PCR test from nasal swabs or tracheobronchial aspirates, serum C-reactive protein (CRP), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), Detection of angiotensin converting enzyme ACE and ACE2 level by ELISA, Genotyping of *ACE I/D* (rs4646994) gene by PCR and *ACE2* (rs2285666) gene variantG8790A detection by Real Time PCR.

2.3. Sample collection

Six milliliter of venous blood were withdrawn and divided into two parts: Four milliliters were collected into a plain sterile tube for performing CRP, LDH and ALT, which were analyzed on Beckman Synchron CX9 Pro Blood Chemistry Auto Analyzer using kits supplied by Beckman coulter, Inc.CA 92821, USA. In addition, serum samples were used for measuring ACE and ACE2 enzyme levels by ELISA method. Another two milliliters were collected into a sterile EDTA tube and was stored at –20ºC to be used for the genotyping techniques.

2.4. Identification of ACE I/D rs4646994 gene variant by polymerase chain reaction (PCR)

This was done in three main steps:

1. Extraction of genomic DNA from peripheral blood leucocytes by Easy Pure Blood Genomic DNA Kit (Trans Gen Biotech Co, Beijing, China). DNA purity and concentration were determined before starting analytical techniques. The ratio of absorbance at 260 and 280 nm was used to assess DNA purity. A ratio of ∼1.8 was accepted as "pure" for DNA [15]. Purity was measured on Nano Drop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA concentration was measured using Qubit 2.0 Fluorometer (Invitrogen, CA, USA).

2. Amplification of the extracted DNA using Taq polymerase enzyme, specific primers and master mix supplied by Invitrogen (Thermo Fisher Scientific, USA). Amplification was performed using Bio Rad thermal cycler T100 (Singapore). Forward (F) and reverse (R) primers sequences were: (F) 5`-CTG GAG ACC ACT CCC ATC CTT TCT-3`. (R) 5`-GAT GTG GCC ATC ACA TTC GTC AGA T-3`. This was done using the protocol proposed by [16] which consisted of initial denaturation at 95ºC for 7 minutes, followed by 35 cycles of amplification consisting of: Denaturation at 94ºC for 45 seconds, annealing at 58ºC for 30 seconds, elongation at 72ºC for 45 seconds and final elongation of 7 minutes at 72ºC.

3. Detection of amplified PCR products was done by using 1.5% agarose gel electrophoresis containing ethidium bromide and Ultraviolet light trans-illumination. The II genotype appeared as a single band at 490 bp. The DD genotype appeared as a single band at 190 bp. The I/D genotype appeared as two bands developed at 190bp and 490bp. Figure (1).

2.5. Identification of genetic variants of ACE2 (rs2285666) by Real-Time PCR

Real-time PCR allelic discrimination assay was designed using Taq-Man SNP Genotyping Assays performed on Step One Real-Time PCR instrument (Applied Bio-systems, MA, USA). Into each well of the reaction plate 10ul of TaqMan Universal PCR Master Mix (2x) and 0.5 ul of 40xworking stock of SNP Genotyping Assay were added. Volume of DNA of each sample was calculated so that each reaction contained 20 ng DNA. DNase-free Water was added to reach a total Volume of 20ul per well. The thermal cycling conditions were programmed as follows: AmpliTaq Gold Enzyme activation at 95°C for 10 minutes. Then 50 cycles of amplification consisted of: Denaturation at 92°C for 15 seconds, an annealing and an extension reaction at 68ºC. Thus, repeating the thermal cycles resulted in a geometric accumulation of amplified target sequences. Allelic Discrimination was performed using the Sequence Detection System Software, which measured and plotted the fluorescence signals from each well to indicate which allele was in each sample. Figure (2) shows heterozygous genotype.

2.6. Detection of Angiotensin converting enzyme ACE and ACE2

This was done using Human ACE Enzyme-linked immunosorbent assay ELISA kit (catalog no. SL0116Hu) and Human ACE2 ELISA kit (catalog no. SL2129Hu) supplied by Sun long Biotech (Hangzhou, China) according to manufacturer`s protocol.

2.7. Statistical methods

Data management and analysis were performed using Statistical Package for Social Sciences (SPSS) v. 28. Numerical data were summarized using means and standard deviations or medians and/or ranges, as appropriate. Categorical data were summarized as numbers and percentages. Estimates of the frequency were done using the numbers and percentages. Numerical data were explored for normality using Kolmogrov-Smirnov test and Shapiro-Wilk test. Chi square or Fisher's tests were used to compare between the independent groups with respect to categorical data, as appropriate. Comparisons between two groups for normally distributed numeric variables were done using the Student's t-test while for non-normally distributed numeric variables, comparisons were done by Mann-Whitney test. Comparisons between more than 2 groups were performed by Kruskal-Wallis for non-normally distributed variables, then followed by post hoc if needed (Post Hoc comparison was done and P value was adjusted using Bonferroni adjustment). To measure the strength of association between non normally distributed measurements, Spearman's correlation coefficients was computed (r is the correlation coefficient & it ranges from -1 to +1). All tests were two tailed & Probability (p-value) ≤ 0.05 is considered significant. Haplotyping analysis was done through https://www.snpstats.net/start.htm.

3. Results and discussion

Table 1 shows demographic, clinical and laboratory data among the three groups. In terms of age, patients with mild disease were significantly younger than those with moderate were and sever disease (P value <0.001). Patients with advanced ages (>50 years) paralleled the severity of COVID-19, where the severe and moderate cases tended to be over the age of 50 years (51.7%, 68.3% respectively), while mild cases were mostly under 50 years (88.3%) (*P* value< 0.001).In regard to the associated comorbidities, the prevalence of diabetes, hypertension and renal disease were significantly higher in moderate (23.3%) and severe cases (30%) than in mild cases (3.3%) (*P* value < 0.001). Moderate and severe groups had significantly higher serum levels of CRP, LDH, ALT and creatinine in comparison to mild group (P value <0.001).Blood level of ACE showed no significant different between the three groups, while blood levels of ACE2 were significantly higher in mild cases in comparison to moderate and severe cases (P value <0.001). ACE1/ACE2 ratio was significantly lower in mild cases in comparison to moderate and severe cases (P value <0.001).Genotype and allele frequencies of *ACE* (rs4646994) I/D polymorphism showed no significant variation among the three groups. While regarding *ACE2* (rs2285666), GG (wild) genotype was significantly more frequent in mild group (71.7%) and AA (variant) genotype was significantly more frequent in severe group (38.3%). G (wild) allele frequency was significantly higher in mild group and A (variant) allele was more frequent among sever group (*P* value <0.001) (Table 1). Table 2 shows comparison between mild versus moderate to severe group, where serum levels of CRP, LDH, ALT and creatinine showed significant higher levels among moderate to severe group(*P* value <0.001). Blood level of ACE showed no difference between the two groups, while ACE2 blood level were higher among mild group (P value <0.001) and ACE1/ACE2 ratio was significantly lower in mild group (P value <0.001). Genotype and allele frequencies of *ACE* (rs4646994) showed no difference between the two groups. However, GG (wild) genotype and G (wild) allele of *ACE2* (rs2285666) were significantly more frequent among mild group, and AA (variant) genotype and A (variant) allele were least frequent among mild group (Table 2). There were no significant associations between the three genotypes frequencies of *ACE* (rs 4646994) neither *ACE2* (rs2285666) genes with their serum enzyme level (Table 3). ACE/ACE2 ratio showed significant positive correlation with age, CRP, ALT and creatinine (*P*=0.021,<0.001,< 0.001 and 0.003 respectively) (Table 4).

3.1. Multivariate analysis (Logistic regression for prediction of COVID severity)

To measure the independent effect of different factors on COVID severity, factors, which had P value less than 0.100, were selected to enter into stepwise logistic regression analysis. The regression coefficient shows the effect of each variable after controlling the effect of other variables in the model. The independent factors that significantly predict COVID severity were: ACE/ACE2 ratio, age ≥50 years and the presence of comorbidities. Regarding ACE/ACE2 ratio, for every unit increase in this ratio there is 8 times increase in the risk of disease severity. Older patients (≥50 years) have 10 times more risk of disease severity.

Patients having comorbidities have 8 times more risk of disease severity compared to patients without comorbidities (Table 5). Haplotype analysis revealed that patients who have D (variant) allele of *ACE* gene combined with A (variant) allele of *ACE2* gene will have nearly double the risk of having severe COVID infection (OR=1.9, P=0.024) (Table 6). The two most important RAS enzymes are angiotensin-converting enzyme (ACE) and ACE2, and SARS-CoV-2 has been shown to be an ACE2-tropic virus [17]. Virus interaction with RAS in cells infected with SARS-CoV-2 can trigger dysregulated immune responses in COVID-19 patients [18]. The aim of the study is to detect the role of *ACE* I/D, *ACE2* G8790A gene variants and serum ACE/ACE2 ratio as a risk factor for severity of COVID-19 infection. In terms of age, current study showed that advanced ages (>50 years) paralleled the severity of COVID-19, where severe and moderate cases tended to be over the age of 50 years (51.7%, 68.3% respectively), while mild cases were mostly under 50 years (88.3% *P*value< 0.001). Besides, age >50 years old was one of independent factors that significantly predict COVID-19 severity (OR=10.4, *P*<0.001) it means that older patients (≥50 years) have 10 times more risk of disease severity. Consistent with this observation, several studies observed that the elderly are not only at increased risk of contracting COVID-19, but also at a severe form of illness [19-20]. Older people were reported to have more difficulty in breathing compared to younger people [21] and are more likely to develop uncommon presentations of disease; for instance, confusion, delirium and dementia, which have also been linked to an increased risk of death [22]. This also can prevent old patients from being diagnosed in the early stages of the disease, which can lead to more fatality. The dysregulated immune system is one of the most important proposed factors, which contribute to an increased risk of COVID-19 infection and severity in older adults [23]. A hallmark of aging is the shift of the innate and adaptive immune responses towards creating inflammatory states in the human [24]. Besides, it has been found that aging is associated with down-regulated functions of cells of the adaptive immune system; for instance, antigen presenting, T-cytotoxic and B cells, and this may counteract the adaptive immune system to control infection and inflammation [23-25]. Nutritional status can also alter the immune responses and may be a contributory factor in disease susceptibility among older adults [26]. Vitamin D acts as an immunomodulatory molecule that can prevent cytokine storm and there is evidence that vitamin D deficiency, which is more common in older adults, is linked to higher severity of COVID-19 symptoms [27]. Importantly, underlying diseases increase the morbidity and mortality of COVID-19, cardiovascular diseases, diabetes, chronic kidney diseases, cancer, and respiratory diseases, which are linked to higher severity [28-29] are more prevalent in elderly people [30]. In our study serum level of CRP, LDH, ALT was significantly higher in moderate and severe groups in comparison to mild group (P value <0.001).Liver dysfunction is likely secondary to the use of hepatotoxic drugs, hypoxia induced liver injury, systemic inflammation, or multi organ failure.

In the current study diabetes, hypertension and renal disease were significantly higher in moderate (23.3%) and severe cases (30%) compared to mild cases (3.3%) (P <0.001). The presence of comorbidities was one of independent factors that significantly predict COVID severity (OR=8.2, *P*=0.012) where patients with comorbidities have 8 times more risk of disease severity compared to patients with no co-morbidity. Previous studies have shown that diabetes and hypertension are the most frequent comorbidities among COVID-19 patients, and have been observed to increase the risk of morbidity and mortality [31-33]. However, Tadic and Cuspidi et al stated it is not clear whether the two comorbidities were independent predictors of COVID-19 severity or might have synergistic effects [34]. The main role of ACE is the conversion of angiotensin I to angiotensin II (Ang-I >Ang-II) the latter being a powerful peptide causing complex processes such as vasoconstriction, inflammation, fibrosis and proliferation via the AT1-receptor. Conversely, ACE2 firstly converts Ang-I to Ang 1–9, that is then converted by ACE to the vasodilator peptide Ang 1–7. Moreover, ACE2 directly converts Ang-II to Ang 1–7 and this latter by acting on MAS-receptor exerts organ protection, antagonizing the biological effects of Ang-II [5], [10]. It has been postulated that an ACE/ACE2 imbalance may play a central role in COVID-19 [35]. In our study ACE2 enzyme level were significantly higher among mild group vs moderate to severe group (P <0.001) and consequently ACE/ACE2 ratio was significantly lower in mild group versus moderate to severe group (P <0.001) and it was one of independent factors that significantly predict COVID-19 severity (OR=8.3, P <0.001) where for every unit increase in ACE/ACE2 ratio there is 8 folds increase in the risk of disease severity. The majority of data are in favor of the idea that a high ACE/ACE2 ratio may be detrimental for Covid-19 infection. ACE/ACE2 ratio is increased in many diseases and conditions such as cardiovascular diseases; obesity and aging that exacerbate Covid-19 symptomatology and worsen outcomes. Moreover, ACE2 is upregulated and the ACE/ACE2 ratio is low in many subjects at low risk for cardiovascular diseases, such as females, exercise-trained individuals, and patients well treated with ACE inhibitors [36]. Since most of Covid-19 patients had hypertension, further consideration is needed for Angiotensin Converting Enzyme inhibitors and Angiotensin II Receptor Blockers (as these drugs upregulate the expression of ACE2). The use of these drugs has been questioned, but the majority of authors are in favor of the use of these drugs [37-39]. We agree that if used correctly they reduce the ACE / ACE2 ratio. Increased ACE2 expression could influence the course of Covid-19 in different ways: increased expression might promote viral entry [17], and at the same time, it may be beneficial as ACE2 has anti-inflammatory effects that could prevent pulmonary edema, ARDS, hypoxia, and oxidative stress development [36]. It is likely that viral load is not strictly related to disease severity, and it is likely that ACE2 over-expression is not responsible for Covid-19 worsening but that there is, rather, some other mechanism within the complex RAS or outside RAS (such as a different macrophages population or a different immune response) which may facilitate the onset of coagulopathies, leading to organ ischemia and multiple pulmonary and cardiovascular complications [40-42]. The virus downregulates ACE2,

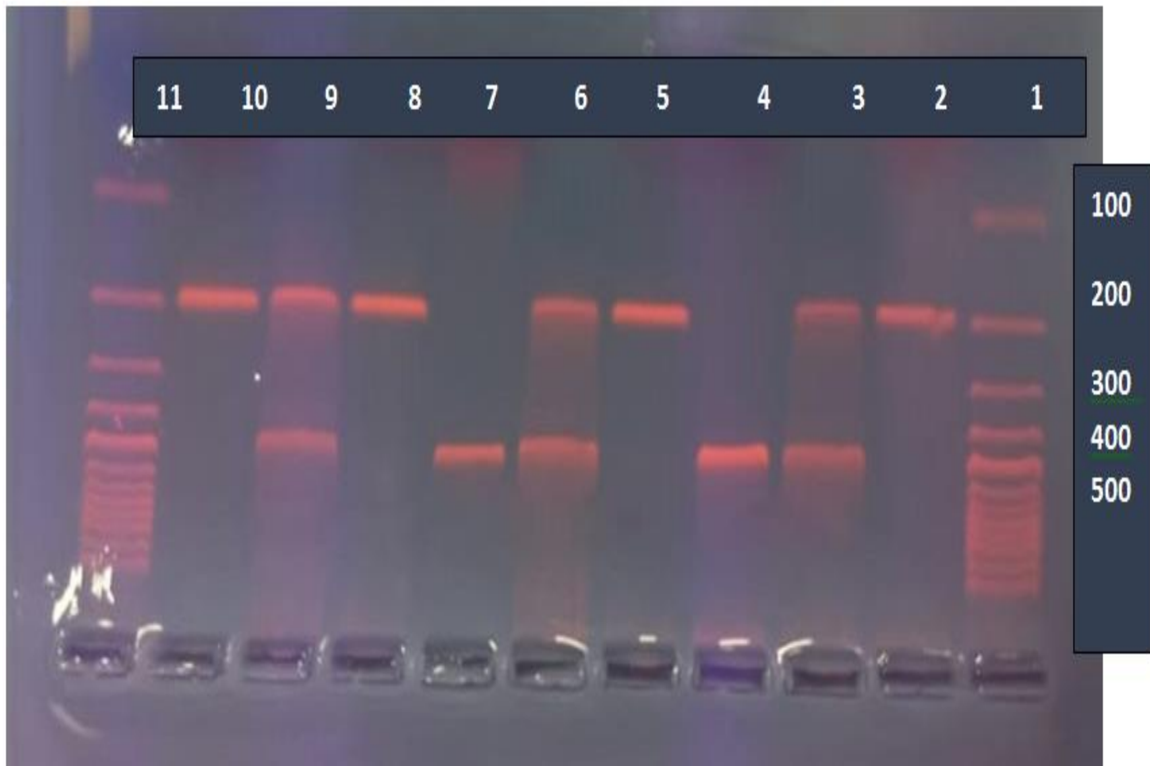

Figure 1. Agarose gel showing PCR analysis of the *ACE I/D* variant

Lanes 1 and 11 show 100 bp DNA ladder. Lanes 2, 5,8,10 show homozygous DD genotype (190 bp).
Lanes 3, 6, 9 show heterozygous ID genotype (190bp and 490bp); lanes 4, 7 show homozygous II genotype (490bp)

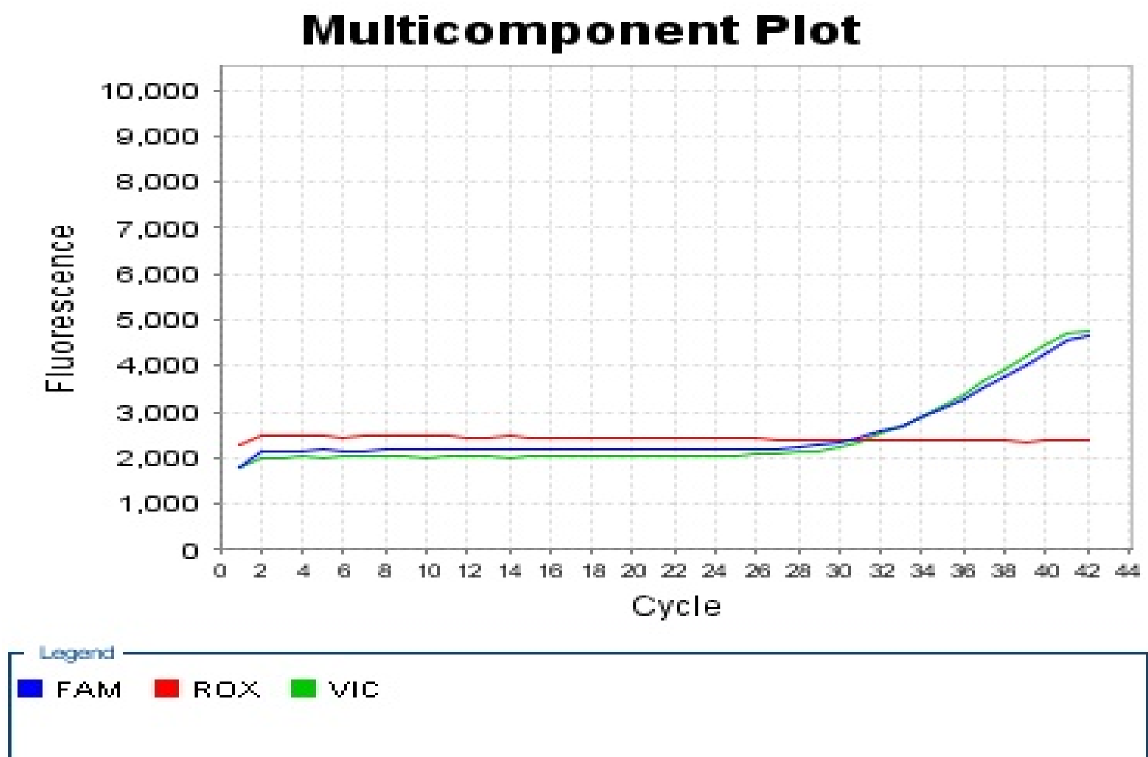

Figure 2. Multicomponent plot shows heterozygous allele A/G

Table 1. Demographic, clinical and laboratory characteristics among the three studied groups

		Mild (n=60)	Moderate (n=60)	Severe (n=60)	P value
Age (years)mean± SD		35 ±12	52±8	55±9	0.12
Age group	**< 50years** n (%)	53 (88.3)	29 (48.3)	19 (31.7)	**<0.001**
	≥50 years n (%)	7 (11.7)	31 (51.7)	41 (68.3)	
Sex	**Female** n (%)	35 (58.3)	36 (60)	32 (53.3)	0.799
	Male n (%)	25 (41.7)	24 (40)	28 (46.7)	
Comorbidities	**Yes** n (%)	2 (3.3)	14 (23.3)	18 (30%)	**0.001**
Types of comorbidities	**Diabetes** n (%)	0 (0)	11 (18.3)	10 (16.6)	**<0.001**
	Hypertension n (%)	2 (3.3)	2 (3.3)	4 (6.7)	
	Renal n (%)	0 (0)	1 (1.7)	4 (6.7)	
CRP* (mg/L)		12 (2-96)	96 (24-132)	96 (12-196)	**<0.001**
LDH* (U/L)		306 (142-650)	524 (189-860)	594 (380-1727)	**<0.001**
ALT*(U/L)		22 (14-32)	38 (12-137)	36 (15-121)	**<0.001**
Creatinine* (mg/dl)		0.9 (0.5-1.3)	1.2 (0.7-8.2)	1.2 (0.8-6.9)	**<0.001**
ACE*(pg/ml)		420 (203-1100)	460 (65-1705)	453 (202-889)	**<0.001**
ACE2*(pg/ml)		755 (232-2895)	290 (105-1700)	326 (86-1440)	**<0.001**
ACE1/ACE2 ratio*		0.6 (0.1-2.9)	1.6 (0.2-5.4)	1.3 (0.2-6.2)	**<0.001**
***ACE* rs4646994 Genotype**	**I/I** (wild) n (%) **I/D**(Heterozygous) **D/D**(variant)	9 (15) 14 (23.3) 37 (61.7)	4 (6.6) 16 (26.7) 40 (66.7)	8 (13.3) 18 (30) 34 (56.7)	0.567
Allele	**I allele** (wild) n (%) **D allele**(variant)	32 (26.7) 88 (73.3)	24 (20) 96 (80)	34 (28.3) 86 (71.7)	0.307
***ACE2*rs2285666 Genotype**	**G/G** (wild) n (%) **G/A**(Heterozygous) **A/A**(variant)	43 (71.7) 12 (20) 5 (8.3)	37 (61.7) 17 (28.3) 6 (10)	17 (28.3) 20 (33.3) 23 (38.3)	**<0.001**
Allele	**G allele** (wild) n (%) **A allele**(variant)	98 (81.7) 22 (18.3)	91 (75.8) 29 (24.2)	54 (45) 66 (55)	**<0.001**

CRP: C reactive protein, LDH: Lactate dehydrogenase, ALT: Alanine transaminase
*Data were represented as Median (range).
P value <0.05 is considered significant

Table 2. Laboratory data of mild versus moderate to severe groups

		Mild (n=60)	**Moderate to severe (n=120)**	**P value**
CRP(mg/L)		12 (2-96)	96 (12-196)	**<0.001**
LDH (U/L)		306 (142-650)	572 (189-1727)	**<0.001**
ALT (U/L)		22 (14-32)	37 (12-137)	**<0.001**
Creatinine(mg/dl)		0.9 (0.5-1.3)	1.2 (0.7-8.2)	**<0.001**
ACE (pg/ml)		420 (203-1100)	456 (65-1705)	0.931
ACE2(pg/ml)		755 (232-2895)	305 (86-1700)	**<0.001**
ACE1/ACE2 ratio		0.6 (0.1-2.9)	1.5 (0.2-6.2)	**<0.001**
***ACE* rs4646994 Genotype**	**I/I**(wild) **I/D** (Heterozygous) **D/D**(Variant)	9 (15%) 14 (23.3%) 37 (61.7%)	12 (10%) 34 (28.3%) 74 (61.7%)	0.524
Allele	**I allele**(wild) **D allele** (Variant)	32 (26.7%) 88 (73.3%)	58 (24.2%) 182 (75.8%)	0.699
***ACE2*rs2285666 Genotype**	**G/G**(wild) **G/A**(Heterozygous) **A/A**(variant)	43 (71.7%) 12 (20%) 5 (8.3%)	54 (45%) 37 (30.8%) 29 (24.2%)	**0.002**
Allele	**G allele**(wild) **A allele**(variant)	98 (81.7%) 22 (18.3%)	145 (60.4%) 95 (39.6%)	**< 0.001**

*Data were represented as Median (range).
*P value <0.05 is considered significant.

Table 3. Association of ACE blood levels and different gene variant

***ACE* rs 4646994**	**I/I**(wild)	**I/D**(Heterozygous)	**D/D**(Variant)	**P value**
ACE (pg/ml)	475 (320-889)	466 (65-1061)	445 (202-1705)	0.876
***ACE2*rs2285666**	**G/G**(wild)	**G/A**(Heterozygous)	**A/A**(Variant)	**P value**
ACE2 (pg/ml)	381 (86-2895)	546 (107-2735)	399 (94-2540)	0.245

*P value <0.05 is considered significant.

Table 4. Correlation of ACE1/ACE2 ratio with other laboratory parameters

Parameters	ACE1/ACE2 ratio
Age (years)	r =0.17
	***P*=0.021**
CRP (mg/L)	r =0.28
	***P*<0.001**
ALT (U/L)	r =0.28
	***P*<0.001**
Creatinine(mg/dl)	r =0.22
	***P* =0.003**
LDH (U/L)	r =0.14
	P =0.054

*CRP: C reactive protein, LDH: Lactate dehydrogenase, ALT: Alanine transaminase
*r is the correlation coefficient & it ranges from -1 to +1
*P value <0.05 is considered significant

Table 5. Results of stepwise logistic regression for prediction of COVID severity

	B	S.E.	OR	95% C.I for OR	P value
ACE/ACE2 ratio	2.1	0.4	8.3	3.7-18.6	**< 0.001**
Age (≥50)	2.3	0.5	10.4	3.8-28.4	**< 0.001**
Comorbidities	2.1	0.8	8.2	1.6-42.1	**0.012**

B=Regression coefficients, SE=Standard error of the coefficient, OR=Odds Ratio, CI = confidence interval.
P-value< 0.05 is considered significant

Table 6. Haplotype analysis among mild group versus moderate to severe group

Haplotype frequencies estimation (n=360)							
Haplotypes	**Total**	**Mild**	**Moderate to Severe**	**Cumulative frequency**	**OR**	**95% CI of OR**	***P* value**
DG (variant/ wild)	0.51	0.59	0.46	0.51	1		
DA(variant/variant)	0.24	0.14	0.29	0.75	1.95	1.1-3.5	**0.024**
IG (wild/wild)	0.17	0.23	0.14	0.90	0.78	0.45-1.37	0.390
IA (wild/variant)	0.08	0.04	0.11	1	2.63	0.94-7.4	0.068

P value < 0.05 is considered significant, OR: Odds ratio, CI: Confidence interval

exacerbating the pro-inflammatory milieu of high ACE/ACE2 ratio [43]. Membrane-bound ACE2 has an anti-inflammatory role and an imbalanced and high ACE/ACE2 ratio is not recommended [44]. It is better to have a low ACE/ACE2 ratio. Whether increasing the ACE2 by pharmacological intervention or by regular exercise may limit Covid-19 severity remains to be ascertained. In current study we compared the genotype and allele frequencies of *ACE2* rs2285666 variant among the three groups to assess their effect on disease severity, our results showed that GG (wild genotype) was more frequent among mild group (71.7%) and AA (variant genotype) was least frequent among mild group (8.3%) (P 0.002) and the allelic frequency of *ACE2* showed G allele (wild) was significantly more frequent in mild group (81.7%), while A allele (variant) was significantly more frequent in moderate to severe group (39.6%) in comparison to mild group (18.3%) (*P* <0.001). The *ACE2* rs2285666 SNP is in the introit-consensus splicing nucleotides and could thus affect the processing of *ACE2* total RNA to mRNA and, eventually, the amount of the protein [11]. Unfortunately, we could not found an association between *ACE2* rs2285666 variant and ACE2 blood level. In Contrary, other studies stated that *ACE2*rs2285666 variants were not associated with COVID-19 severity [45-47]. This inconsistency across studies around the world may be related to ethnic variations between populations as these variants show some population-based differences [48-49]. Several studies showed that genotypic and allelic frequencies of *ACE* rs4646994 *I/D* variant has no association with COVID-19 severity [45-46]. Similarly, in the current study, genotypic and allelic frequencies of *ACE* rs4646994 *I/D* variant showed no difference among the three groups. However haplotype analysis showed that patients who have D (variant) allele of *ACE* gene combined with A (variant) allele of *ACE2* gene (D/A) will have nearly double the risk of having severe COVID infection (OR=1.9, *P*=0.024). Therefore, we may encounter conflicting results but this should not underestimate the role of variants in the *ACE* and *ACE2* genes in susceptibility to COVID-19 or disease severity. This is because the two enzymes have been implicated in causing severe lung injury and organ regression in COVID-19 patients. Besides, their receptors are key factors for the entry of SARS-CoV-2 into cells, retention of sodium and water with hypertension, and the promotion of fibrotic and inflammatory conditions that lead to cytokine release syndrome (or cytokine storm) [50]. We recommend a prospective study with larger sample size and diverse populations to verify any relationships between ACE1 I/D and ACE2 rs2285666 variants and COVID19 severity.

4. Conclusion

Critical factors included age (>50 years), presence of co-morbidities, high ACE/ACE2 ratio, and presence of D/A (variant/variant) allelic haplotype of *ACE* and *ACE2* genes are recognized as pivotal mediators for COVID-19 severity. Additionally, our results support the notion that host genetic variants may affect clinical outcomes of COVID-19-2 infection, which may pave the way for personalized medicine.

Declarations

Ethics approval and consent to participate

The study protocol conformed to ethical guidelines of the Declaration of Helsinki, 1975(14) and was approved by the Research Ethics Committee, Faculty of Medicine, Cairo University (MD-299-2020). Policy of data confidentiality was strictly followed. The aim was explained clearly, and informed consent was obtained from all participants before enrolment.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The manuscript is original work of the authors. Authors prepare all data used in the manuscript originally. The manuscript has not been and will not be published elsewhere or submitted elsewhere for publication.

Competing interests

There is no conflict of interest.

Funding

This study was partially funded by the Faculty of Medicine, Cairo University by purchasing the kits of laboratory work. The entire work was independent of the funder.

Authors' contributions

All the authors contributed to this manuscript. Mona Hegazy and Mona Fathy contributed to the conception and design of the work. Samar Moemen, WafaaAshour, Ahmed Abdelghani, and Omar ashoush: recruited the patients with collection of demographic and clinical data. Samar Moemen, Shadia Hussein, Naglaa Elsalawy and Mona Fathy: performed the chemical and genetic assays. Dalia Abdelfatah: performed the statistical analysis. Mona Fathy, Dalia Hamed and Dalia Abdelfatah: wrote the manuscript. All the authors revised and approved the submission of this manuscript.

Acknowledgments

Not applicable

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