Assessment of *ACE1* variants and *ACE1/ACE2* expression in COVID-19 patients

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ABSTRACT

Contribution of the renin-angiotensinogen system in the risk of COVID-19 and related complications have been assessed by several groups. However, the results are not consistent. We examined levels of *ACE1* and *ACE2* in the circulation of two groups of COVID-19 patients (ICU-admitted and general ward-admitted patients) compared with healthy controls. We also genotyped two polymorphisms in *ACE1* gene (the *ACE1*-I/D polymorphism rs1799752 and rs4359) to appraise their association with expression levels of *ACE1* and *ACE2*. Expression level of *ACE1* was significantly higher in ICU patients compared with non-ICU patients (P value = 0.02). However, its expression was not significantly different between total COVID-19 patients and total controls (P value = 0.34). *ACE2* expression was not different ether between two groups of COVID-19 patients (P value = 0.12) or between total COVID-19 patients and total controls (P value = 0.79). While distribution of rs1799752 and rs4359 alleles was similar between study groups, genotype frequencies of rs1799752 were differently distributed among total COVID-19 patients and controls (P value = 0.00001). Moreover, genotypes of the other polymorphism tended to be distinctively distributed among these two groups (P value = 0.06). In the total population of patients and controls, different *ACE1* mRNA levels were observed among carriers of different rs1799752 genotypes; of note, ID genotype carriers showed a higher expression of *ACE1* compared with II genotype carriers ($P = 0.01$). *ACE1* polymorphisms might affect risk of COVID-19 and expression of *ACE* transcripts.

1. Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as a global health problem since late 2019. This disorder has been associated with abnormal immune responses in some patients. Adult stem cells which have immunomodulatory and pro-reparative activities in the local environment [1,2], might be used as modulators of these response. Several research groups have aimed at identification of the underlying mechanism of susceptibility to this infection and development of severe forms of COVID-19 pointing to changes in the expression of certain genes or the presence of polymorphisms in genes mediating virus entry

to target cells. *Angiotensin-converting enzyme* (*ACE2*) as the gene encoding the specific receptor of the SARS-CoV-2 has been at the center of attention. Expression level of ACE2 in the epithelial cells have been suggested as a factor for increasing susceptibility to COVID-19 [3]. Others have linked the lower risk of COVID-19 among children to the decreased levels of ACE2 receptor in them compared with adults [4].

Meanwhile, the presence of polymorphisms in renin-angiotensin system has been associated with risk of COVID-19 and disease course. For instance, *ACE1*-insertion/deletion (I/D) polymorphism has been shown to be strongly associated with COVID-19. Carriers of DD genotype have exhibited higher ACE1 levels and higher risk for development of acute respiratory distress syndrome and mortality [5,6]. In fact, D allele

of this polymorphism has been found to be associated with progression of COVID-19 [7] and mortality rate from this infection [6].

Evidence suggests that SARS-CoV-2 interferes with normal balance of ACE1/ACE2 and induces the angiotensin II (Ang II)/Angiotensin II type 1 receptor (AT1R) pathway, resulting in severe COVID-19 consequences [8]. In addition, down-regulation of ACE2 and the imbalance between the renin-angiotensin system and ACE2/angiotensin- [1–7]/MAS following COVID-19 infection has been proposed to participate in the pathogenesis of multiple organ damage in this disorder [9].

In this study, we examined levels of *ACE1* and *ACE2* in the circulation of two groups of COVID-19 patients (ICU-admitted and general ward-admitted patients) compared with healthy controls. Moreover, we genotyped two polymorphisms in *ACE1* gene (the *ACE1*-I/D polymorphism rs1799752 and rs4359) to appraise their association with expression levels of *ACE1* and *ACE2*.

2. Material and methods

2.1. Cases and controls

The present genotyping and expression assay project was performed on 91 COVID-19 cases admitted to Nikan Hospital, Tehran, during 2020. COVID-19 was confirmed in all cases through RT-PCR method on nasopharyngeal swab samples. In addition, 91 control specimens were obtained from unaffected individuals without history of exposure to COVID-19 cases. The presence of any infectious or immune-related disorder was considered as criterion for exclusion of controls. The study protocol was approved by ethical committee of Shahid Beheshti University of Medical Sciences. Informed consent was obtained from all COVID-19 cases and controls. Laboratory parameters were gathered from all COVID-19 cases.

2.2. Genotyping

Tetra-primer amplification-refractory mutation system-PCR method was used for identification of rs4359 genotypes according to our former research [10]. Primers were designed using Primer1 software. The sequences of primers were as follow: Forward inner primer (T allele): GGGTCAGACAGAACTGGGTTCAATCT, Reverse inner primer (C allele): TTCTCTAGGAAACAAAGTAATGGAGACTGG, Forward outer primer: TGGCTAATGGTTACCTGACCTTGGTTAA and Reverse outer primer: TAGAGAGTGATGAATAGTGGGGTCCTGG. Annealing step was set at 62 °C.

Two rounds of PCR and electrophoresis were used for genotyping of the rs1799752 (I/D) polymorphism. First round of PCR was accomplished using the following primers: TGGAGAGCCACTCCCATCCTTTCT and GACGTGGCCATCACATTCGTCAGAT. Second round of PCR was performed using TGTAAGCCACTGCTGGAGAG and TGGCCATCA-CATTCGTCAGA as forward and reverse primers, respectively. The PCR program consisted an initial denaturing phase at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, specific annealing temperature for 30 s and extension at 72 °C for 60 s. Finally, microtubes were incubated at 72 °C for 5 min. Genotyping results with confirmed with Sanger sequencing of a number of samples.

2.3. Expression assays

Blood samples were collected from COVID-19 patients and healthy controls in EDTA-containing tubes. Total RNA was extracted from all samples using GeneAll RNA extraction kit (Seoul, South Korea). Then, RNA was converted to complementary DNA using the BioFact™ kit (Seoul, South Korea). Levels of *ACE1* and *ACE2* genes were quantified in all samples in relation with expression of *B2M* gene. The RealQ Plus 2× Master Mix (Amplicon, Denmark) was used for preparation of reactions. Table 1 demonstrates the information about primers sequences and amplicons.

Table 1
Sequence of primers used in expression assays.

Gene	Primer sequence		Primer length	Product size
ACE1	Forward primer	ACGTGAGGATACAGCAAGGC	20	75
	Reverse primer	AGAGTTCCTGCATGGTCTGG	20	
ACE2	Forward primer	ATCTACTCCACCGCCAAGGT	20	187
	Reverse primer	TGCTGAGGGCAGTGAAATCC	20	
B2M	Forward primer	AGATGAGTATGCCTGCCGTG	20	105
	Reverse primer	GCGGCATCTTCAAACCTCCA	20	

2.4. Statistical methods

The Statistical Package for the Social Sciences (SPSS) v.22.0 (SPSS Inc., Chicago, IL) and SNP Analyzer 2.0 were used for statistical assessments. Graphics were created using GraphPad Prism version 9.0 for Windows (La Jolla California, USA). Expressions of *ACE1* and *ACE2* genes in each sample were calculated using the Efficiency adjusted Ct of normalizer gene (*B2M*) - Efficiency adjusted Ct of target genes (comparative –delta Ct method). Student *t*-test was used to compare expression levels of *ACE1* and *ACE2* between groups (COVID-19 patients vs. controls, and ICU patients vs. non-ICU patients). Mann Whitney's *U* test or student t-test was used to compare laboratory data between subgroups of COVID-19 patient (ICU patients vs. non-ICU patients).

Allele and genotype frequencies were compared between groups by the chi-squared test. Relative risk (odds ratio (OR)) for effect alleles and genotypes was calculated by logistic regression. Adjusted relative risks were calculated considering gender and age as covariates. Associations between genomic variants and COVID-19 risk were assessed in codominant, dominant, recessive and over-dominant models. The results of association analysis were described as OR and 95% confidence interval of OR (95% CI), *P*-value and FDR adjusted q-values. The FDR adjusted q-values were calculated through analyzing a stack of *p* values in column analyses by GraphPad Prism version 9.0. *P*-values less than 0.05 were considered as statistically significant. Estimation of accordance of genotype distributions with Hardy–Weinberg equilibrium, haplotype estimation, linkage disequilibrium (LD) blocking and were performed in SNP Analyzer 2.0.

The correlation of *ACE2* and *ACE1* expression levels with age, complete blood cells, ESR and CRP were analyzed using nonparametric spearman correlation test.

3. Results

3.1. General data of patients

First, we compared general laboratory data of ICU-admitted and general ward-admitted patients. This comparison showed higher levels of ESR and CRP in ICU-admitted group (*P* values = 0.011 and 0.000001, respectively). Moreover, ICU-admitted patients had lower lymphocyte count while higher neutrophil count (P values = 0.000492 and 0.000086, respectively) (Table 2).

3.2. Genotyping and expression assays

Expression level of *ACE1* was significantly higher in ICU patients compared with non-ICU patients (*P* value = 0.02). However, its expression was not significantly different between total COVID-19 patients and total controls (*P* value = 0.34). *ACE2* expression was not different ether between two groups of COVID-19 patients (*P* value = 0.12) or between total COVID-19 patients and total controls (P value =

Table 2
General laboratory data of COVID-19 patients' subgroups.

Parameters	ICU (*N* = 37)	Non-ICU (*N* = 54)	p[a]
WBCs	10,316.48 ± 1980.19	6613.3 ± 598.21	0.027
RBC	4.6 ± 0.14	4.73 ± 0.091	0.29
HB	12.29 ± 0.41	12.98 ± 0.26	0.133
HCT	38.21 ± 1.25	39.98 ± 0.78	0.23
MCV	83.08 ± 0.9	84.6 ± 0.79	0.32
MCH	26.76 ± 0.39	27.41 ± 0.31	0.106
MCHC	32.24 ± 0.25	32.42 ± 0.16	0.317
PLT	225,718.91 ± 17,222.78	199,827.77 ± 12,044.75	0.17
LYM	16.35 ± 1.68	24.25 ± 1.5	0.000492
NEU	75.21 ± 1.93	64.90 ± 1.69	0.000086
ESR	54.21 ± 5.56	37.22 ± 4.16	0.011
CRP	115.29 ± 12.21	44.45 ± 6.76	0.000001

[a] Mann Whitney's *U* test or student t-test was used to analyze data between subgroups of COVID-19 patients.

0.79). While distribution of rs1799752 and rs4359 alleles was similar between study groups, genotype frequencies of rs1799752 were differently distributed among total COVID-19 patients and controls (*P* value = 0.00001). Moreover, genotypes of the other polymorphism tended to be distinctively distributed among these two groups (P value = 0.06). Table 3 shows demographic data, relative expressions of *ACE1* and *ACE2* and distribution of *ACE1* variants in COVID-19 patients and matched controls.

Distribution of genotype frequencies of both polymorphisms were in accordance with Hardy-Weinberg equilibrium in control subjects (*P* values = 0.77 and 0.1, respectively), but not in patients (P values = 0.0018 and < 0.0001, respectively) (Table 4).

Fig. 1 shows distributions of alleles and genotypes of rs4359 and rs1799752 variants among normal controls, all COVID-19 patients, non-ICU and ICU-admitted cases.

Fig. 2 demonstrates relative expressions of *ACE1* and *ACE2* in COVID-19 patients compared with healthy controls.

3.3. Association between ACE1 genotypes and ACE1 and ACE2 expressions

The *ACE1* rs1799752 polymorphism was associated with a high risk of COVID-19 in dominant and co-dominant models (Fig. 3). In the

Table 4
The results of exact-test for Hardy-Weinberg equilibrium (P values and genotype distributions are shown).

Variants	rs4359				rs1799752			
	CC	CT	TT	Hardy-Weinberg P-value	DD	ID	II	Hardy-Weinberg P-value
COVID-19 patients	19	60	12	0.0018	17	70	4	<0.0001
Normal controls	32	45	14	0.77	33	37	21	0.1

dominant model, the presence of at least one mutated (−) allele was tested against the homozygous wildtype genotype (wt/wt). The *ACE1* rs1799752 polymorphism showed a significant protective effect against COVID-19 risk in over-dominant model. In the total population of patients and controls, different *ACE1* mRNA levels were observed among carriers of different rs1799752 genotypes; of note, ID genotype carriers showed a higher expression of *ACE1* compared with II genotype carriers (*P* = 0.01).

ACE2 expression was significantly higher in ID genotype carriers than the II genotype carriers in total population. In fact, increase in *ACE1* expression was associated with increase in the *ACE2* expression in ID genotype carriers (Fig. 4).

The *ACE* rs4359 polymorphism was associated with a higher risk of COVID-19 in dominant and co-dominant models. The *ACE1* rs4359 polymorphism showed a significant protective effect against the risk for COVID-19 in over-dominant model. However, in contrast to the rs1799752 genotypes, there was no significant difference in the *ACE1* expression level between carriers of different rs4359 genotypes in the total population of patients and controls (Fig. 5).

There was no significant difference in *ACE2* expression level among the rs4359 genotype carriers which was consistent with *ACE1* expression levels among the rs4359 genotype carriers (Fig. 6).

There was no significant difference in distribution of rs4359 and rs1799752 variants between CIVID-19 cases and control in allelic model.

Then, we appraised association between estimated haplotypes and COVID-19 risk. The results showed similar distribution of *ACE1* haplotypes between COVID-19 cases and healthy controls (Table 5).
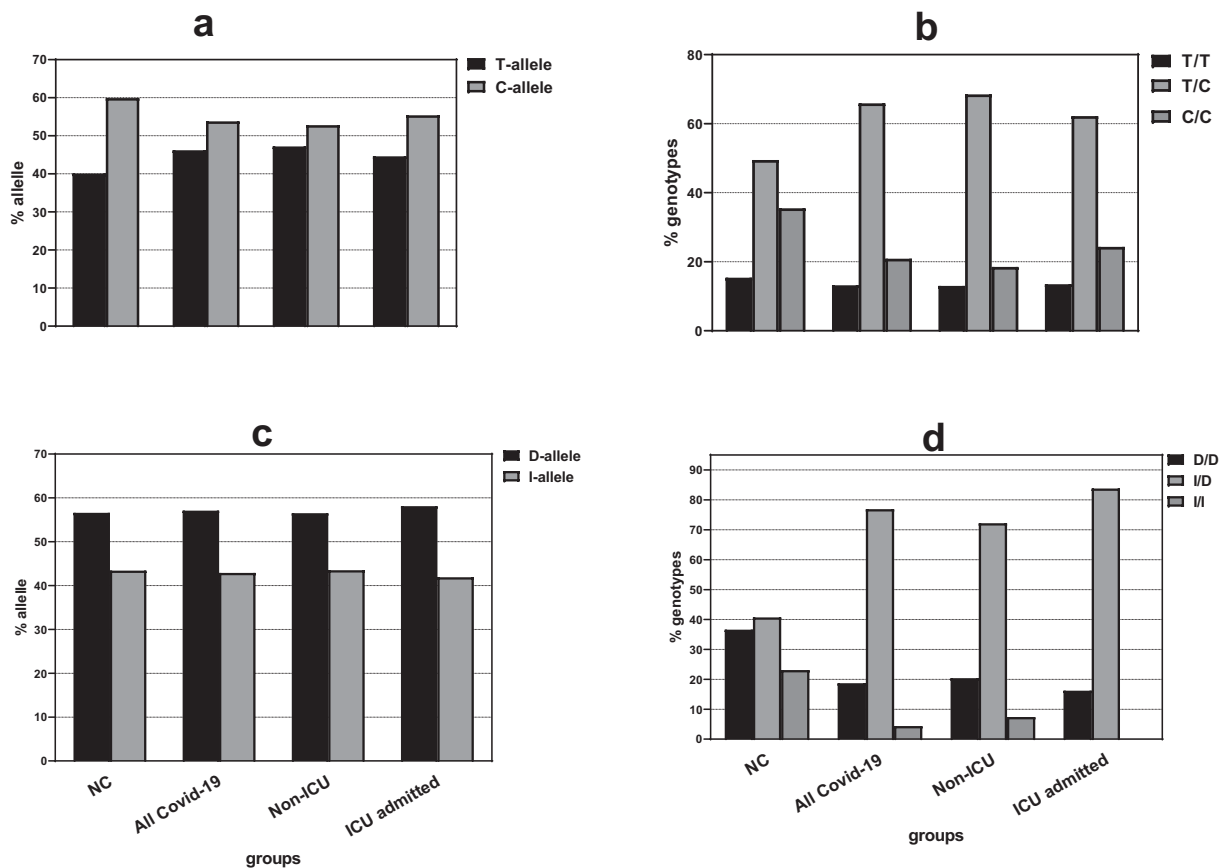
Table 3
Demographic data, *ACE1* and *ACE2* relative expressions and distribution of *ACE1* variants in COVID-19 patients and matched controls.

Parameters	ICU patients (*N* = 37)	Non-ICU patients (*N* = 54)	p[a]	All COVID-19 patients (*N* = 91)	Controls (N = 91)	p[a]
Gender						
Male	24 (64.9)	29 (53.7)	0.28	53 (58.2)	52 (57.1)	Adjusted
Female	13 (35.1)	25 (46.3)		38 (41.8)	39 (42.9)	variable
Age (year)	58.62 ± 2.66	56.18 ± 2.39	0.50	57.17 ± 1.78	51.82 ± 1.5	Adjusted variable
ACE1 Expression (−delta CT)	−3.63 ± 1.08	−6.43 ± 0.65	0.02	−5.29 ± 0.6	- 4.85 ± 0.5	0.34
ACE2 Expression (−delta CT)	−0.61 ± 0.57	−2.06 ± 0.66	0.12	−1.47 ± 0.46	−1.28 ± 0.58	0.79
Allele (n %)						
rs1799752						
I*	31 (41.9)	47 (43.5)	0.82	78 (42.9)	79 (43.4)	0.91
D*	43 (58.1)	61 (56.5)		104 (57.1)	103 (56.6)	
rs4359						
T*	33 (44.6)	51 (47.2)	0.72	84 (46.2)	73 (40.1)	0.24
C*	41 (55.4)	57 (52.8)		98 (53.8)	109 (59.9)	
ACE1 Genotypes (n %)						
rs1799752						
II	0 (0)	4 (7.4)	0.09	4 (4.4)	21 (23.1)	<0.00001
ID	31 (83.8)	39 (72.2)		70 (76.9)	37 (40.7)	
DD	6 (16.2)	11 (20.4)		17 (18.7)	33 (36.6)	
rs4359						
TT	5 (13.5)	7 (13)	0.78	12 (13.2)	14 (15.4)	
TC	23 (62.2)	37 (68.5)		60 (65.9)	45 (49.5)	0.06
CC	9 (24.3)	10 (18.5)		19 (20.9)	32 (35.5)	

Data are shown as mean ± standard error of mean or percentage. Alleles*, total number of chromosomes.
[a] student's *t*-test, Mann Whitney's *U* test or Chi square test as appropriate.

Fig. 1. Distributions of alleles (a, c) and genotypes (b, d) of rs4359 and rs1799752 among normal controls (NC), COVID-19 patients, non-ICU and ICU-admitted cases. There was significant difference between genotypes distribution of rs1799752 among COVID-19 patients compared to matched controls.
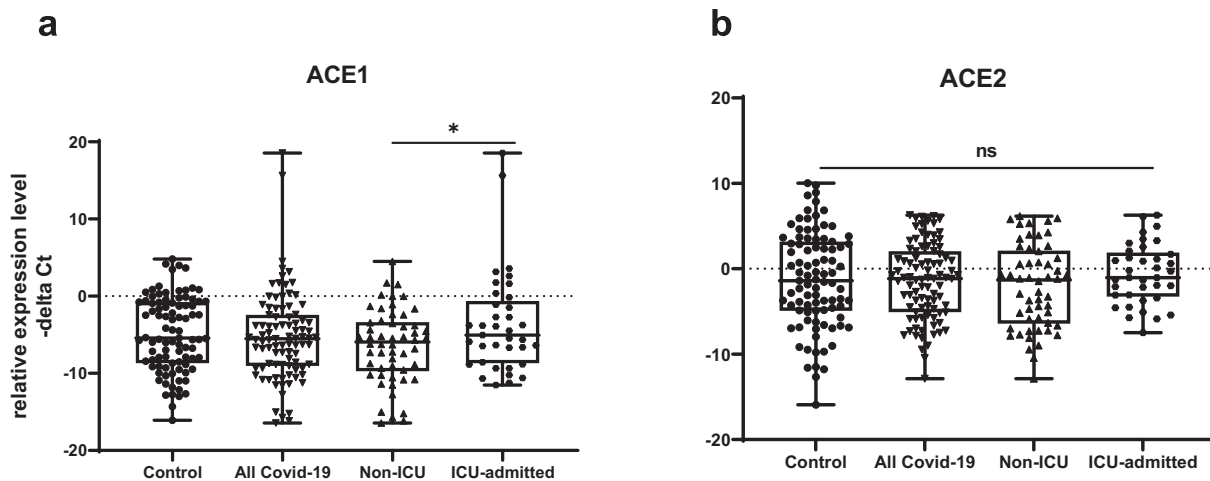

Fig. 2. Relative expressions of *ACE1* and *ACE2* in COVID-19 patients ($n = 91$) compared with healthy controls (n = 91). There was no significant difference in expression of *ACE1* or *ACE2* between COVID-19 patients and healthy controls. However, a moderate increase in *ACE1* expression was observed in ICU-admitted COVID-19 patients compared to other group of COVID-19 patients. – delta Ct values in the figures were plotted as box and whisker plots (showing the median [line], interquartile range [box], and minimum and maximum values).

A strong positive correlation has been detected between *ACE1* and *ACE2* expression in COVID-19 patients, healthy controls as well as total population (Fig. 7).

Finally, the correlation of *ACE2* and *ACE1* expression levels with age, blood cells counts, ESR and CRP was analyzed using nonparametric spearman correlation test. – delta CTs of *ACE1* and *ACE2* genes and log 2 of age and blood cells parameters were used for correlation tests. There was a significant positive correlation between *ACE2* expression levels and RBC, HB and HCT levels in COVID-19 patients. However, *ACE2* expression levels had a negative correlation with ESR in COVID-19 patients (Fig. 8). In contrast to *ACE2*, *ACE1* expressions had no correlations with assessed factors (except for HCT ($r = 0.2$, $p = 0.04$)).
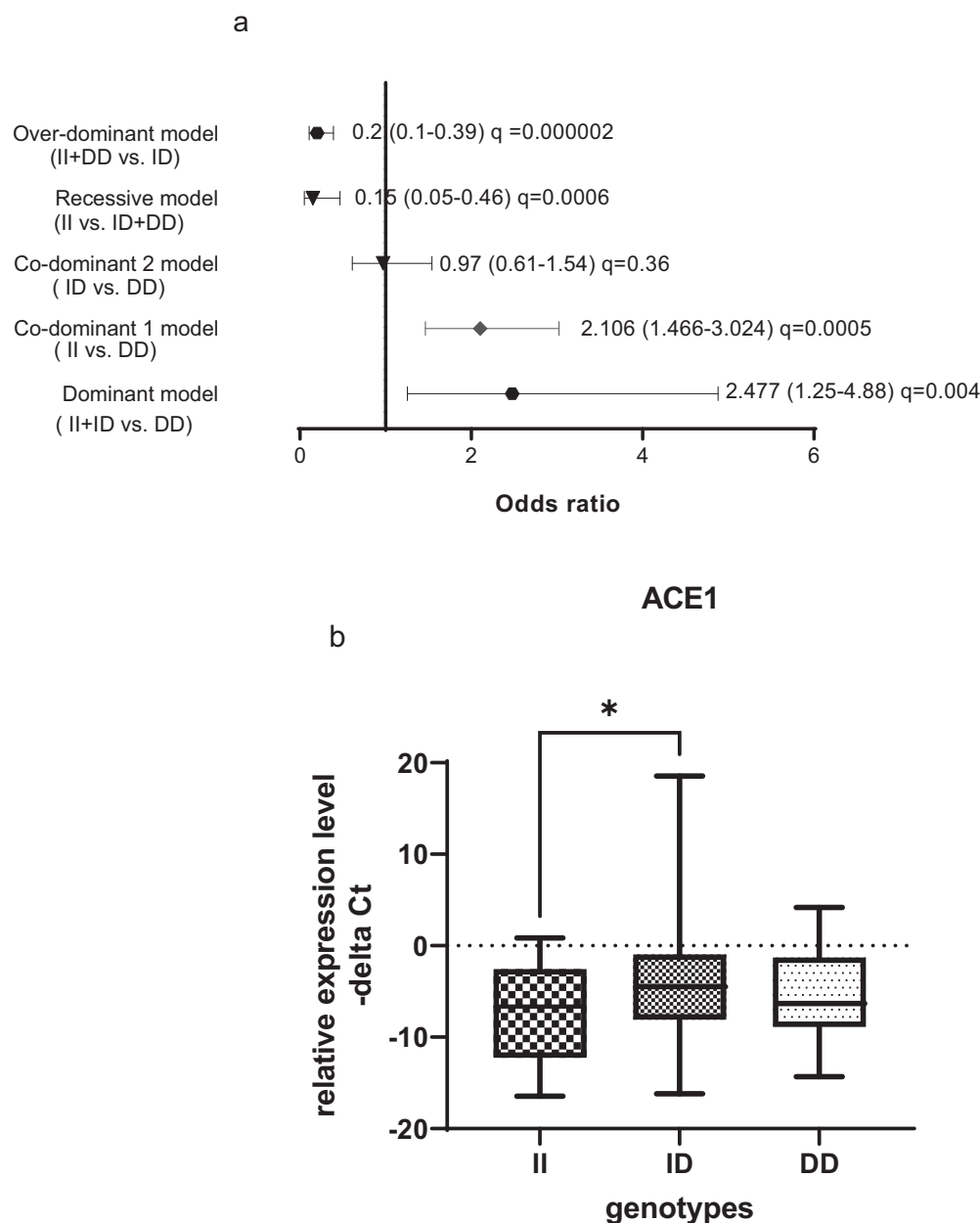
a

Over-dominant model
(II+DD vs. ID) 0.2 (0.1-0.39) q =0.000002

Recessive model
(II vs. ID+DD) 0.15 (0.05-0.46) q=0.0006

Co-dominant 2 model
(ID vs. DD) 0.97 (0.61-1.54) q=0.36

Co-dominant 1 model
(II vs. DD) 2.106 (1.466-3.024) q=0.0005

Dominant model
(II+ID vs. DD) 2.477 (1.25-4.88) q=0.004

Odds ratio

ACE1

b

Fig. 3. Association between *ACE1* rs1799752 polymorphism and COVID-19 infection as well as *ACE1* mRNA levels by rs1799752 genotypes. (a) The results of association tests under five different inheritance models are shown. The Odds Ratios (plus Confidence Intervals) are reported on the X axis in a linear scale. Data on the right of Y axis indicates causative effects toward the risk and the data on the left indicates protective effects. The *ACE1* rs1799752 polymorphism was associated with a high risk of COVID-19 in dominant and co-dominant models. In the dominant model, the presence of at least one mutated (−) allele was tested against the homozygous wildtype genotype (wt/wt). The *ACE1* rs1799752 polymorphism showed a significant protective effect against COVID-19 risk in over-dominant model. (b) In the total population of patients and controls, different *ACE1* mRNA levels were observed among carriers of different rs1799752 genotypes; of note, ID genotype carriers showed a higher expression of *ACE1* compared with II genotype carriers (*P* = 0.01). The level of Odds Ratios was showed as FDR adjusted q-values. MAF: minor allele frequency.

4. Discussion

The importance of renin-angiotensin system in the pathogenesis of COVID-19 has been assessed by several studies. Over-activation of the renin-angiotensin-aldosterone has been suggested to participate in abnormal biochemical and clinical manifestations of SARS-CoV-2 infection [11]. The protective renin-angiotensin system medicated by ACE2 might be inhibited in COVID-19 [11]. ACE2 is regarded as a negative regulator of renin angiotensin system which converts Ang II to angiotensin 1–7 [12]. On the other hand, ACE1 catalyzes biogenesis of Ang II from Ang I [13]. Thus, ACE1/ACE2 level has a critical significance in the pathogenesis of disorders associated with renin-angiotensin system [14].

In the current study, we demonstrated higher levels of *ACE1* in ICU patients compared with non-ICU patients. However, its expression was not significantly different between total COVID-19 patients and total controls. *ACE2* expression was not different ether between two groups of COVID-19 patients or between total COVID-19 patients and total

controls. These findings indicate imbalance in *ACE1/ACE2* level in severely affected COVID-19 patients.

The *ACE1* rs1799752 polymorphism was associated with a high risk of COVID-19 in dominant and co-dominant models. In the dominant model (II + ID versus DD), the presence of at least one mutated allele has been shown to increase risk of COVID-19. This polymorphism showed a significant protective effect against COVID-19 risk in over-dominant model (II + DD versus ID). Yet, due to small sample size, this result is not conclusive. In the total population of patients and controls, different *ACE1* mRNA levels were observed among carriers of different rs1799752 genotypes. Most notably, ID genotype carriers showed a higher expression of *ACE1* compared with II genotype carriers. Supposing the impact of ACE1 up-regulation in COVID-19, this finding is in accordance with the observed association between this polymorphism and risk of COVID-19 in dominant and over-dominant models in the current study. However, these findings are in contrast with the previously reported higher levels of ACE1 in carriers of DD genotype [5,6]. *ACE2* expression was also higher in ID genotype carriers than the II genotype carriers in total
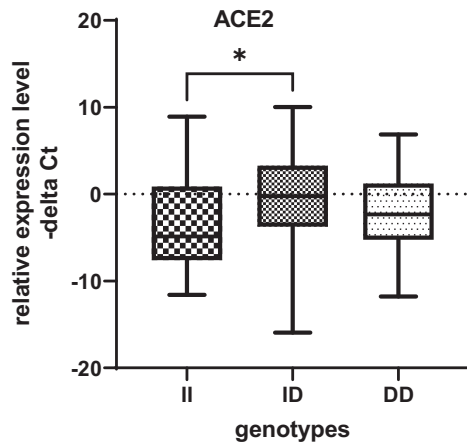
Fig. 4. *ACE2* expression was significantly higher in ID genotype carriers than the II genotype carriers in total population.

population.

The *ACE1* rs4359 polymorphism was associated with a higher risk of COVID-19 in dominant and co-dominant models. The *ACE1* rs4359 polymorphism showed a significant protective effect against the risk for
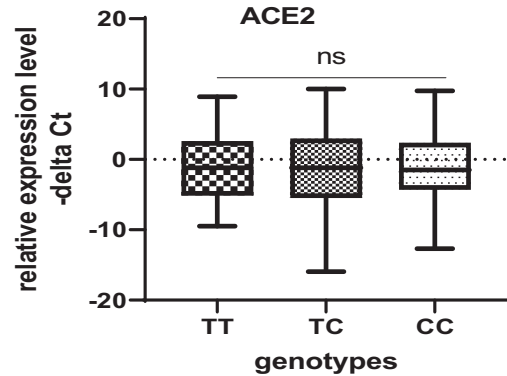

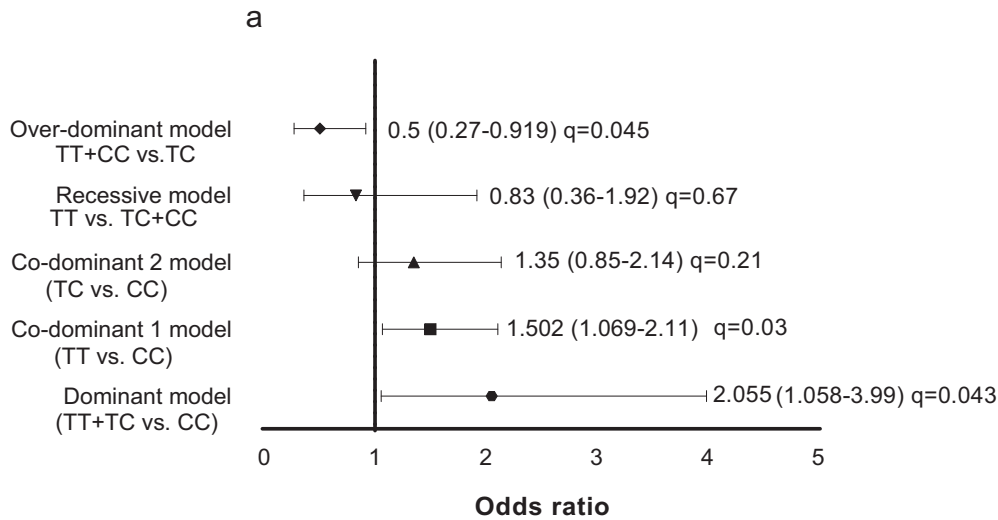**Fig. 6.** *ACE2* expression in carriers of different rs4359 genotypes in total population.

Fig. 5. Association between *ACE1* rs4359 gene polymorphism and COVID-19 risk as well as *ACE1* mRNA levels by rs4359 genotypes. (a) The results of association tests under five different inheritance models are shown. The Odds Ratios (plus Confidence Intervals) are reported on the X axis in a linear scale. Data on the right of Y axis indicates causative effects toward the risk and the data on the left indicates protective effects. The *ACE1* rs4359 polymorphism was associated with a higher risk of COVID-19 in dominant and co-dominant models. The *ACE1* rs4359 polymorphism showed a significant protective effect against the risk for COVID-19 in over-dominant model. (b) There was no significant difference in the *ACE1* expression level between rs4359 genotypes in the total population of patients and controls. Odds Ratios are shown as FDR adjusted q-values. MAF: minor allele frequency.
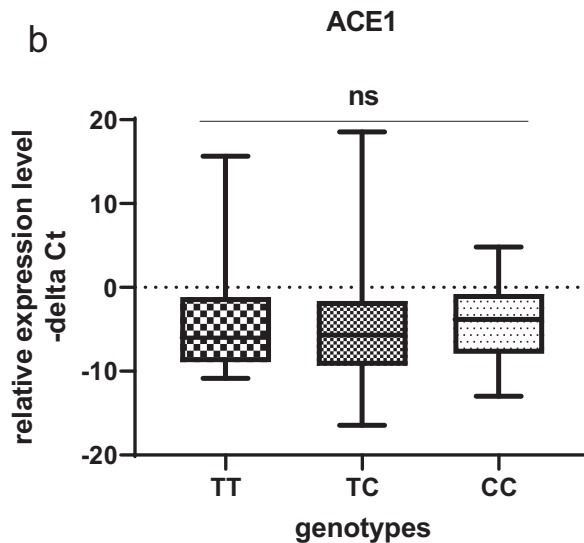
Table 5
The results of haplotype analysis in COVID-19 patients and controls.

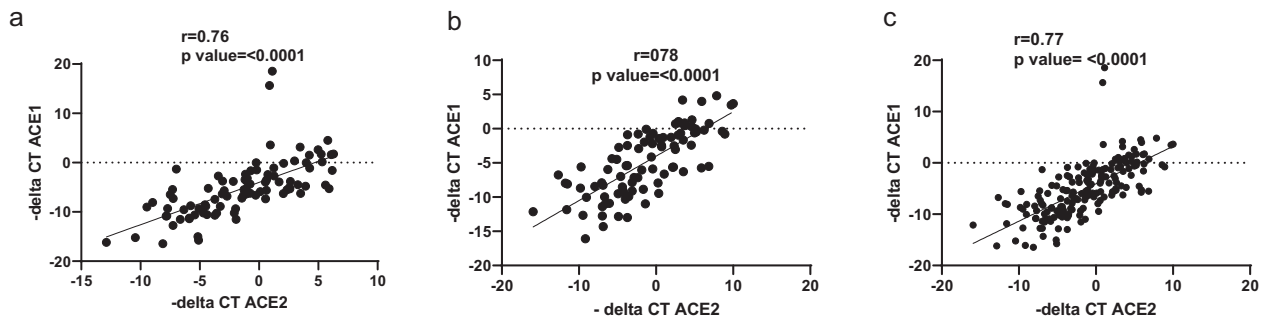| rs4359	rs1799752	Case	Control	Total	OR (95% CI)	P-value	FDR q-Value
C	D	0.22	0.35	0.32	1.14 (0.75–1.74)	0.52	0.525
C	I	0.31	0.24	0.23	0.48 (0.26–0.87)	0.014	0.057
T	D	0.34	0.21	0.23	0.81 (0.46–1.43)	0.47	0.525
T	I	0.11	0.19	0.19	1.55 (0.97–2.48)	0.06	0.121

Fig. 7. Correlation between *ACE1* and *ACE2* expression levels. There was a strong positive correlation between *ACE1* and *ACE2* expression in COVID-19 patients (a), healthy controls (b), and total population (c).
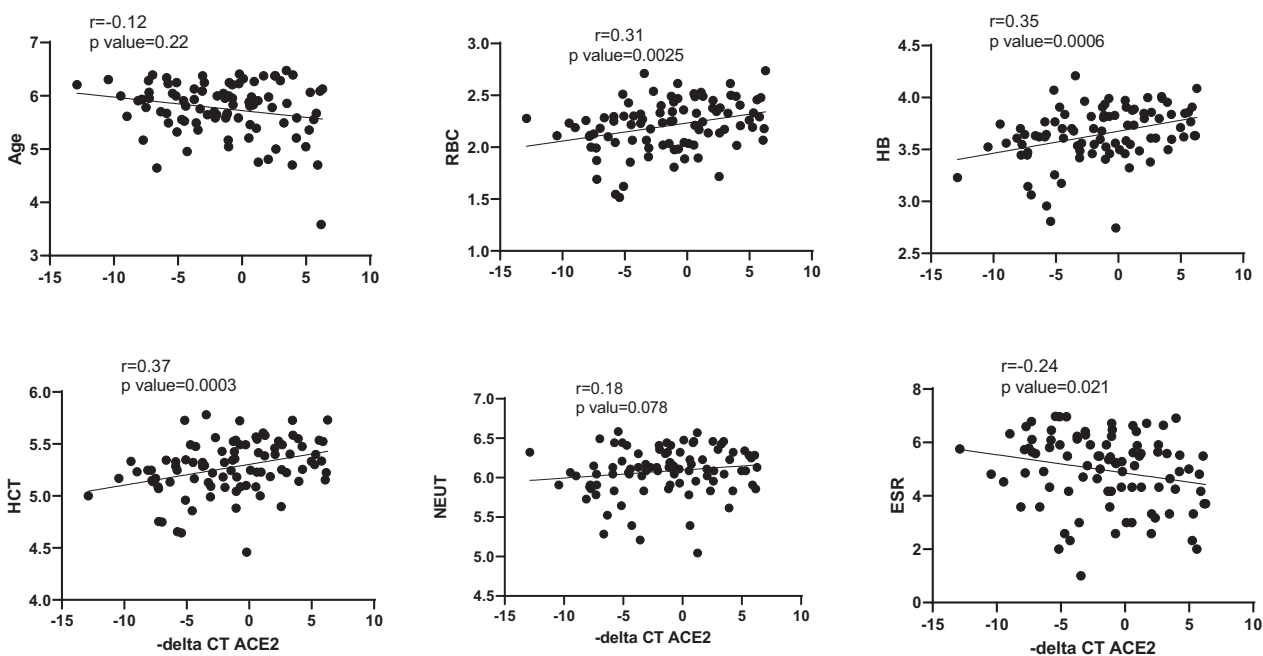

Fig. 8. Correlation of *ACE2* expression levels with age, RBC, HB, HCT, NEUT and ESR of COVID-19 patients. – delta CTs of *ACE2* and log 2 of age and blood cells parameters were used for correlation tests.

COVID-19 in over-dominant model. However, in contrast to the rs1799752 genotypes, there was no significant difference in the *ACE1* or *ACE2* expression level between carriers of different rs4359 genotypes in the total population of patients and controls. Thus, this polymorphism can be regarded as a non-functional polymorphism in this regard, although a previous study had reported the role of this polymorphism in modulation of response of patients to a certain ACE inhibitor medication [15].

A strong positive correlation has been detected between *ACE1* and *ACE2* expression in COVID-19 patients, healthy controls as well as total population. Thus, one can deduce that the balance between these two transcripts is only impaired in severely affected COVID-19 cases.

Finally, *ACE2* expression levels were positively correlated with RBC,

HB and HCT levels in COVID-19 patients. However, *ACE2* expression levels had a negative correlation with ESR in COVID-19 patients. In contrast, *ACE1* expression was only correlated with HCT. A previous study has demonstrated the impact of Ang II on enhancement of erythropoiesis [16]. In fact, ACE1 knockout mice have exhibited anemia in spite of having normal kidney function [16]. Considering the opposite impacts of ACE1 and ACE2 on Ang II levels [13,14], *ACE1* correlation with HCT can be contributed to the impact of this enzyme on erythropoiesis. However, the underlying mechanism for the observed correlation between *ACE2* levels and the mentioned parameters should be clarified in future.

Taken together, *ACE1* polymorphisms might affect risk of COVID-19 and expression of *ACE* transcripts.

Authors statement

SGF wrote the draft and revised it. MT designed and supervised the study. SE analyzed the data. MA, GA, NA and BMH performed the experiment and collected the clinical data and samples. All the authors read and approved the submitted version.

Declaration of competing interest

The authors declare they have no conflict of interest.

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