ACE-2 Expression and Methylation Pattern in Bronchoalveolar Lavage Fluid and Bloods of Iranian ARDS Covid-19 Patients

Reza Najafipour[1] , **Davood Mohammadi[2]** , **Mabood Momeni[3]** , **Sahar Moghbelinejad*[4]**

1. Genetics Research Center, the University of Social Welfare and Rehabilitation Science, Tehran, Iran.

2. Department of Surgery, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran.

3. Khatam Pathobiology and Genetic Lab, Qazvin, Iran.

4. Research Institute for Prevention of Non-Communicable Diseases, Cellular and Molecular Research Centre, Qazvin University of Medical Sciences, Qazvin, Iran.

Article type:	**ABSTRACT**
Original Article	The aim of the present study was to investigate the expression and methylation pattern of the angiotensin I converting enzyme 2 (ACE-2) in acute respiratory distress syndrome (ARDS) covid-9 patients. A total of 25 patients with covid-19 ARDS and 20 controls were recruited. Expression of the ACE-2 gene was evaluated by quantitative real time PCR, and methylation of CpG dinucleotides, in the ACE-2 promoter, was quantified using bisulfite pyro-sequencing. Our results showed high expression of the ACE-2 gene in the blood samples of ADRS patients (1.93 ± 0.67) in comparison to controls (0.62 ± 0.35) (P = 0.03). Correspondingly, in ARDS bronchoalveolar lavage fluid (BALF) samples, there was a high expression of this gene (1.8 ± 0.78) in comparison to controls (0.58 ± 0.2) (p <0.05). Moreover, the methylation rate of the ACE-2 gene in blood samples of ARDS patients was 64.07 ± 6.1 in comparison to controls (80.3 ± 7.3) (p<0.0001). In BALF samples, there was this pattern too (55.07 ± 3.1 vs. 72.35 ± 5.1) (p<0.0001). Finally, a significant correlation was found between expression and methylation in BALF (R= -0.54, P= 0.002) and blood (R= -0.321, P= 0.013) samples. These results indicated that aberrant methylation of the ACE-2 promoter might be associated with high expression of this gene and the occurrence of ARDS in covid-19 patients.
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*Corresponding Author: Sahar Moghbelinejad
Address: Research Institute for Prevention of Non-Communicable Diseases, Cellular and Molecular Research Centre, Qazvin University of Medical Sciences, Qazvin, Iran.
E-mail: smoghbelinejad@qums.ac.ir

Introduction

Coronaviruses (CoV) are a major family of human and animal pathogens. The COVID-2019 pandemic emerged in Wuhan, China, in December 2019 due to a highly contagious coronavirus capable of human infection and transmission. The rapid spread and the host diversity of severe acute respiratory syndrome coronaviruses-2 (SARS-CoV-2) highlighted the need for in deep understanding of its structure and biological function (1). Emerging experimental evidence reveals an interplay of genetic and epigenetic alterations in host response (2). Angiotensin-converting enzymes (ACEs) play an essential role in the regulation of blood pressure as well as electrolyte and fluid homeostasis. These enzymes are parts of the renin-angiotensin (Ang) system. The function of ACE is the cleavage of AngI into AngII. Then, AngII degrades into small peptides by the ACE-2 enzyme (3, 4). SARS-CoV-2 enters the host cells through the ACE-2 receptor (4). The *receptor binding* motif of *S protein* interacts with ACE2, resulting in endocytosis by *host cells.* Studies have shown an imbalance of ACE to ACE-2 ratio involved in the pathophysiology of lung injury in SARC-COV-2 infection (5, 6).

One of the effects of the virus on humans, which often leads to death, is acute respiratory distress syndrome (ARDS), and there are still few studies on the molecular mechanisms involved in the outbreak of ARDS. On the other hand, studies emphasize the significant role of the host ACE-2 in SARS-CoV-2 entering the cells and finally cytokine storms (7, 8). In general, few studies have been done on the expression of ACE-2 on lung and blood samples of patients with ARDS, and the results are slightly contradictory. There are also very few studies on the regulation mechanisms of ACE-2 expression in ARDS patients. Since one of the mechanisms of regulating the expression of different genes is epigenetic variation and methylation, in this study, we decided to examine the pattern of expression and methylation of the ACE-2 gene in the bronchoalveolar fluid (BALF) and blood samples of ARDS patients

Materials and Methods

Sample collection

BALF and whole blood samples were obtained from 25 severe ARDS COVID-19 patients at Velayat Hospital, Qazvin, Iran, from September 2020 to September 2021. The patients were informed about the sample collection process and had signed informed consent forms. The recruited patients were men between 50 to 55 years old with severe shortness of breath or breathlessness, rapid and labored breathing, extreme tiredness, and muscle fatigue admitted to the ICU section. These patients had a positive result in the COVID-19 nasopharyngeal swab Reverse transcription PCR (RT-PCR) test. Patients with obesity, diabetes, heart, kidney, and lung diseases were not included in the study due to many factors involved in the expression and methylation of ACE-2. For BALF preparation, local anesthesia was done with injecting 2% lidocaine into the lung segment. 100 ml fractions of room temperature sterile saline were instilled into the right middle lobe of the left lingula part of the lung. BALF was retrieved by gentle syringe suction and put into sterile containers.

Control samples were candidates for BALF preparation for any medical reason; their RT-PCR test result for COVID-19 was negative, and they had no symptoms of ARDS. The controls were selected in such

a way as to match the gender and age distribution of test patients. 20 BALF and blood samples were collected from the control candidates. 5 cc blood samples were taken from patient and control candidates in venoject tubes with ethylene diamine tetraacetic acid (EDTA). Evaluation of blood parameters results such as WBC, RBC, HB, HCT, MCV, MCHC, NEU, ESR, CRP, and PLT were collected from patients' laboratory medical documents, Table (1). The Ethics Committee of the Qazvin University of Medical Sciences approved the study IR.QUMS.REC.1399.003.

DNA extraction and bisulfite modification

In this part, the standard phenol-chloroform method was used to extract DNA from the BALF and blood samples (9). Then, for the methylation study, 10 ng of genomic DNA was bisulfited using the EpiJET™ Bisulfite Conversion kit (Thermo Fisher Scientific, Inc) according to the kit instructions. Bisulfite treatment converted non-methylated cytosine 'C' bases to thymine 'T' bases; however, the methylated cytosine 'C' was unchanged. The methylation levels were determined by using the Pyrosequencing method. In this regard, CpG islands of the ACE-2 gene were identified using MethPrimer (www.urogene.org/methprimer). Then, CpG sites of interest were ChrX: 15621790-15621942, and PCR primers were selected according to the general rules and advice of the primer design. After the bisulfate treatment procedure, the target sequence of the ACE-2 gene was amplified by polymerase chain reaction with these primers: F 5'-GGGTAG ATTAAGAGGTTAGAAG-3', R 5'-Biotin-ATTCACCCC ATTCTCCTA-3', and this condition: 95 °C for 15 min (Hot start), followed by 35 cycles at 95 °C for 20 seconds (denaturation), 56.5 °C for 45 seconds (annealing), and 72 ℃ for 45 seconds (extension). Pyrosequencing was also done using Pyromark Gold Q96 (Qiagen GmbH) with this primer sequence: 5'-TTATTA AAAATATAAAAATATTAG-3'. Subsequently, complete cytosine conversion at a non-CpG site ensured successful bisulfite conversion. To evaluate the overall ACE-2 methylation level in BALF and blood samples, the amount of C relative to the amount of C and T at each CpG site was calculated as thepercentage.

RNA extraction and qRT-PCR

RNAs were extracted using RNeasy Mini Kit (Qiagen, Germany), and then they were frozen at -80 °C. We used NanoDrop 2000c (Thermo, USA) to evaluate the quality and quantity of isolated total RNAs. In this regard, RNA samples with A260/A280 ratios of ≥2 were selected for quantitative analysis. First-strand complementary DNA (cDNA) synthesis was initially performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA). Then, Real time quantification was performed using Rotor gene-Q real time PCR system (Qiagen, Germany). Each real time PCR (10 µL) included 1 µL of reverse and forward primers (Exiqon, Denmark), 5 µL of Amplicon real Q plus 2x master mix green (Ampliqone, Denmark), and 4 µL of diluted cDNA. Primer sequences for the ACE-2 gene are F: 5´-TCCATTGGTCTTCTGTCACCCG-3´, R: 5´-AGACCATCCACCT CCACTTCTC-3´. The reactions were incubated in a 72-well optical strip at 95°C for 15 min (enzyme activation), followed by 95 °C for 20 s and 60 °C for 60 s (40 cycles). All reactions were run in triplicate. After the reactions, the mean Ct was determined from the triplicate PCRs. We used Ct values to evaluate the expression levels of the ACE-2 gene. It should be noted that Beta-actin was the endogenous control gene to normalize RNA contents among different samples. The expression value of the ACE-2 gene relative to internal controls was determined using the $2^{-\Delta Ct}$ method.

Statistical analysis

The results of this research were analyzed by Graph Pad software (GraphPad PRISM V 5.04 Analytical software). The difference in the expression and methylation levels of ACE-2 between covid-19 patients and controls was calculated with a student t-test. For further investigation, the correlation between the prevalence of the ACE-2 expression and methylation with other parameters was evaluated by Pearson and Spearman correlation. All p-values were two-tailed, with $p < 0.05$ considered statistically significant.

Results

The mean age of the ARDS patients with covid-19 was 52.0 years which was not significantly different from the controls' mean of 51.8 years ($P = 0.116$). Because age and sex affect the methylation pattern ACE-2 gene, all patients and controls were males. There was no significant difference in their age range, ethics, and other factors such as diabetes and hypertension. Also, we evaluated the laboratory parameters of the studied samples; we saw a significantly high level of WBC ($P < 0.01$), NEU ($P = 0.0021$), ESR ($p < 0.0001$), and CRP ($p < 0.001$) in ARDS patients in comparison to controls, Table 1.

Table 1. Laboratory parameters of studied samples.			
Parameters	**Cases**	**Controls**	**P-value**
WBC	9543± 123.2	5432± 232.2	**<0.01**
RBC	5.2±1.2	5.3±0.99	0.22
HB	13.9±2.1	12.8±1.9	0.34
HCT	37.2±1.3	36.9±1.8	0.12
MCV	83.2±0.99	82.1±0.87	0.33
MCHC	27.2±1.2	26.6±0.75	0.432
PLT	224.343±12.3	211.165±10.1	0.11
NEU	72.1±5.2	63.1±3.8	0.003
ESR	49.1±3.1	15.2±2.1	0.0021
CRP	112.1±7.9	10.6.3±0.99	<0.0001

WBC: white blood cells, RBC: Red blood cells, HB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, PLT: Placate, NEU: Neutrophil, ESR: Erythrocyte sedimentation ratio, CRP: C reactive protein.

ACE-2 expression and methylation rate in ARDS patients and controls

BALF ACE-2 level significantly increased in covid-19 patients (1.8±0.78) as compared to controls (0.58±0.2) ($P < 0.03$) (Fig. 1(a)). We, moreover, noticed that ACE-2 levels significantly increased in blood samples of covid-19 (1.93± 0.67) as compared to controls (0.62±0.35) ($p < 0.05$) (Fig. 1(b)). We examined the ACE-2 methylation rate in 25 covid-19 BALF and blood samples compared to non-covid-19 samples. The covid-19 BALF samples exhibited a significantly lower rate of ACE-2 methylation (55.07 ±3.1) (%) than matched control samples (72.3±5.1) (%) ($p < 0.0001$) (Fig. 2a). In blood samples of covid-19 patients,

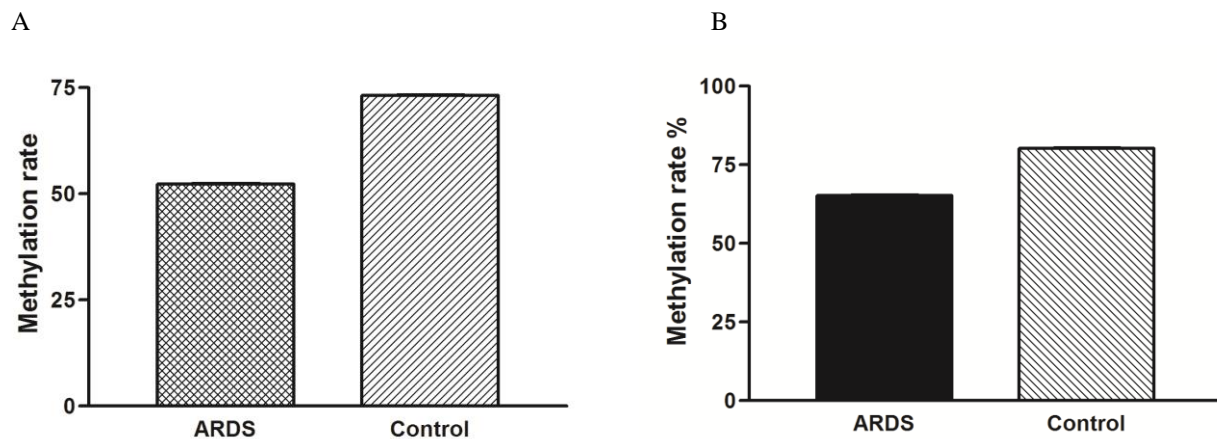a significant hypo-methylation of the ACE-2 gene (64.07 ±6.1) (%) was observed in comparison to control samples (80.3 ±7.3) (%) (p<0.0001) (Fig. 2b). The results also showed a significant negative correlation between ACE-2 expression and methylation in BALF (R= -0.454, P= 0.002) and blood (R= -0.321, P= 0.013) samples.

Correlation between ACE-2 hypo-methylation with laboratory parameters

As mentioned in Table 2, different laboratory parameters were compared in ARDS patients and controls. Then, the parameters that had significant differences were selected and their correlation with the rate of hypomethylation was investigated. The results showed that there was a significant correlation between increased WBC (R= -0.387, P= 0.0012), NEU (R= -0.478, P= 0.001), ESR (R= -0.321, P= 0.001), and CRP (R= -0.511, p< 0.001) with decreased methylation in BALF samples. Furthermore, there was a significant correlation between laboratory parameters such as WBC (R= -0.452, P= 0.0045), NEU (R= -0.628, P= 0.0013), ESR (R= -0.821, P= 0.00001), and CRP (R= -0.768, P< 0.001) in blood samples and hypomethylation of ACE-2 gene.
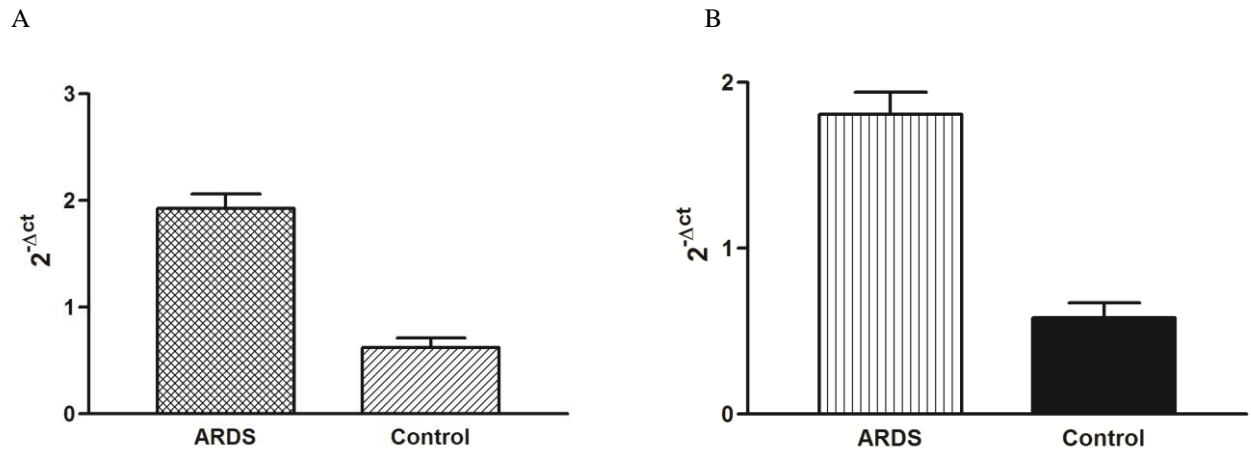
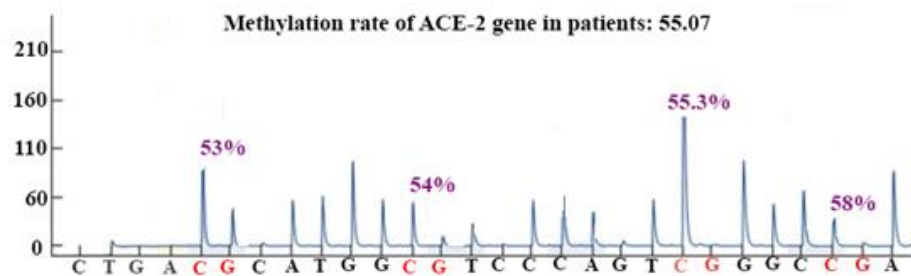**Fig. 1. a)** Shows high expression rate of ACE-2 gene in balf samples of ADRS patients in comparison to controls (p<0.03). **b)** shows comparison of expression of ACE-2 gene in blood samples of ARDS patients in comparison to controls (p<0.05).
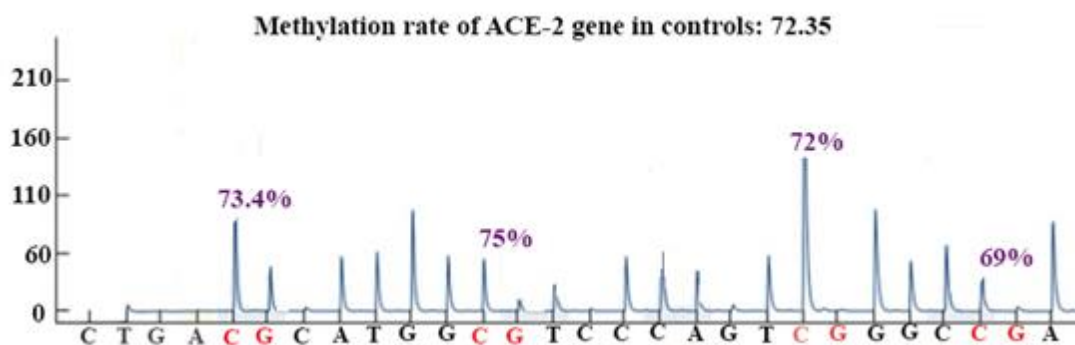

Fig. 2. a) Showed significant low methylation frequency of ACE-2 gene in BALF of ARDS patients (p<0.0001). **b)** and in blood samples (p<0.0001).

A

B

Fig. 3. Pyrosequencing method was used to measure the ACE-2 methylation level. a) ACE-2 methyalation rate in BALF samples of ADRS covid-19 patients (55.7%). b) and in controls (72.35%). The percent (%) is the methylation level of each CpG site was estimated by the proportion of C (%), after bisulfite conversion.

Discussion

This research showed overexpression and hypomethylation of the ACE-2 gene in the BALF and blood samples of covid-19 ADRDS patients. A novel coronavirus previously named 2019.

nCoV, now known as SARS-CoV-2, was the cause of the ARDS disease [10]. A coronavirus has a spike protein, commonly called S-protein, distributed on its lipid layer.

The virus binds to the specific surface receptors on the target cell's membrane (11). Coronavirus enters cells via membrane-bound ACE-2. The ACE-2-mediated coronavirus entry into the host cell can happen in two independent ways, (a): the virus enters the cell via endocytosis; (b): the second way is membrane fusion (12). Studies have shown that ACE-2 expression is tissue-specific, and lung tissue is one of the cases where ACE-2 is expressed high. In this regard, Zhao *et al*. (2020) showed that about 83% of ACE-2 is expressed in Alveolar type 2 (AT2) cells of lung tissue, which may be the reason for severe alveolar injury following the initial infection. It seems that SARS-CoV-2 utilizes AT2 cells for their reproduction and spread (13).

Research studies have recently implicated DNA methylation in the regulation of the ACE-2 gene expression, indicating that the host epigenome may represent a risk factor for covid-19 infection (14). On the other hand, ACE-2 methylation in the lungs of covid-19 patients has been studied very little. About the ACE-2 methylation pattern in the lung, studies have shown that methylation near the transcription starting

site of the ACE-2 gene was significantly associated with biological age and DNA methylation analysis at a CpG of the ACE-2 gene, which revealed a significant degree of variability in both men and women (15,16). In another study, Cardenas et al. showed that nasal ACE-2 DNA methylation reflects differences in sex, race/ethnicity, and biological aging (17). For these reasons, we selected samples from men with the same range in this research. Our results showed high expression and hypo-methylation of ACE-2 in BALF and blood samples of covid-19 ARDS patients compared to controls. In line with our study, Sawalha *et al*., (2020) based on whole-genome DNA methylation data, found the hypo-methylation and overexpression of the ACE-2 gene in the T cells of lupus patients with covid-19 .They concluded that oxidative stress induced by viral infections could strengthen the DNA methylation defect in lupus, leading to further ACE-2 hypo-methylation and enhanced viremia (18).

In another study, Gerad *et al*. (2020) showed ACE-2 up regulation in lung tissue and serum of COVID-19 ARDS patients (19). Likewise, Bruna *et al*. (2020) indicated high expression of ACE-2 in lung tissue of severe covid-19 patients who had comorbidities (20). On the other hand, Pratibha *et al*., (2020) showed a significant difference in ACE-2 gene expression in saliva samples of covid-19 patients (21).

Interestingly, in a study on the reasons for low involvement of children with covid-19, high methylation rate and low expression of ACE-2 gene are reported (22). Ackermann et al. (2020) showed a significant increase in the numbers of ACE-2-positive cells in the lungs of covid-19 patients (23). In contrast, the animal model study showed that in inducing ARDS mice, ACE-2 expression dramatically reduced in the lung, suggesting ACE-2 down regulation might be causing the severe acute lung pathologies in SARS-CoV2 (24). The justification for this discrepancy is the difference in the pathogenesis of the disease between humans and animals. If we want to explain the mechanism of ARDS by increasing the expression of ACE-2, it is as follows: during the occurrence of ARDS, high expression levels of ACE-2 and ANG II type 1 receptor are responsible for the progression of lung injury (25). In contrast, it has been shown that ACE-2 and ANG II type 2 receptors can improve ARDS symptoms (25). Since SARS-CoV-2 uses ACE-2 for cell entry, we can speculate that ANG II degradation by ACE-2 will be down-regulated. As a result, ANG II type 1 receptor levels will exacerbate the promotion of lung injury. In this regard, for the treatment of ARDS patients, the use of soluble recombinant ACE-2 has been suggested (25). If used in excess, however, recombinant ACE-2 can bind competitively with SARS-CoV-2 and neutralize the virus and rescue the ACE-2 cellular activity that negatively regulates the Renin-Angiotensin-Aldosterone System (RAAS) to protect the lungs from injury; it also slows the viral entry into the cells, hence decreasing viral spread. In conclusion, we showed up regulation and hypo methylation of the ACE-2 gene in ARDS patients, which can be one of the mechanisms of cytokine storm and lung injury in this research.

Acknowledgment

Conflicts of Interest

None.

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