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Evaluation of Serum Levels of *MicroRNA-200C* and *ACE2* Gene Expression in Severe and Mild Phases of Patients with COVID-19

Hadi Sodagar[1], Mohammad Hasan Khadem Ansari[1], Rahim Asghari[2], and Shahriar Alipour[1,3]

[1] Department of Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[2] Department of Internal Medicine, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[3] Student Research Committee, Urmia University of Medical Sciences, Urmia, Iran

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ABSTRACT

The role of microRNA (miR)200c-3p in regulating *ACE2* gene expression in viral and bacterial respiratory diseases has been established. Since *ACE2* reduces the acute inflammatory effects in lung diseases and acts as a coronavirus receptor to invade the lung cells, this study investigates the relationship between miR-200c-3p and *ACE2* expression in COVID -19 patients.

In this study, COVID-19 patients were divided into two groups: mild phase (PCR-positive and mild symptoms) and severe phase (PCR-positive with acute pulmonary symptoms and inflammation). Then, the subjects' demographic, clinical, and paraclinical characteristics were recorded using a prepared checklist. Total RNA was isolated from all samples according to the Trizol kit protocol to evaluate gene expression. Subsequently, the extracted product was analysed for miR-200c expression and *ACE2* target gene expression by real-time PCR.

The results of the checklist data showed that smoking, cough, and the factors ESR and HCT were statistically significant between the two groups of patients in the mild and acute phases. Also, the mean expression of the *miR-200c* gene in the mild and acute patients was 1.87±0.70 and 1.87±0.62, respectively, which was not statistically significant. Still, the mean expression of the *ACE2* gene, which was 3.96±0.76 and 3.28±0.52 in the mild and acute disease groups, respectively, showed a significant difference between the two groups.

This study showed that the expression levels of *ACE2* were significantly reduced in people with severe inflammation compared to people with mild inflammation.

Keywords: *ACE2* protein; miR200c, human; Immune system; Inflammation

INTRODUCTION

The emergence of the COVID-19 or SARS-Cov-2 virus began in Wuhan, China, and its rapid spread to more than 200 countries prompted the World Health Organization to declare an epidemic on Jan. 30, 2020. Coronavirus (COVID-19) is caused by a modern mutant coronavirus. SARS-CoV-2 is a giant, enveloped virus with an RNA strand about 80-160 nm in size and a genome size of about 27-35 kb causes COVID-19 disease.[1,2]

Corresponding Author: Shahriar Alipour, PhD;
Department of Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran Tel: (+98 914) 4410 298, Fax: (+98 44) 3278 0803, E-mail: alipourshahriar17@gmail.com, alipour.sh@umsu.ac.ir

Inflammation plays a vital role in the pathophysiology of pulmonary diseases. Airway inflammation is associated with the response of various immune cells and mediators, ultimately leading to pathophysiological changes in the condition.[3] With the initial invasion of neutrophils, the release of elastase and myeloperoxidase, the increase in inflammatory cytokines and chemokines, and the increase in the number of active T lymphocytes, a wave of cytokine responses and proinflammatory chemokines, including interferon-gamma, IL -1α, MCP-1, and IL-6 produced, termed the cytokine storm, which is an important cause of lethal immunopathogenesis.[4,5] Both insufficient and high levels of specific cytokines may be associated with adverse effects.[6-8]

ACE2 inactivates angiotensin II (Ang II) by cleavage and produces Ang 1-7. Angiotensin II binds to high-affinity Ang II type 1 and 2 receptors and is involved in regulating fluid balance, inflammation, cell proliferation, hypertrophy, and fibrosis. The enzyme ACE2 has been shown to neutralize the development of severe ARDS caused by the avian influenza virus, coronavirus, and sepsis in mice.[9,10]

One factor controlling epigenetic changes in different races is non-coding RNA, the most important of which are miRNAs. MiRNAs are regulatory molecules with an average length of 22 nucleotides that stop their translation process by incompletely pairing with only 6 or 8 nucleotides of the target mRNA. When the miRNA pairs to the target sequence are complete, it destroys the target mRNA.[8,11] Research has shown that miRNAs are involved in growth processes such as temporal growth control, cell proliferation, neuronal cell fate, lipid metabolism, gene expression, brain development, muscle differentiation, and embryonic stem cell division, as well as in processes of apoptosis, cellular immunity, and neurotransmitter synthesis.[12,13] Decreased regulation of miRNA expression contributes significantly to the development and progression of many diseases, particularly cancer, diabetes, immune system disorders, and neurodegenerative diseases.[14-16]

Altered expression of miRNAs has also been observed in various respiratory diseases[17] In the study by Liu Q, the expression of miRNA-200c-3p was found to be increased in viral and bacterial respiratory diseases, suggesting that this miRNA is involved in the exacerbation of these respiratory diseases through various signaling pathways and hurts the expression of the ACE2 protein gene. The miR-200 family miRNA-200c-3p plays a crucial role in acute respiratory distress syndrome and is considered a potential factor for research related to SARS-CoV-2.[18]

In a study of the H5N1 avian influenza virus, serum levels of miRNA-200c-3p were found to be elevated in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) caused by the virus. This miRNA binds to the 3'-UTR locus of the *ACE2* gene and inhibits the expression of this protein, thereby exacerbating the disease and causing ARDS.[18] Also, in another study of COVID-19 disease, miRNAs regulating ACE2 protein expression were investigated. Finally, miRNA-200c-3p was found to negatively correlate with protein expression, which may be considered for therapeutic cases and a new treatment method. This study investigated the relationship between miRNA-200c-3p and *ACE2* protein expression in COVID-19, a global epidemic. Therefore, this study examined the serum level of miRNA-200c-3p and the expression level of the *ACE2* gene in two phases of the disease (mild phase and severe phase) to investigate the relationship between the levels of these factors and the clinical and paraclinical characteristics of the subjects.

MATERIALS AND METHODS

Sampling

This prospective case-control study included individuals with COVID-19 (acute and mild phases) referred to health centers (university) in West Azerbaijan province. This study was conducted at Urmia University of Medical Sciences with the code of ethics IR.UMSU.REC.1399.374 was approved and then worked at the Department of Clinical Biochemistry. To determine the sample size in this study, the results of the survey by Y Liu et al were used,[18] the correlation of values in patients with COVID-19 was approximately 68%, and considering α=0.05 and 80% power, 85 samples were selected. Finally, 40 subjects were considered for the control group and about 45 issues for the case group. The sampling was random and among the available samples. In this study, the inclusion criteria for the acute phase group included a positive PCR test for COVID-19, acute respiratory symptoms, and pneumonia.[8] Inclusion criteria for people in the mild phase also had a positive PCR test for COVID-19 and no acute pulmonary symptoms. Also, in this study, people with chronic respiratory disease, other underlying conditions (hypertension, diabetes, cancer,

cardiovascular disease), and autoimmune inflammatory diseases such as rheumatoid arthritis were excluded.

Subsequently, a questionnaire and a prepared checklist collected demographic, clinical, and paraclinical information about the individuals. In addition, the laboratory and imaging findings and the contents of the medical record with a specialist's opinion were included in the list. Samples were collected by the staff of medical centers from patients with acute phases and individuals without respiratory symptoms, referring to medical training centers affiliated with Urmia University of Medical Sciences. These centers forwarded these samples to the molecular laboratory of the College for molecular testing.

Total RNA Extraction and RT-PCR

This study extracted total RNA from individual samples in cell plates (PBMC) and serum. To isolate mononuclear cells, approximately 4 CC of whole blood (EDTA tubes) was taken from individuals. Then PBMC cells were isolated from cell samples collected as a cell plate using the Ficoll method (Lymphodex, Inno-Train, Germany). Also, serum from each piece was collected in separate microtubes. To separate the RNA from the cell plate, approximately 1 cc of TRizol solution was poured into the samples. The rest of the steps were performed according to the manufacturer's protocol (GeneAll). To separate microRNAs from serum, 0.75 cc of LX -TRizol solution was poured onto 0.25 cc of serum, and the rest of the steps were performed according to the manufacturer's protocol (General). Finally, after RNA extraction from the samples, gel electrophoresis and nanodrop method was used to evaluate the quality and quantity of the pieces. All isolated models were 1.8-2.1 at the 260/230 and 260/280 levels.

At this stage, we used two methods to synthesize the samples' cDNAs (synthesis of specific cDNA for PBMC samples and specific cDNA for serum samples), which used the same amount of RNA (1 μg) to synthesize cDNA in PBMC samples. Then, different stages of RT-PCR were used according to the kit manufacturer's protocol (Thermo Fisher Scientific, USA). The stem-loop method synthesized serum-specific cDNA (evaluation of microRNAs). In this method, only specific primers coated inside the vial were used in the step of cDNA synthesis to synthesize only the particular sequences of miR-200c and U6. Finally, the synthesized samples were used to measure the expression of the target genes. For the synthesis of the specific cDNA for the desired microRNAs, specific primers for each miR (miR200c, U6) were used as a stem-loop instead of oligo and random hexamer primers.

Primer Design

The ACE2 gene sequence and miR-200c data were taken from the National Center for Biotechnology Information (NCBI) and mirVana. For the ACE2 mRNA sequence, primer pairs were designed using OLIGO7 software (Molecular Biology Insights, Inc., Cascade, CO., USA). In addition, miR-200c was predicted using the mirVana site (Table 1).

Expression of *MiR-200c* and ACE2 Gene

Peripheral blood mononuclear cells (PBMCs) were extracted from EDTA blood tubes by Ficoll density gradient centrifugation (Lymphodex, Inno-Train, Germany). Total RNA was extracted from PBMCs using TRIzol (Invitrogen, San Diego, CA), followed by reverse transcription using the Reagent Kit for Reverse Transcription (Thermo Fischer Scientific, USA). Then the samples were stored in the freezer (-70˚C) until the subsequent use.

The relative expression of ACE2 was measured using a MIC instrument (BioMolecular Systems, AUSTRALIA). The following sequences of the forward and reverse primers of ACE2 were used: forward 5'- TATCAATGATGCTTTCCGTCT-3' and reverse 5'- GATGACAATGCCAACCACT -3'. Beta-actin was chosen as an internal reference for detecting expression and copy number variations, and its expression was assessed using the following primers in Supplementary Table 1 (Table S1). The relative expression levels of ACE2 and miR-200c were calculated using the ΔΔCt formula. All tests were performed in three biological replicates.

Statistical Analysis

All data were expressed as mean±standard error for at least three separate experiments for each treatment. Statistical significance of differences between means was analyzed using SPSS 16 statistical analysis software (SPSS Inc. Chicago, IL) by one-way analysis (ANOVA) followed by Tukey's HSD posthoc test. All analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA). The level of significant differences was set at $p<0.05$.

Fold differences in gene expression normalized to control were plotted graphically as histograms using a Microsoft Excel computer program.

RESULTS

The results of the quantitative variables of the demographic characteristics are expressed as mean ± standard deviation (SD) and the qualitative variables as percentages. The demographic and clinical characteristics of the two groups are summarized in Table 2, and the results and comparisons of the variables are listed for each.

The mean age in the mild disease group was 62.25 years, and in the acute disease group, 60.76 years. In both groups, 43.5% (27 subjects) were men, and 56.5% (35 issues) were women. There was no significant difference between the two groups regarding mean age (p=0.69) and sex (p=0.17). Also, the mean BMI in the mild group was 27.31 and in the acute group was 26.08 kg/m2, but the two groups, mild and acute, showed no significant difference in mean BMI (p=0.33). As shown in the Table, smoking, cough, ESR, and HCT factors showed a statistically significant difference between the two groups of patients in the mild and acute phases (p<0.05).

After real-time PCR, diagrams and shapes related to fluorescence radiation, CT number diagrams, and melting curve diagrams were used for detailed analysis. Finally, information about patients' characteristics was analyzed after calculating the gene expression level. The mean expression of the *miR-200c* gene in mild and acute patients was 1.87 70 0.70 and 1.87 62 0.62, respectively, which was not statistically significant between the two groups (p=0.544) (Figure 1). However, the mean expression of the *ACE2* gene in the mild and acute disease groups was 3.96 76 0.76 and 3.28 52 0.52, respectively, which was a significant difference between the two groups (p<0.0001) (Figure 2).

Also, in this study, after dividing patients into two age groups, under 60 years and over 60 years, and examining the association with disease severity (acute and mild), no association was found between age and disease severity in people with coronary artery disease. (p>0.05). No association was also found between age and ACE2 and *miR-200c* gene expression, sex, cough, and smoking (p> 0.05).

ESR (p=0.023) and HCT (p=0.018) were paraclinical parameters that revealed a significant difference between the two groups of acute and mild patients. Then, by analyzing the correlation between these variables and the mean expression of the miR-200c and *ACE2* genes, it was determined that only the *miR-200c* gene expression and HCT (p<0.05) had a statistically significant difference.

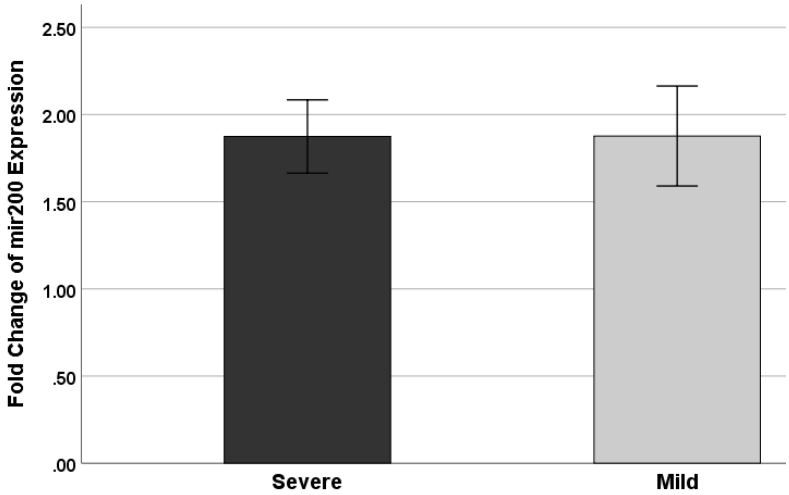

Figure 1. miR-200c expression in the mild and acute disease groups Altered expression of *miR-200c* genes in the acute and mild disease groups; As shown in the figure, *miR-200c* gene expression did not show a significant increase in the mild group compared with the acute group (p>0.05).

Table 2. Baseline Characteristics Between mild/severe Group Demographic, clinical, and paraclinical characteristics of the subjects. Individuals are divided into two groups, acute phase, and mild phase, and compared.

Variable	Total	Mild (n=36)	Severe (n=46)	*p*
Age	61.6	62.25	60.76	0.69
Weight	79.9	81.09	78.05	0.5
BMI**	26.85	27.31	26.08	0.33
Sex				
Male	37(45)	14(17)	22(27)	0.17
Female	45(55)	22(27)	24(29)	
Smoke	8(13.1)	2(3.3)	6(9.8)	***0.002***
Clinical				
Oxygen saturation	85.27	85.55	84.88	0.24
Cough	59(72)	23(28)	36(44)	***0.001***
Dry cough	7(41.2)	5(29.4)	2(11.8)	
Sputum cough	10(58.8)	3(17.6)	7(41.2)	
Fever	30(48.4)	14(22.6)	16(25.8)	0.31
Sore throat	3(4.8)	2(3.2)	1(1.6)	0.37
Headache	17(27.4)	9(14.5)	8(12.9)	0.2
Gastrointestinal symptoms	19(30.6)	8(12.9)	11(17.7)	0.6
Chest graph	59(95.2)	25(40.3)	34(54.3)	0.6
lung involvement				
0-20%	1(5)	1(5)	0	
20-40%	5(25)	2 (10)	3(15)	
40-60%	5(25)	3(15)	2 (10)	
60-80%	6(30)	3(15)	3(15)	
80-100%	3(15)	0	3(15)	
Paraclinical profile				
WBC**	10592.58	8841.6	13016.9	0.19
RBC**	4.23	4.23	4.21	0.91
ESR**	50.01	53.5	45.8	***0.023***
CRP**	38.35	38	38.8	0.55
HCT**	43.00	36.56	51.92	***0.018***
Hb**	12.39	12.18	12.68	0.37

* Values less than 0.05 are significant for *p*-value. **body mass index, White blood cell, Red blood cell, Erythrocyte sedimentation rate, c-reactive protein, Hematocrit, Hemoglobin

WBC: White blood cell; RBC: Red blood cell; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; HCT: Hematocrit; Hb: Hemoglobin

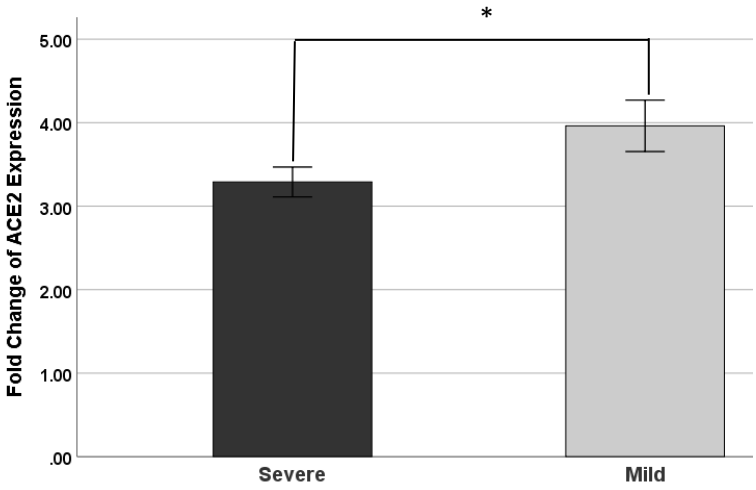

Figure 2. ACE2 expression in the mild and acute disease groups. Altered expression of *ACE2* genes in the acute and mild disease groups; As shown in the figure, *ACE2* gene expression showed a significant increase in the mild group compared with the acute group. *$p<0.05$

DISCUSSION

Recent advances in understanding the molecular mechanisms underlying respiratory infection have suggested the role of miRNAs in these lung infections.[19] The expression of *miR-200c* and the *ACE2* gene, the microRNA's target gene, was compared in serum samples from patients with COVID-19 in both acute and mild stages of the disease. In several cancer-related mechanisms, miR-200c is measured as a biomarker to predict disease progression, diagnosis, and response to therapy, both in tissue and body fluids (blood, urine).[20] Furthermore, miR-200c-3p has been shown to play a significant role in acute respiratory distress syndrome (ARDS) and is a potential contributor to SARS-CoV-2 research.[18] The SARS-CoV-2 virus has been found to use a pulmonary angiotensin-converting enzyme (ACE2) as a receptor to enter lung cells.[21-23] Demographic, clinical, paraclinical, and miR-200c and ACE2 expression factors were compared and evaluated among the mentioned subgroups (acute and mild).

The present study's findings revealed that the expression of the *ACE2* gene was significantly different between the acute and mild groups. In addition, the acute group had a 1.2-fold lower mean fold than the mild group. Additionally, decreased *ACE2* gene expression was observed as disease severity increased. In a study published in 2021 by Papannarao et al., they looked at changes in the expression of miR-200c as well as changes in the amount of ACE2 enzyme as a specific functional receptor for the SARS-CoV2 virus; the results showed a significant reduction in the level of ACE2 secreted in two groups of obese and healthy people, as a direct target of miR-200c in obese individuals. The findings of this study were consistent with the results of our research. The difference observed in this study was related to the measurement of the ACE2 enzyme, which was measured at the serum level by the ELISA method. Still, its amount was examined at the *gene* expression level in our study.[21] The ACE2 enzyme, which is the main SARS-CoV2 receptor, is thought to be a promising therapeutic target for COVID-19 treatment.[24] ACE2 is expressed to varying degrees in almost all human organs and plays a vital role in cellular homeostasis. ACE2 is primarily expressed in type II alveolar epithelial cells in the lungs. It is a critical component of the renin-angiotensin system throughout the body, and it has anti-inflammatory, anti-regenerative, and anti-proliferative properties by lowering angiotensin II levels.[25] It is important to notice that COVID-19 patients who take angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, which raise ACE2 levels, do not have a higher mortality or disease severity.[26] As a result, low ACE2 levels are unlikely to benefit COVID-19 disease. When people with low ACE2 levels, such as obese people in a recent study, are exposed to COVID-19 disease, SARS-CoV-2 binding to ACE2 is likely to reduce ACE2 cell levels.

As a result, as a proinflammatory factor, very low levels of ACE2 and elevated angiotensin II can worsen the disease.[27]

So ACE2 acts as a SARS-CoV-2 cellular receptor, and increased expression and activity may contribute to increased COVID-19 mortality in cardiovascular disease patients. Serum ACE2 activity increased significantly in people with hypertension and patients with end-stage heart failure; according to a study published in 2021 by Fagyas et al. overweight people, and the elderly with high blood pressure all had higher serum ACE2 activity. The study's main finding is that a wide range of cardiovascular and pathological risk factors, including hypertension, aging, obesity, and heart failure, increase the expression of ACE2, a SARS-CoV-2 receptor. Provides a possible explanation for COVID-19 higher mortality rate in cardiovascular patients.[28]

Our findings also revealed that the expression of miR-200c in the acute and mild groups was not significantly different. While the findings of Pimenta et al revealed a significant increase in the expression level of miR-200c-3p in both acute and severe patients. And observed that due to the severity of the disease, the expression pattern also increased. Furthermore, based on their findings, it was confirmed that patients over the age of 42 had higher expression of this miRNA.[29] Perhaps the differences in our results from those of other studies can be attributed to the type of sample (saliva, serum) and the grouping of individuals, in which we divided the entire population into acute and mild groups. In our study, no correlation was found between age and disease severity, nor between age and miR-200c expression in people with coronary heart disease. Significant differences between miR and age were observed in the Pimenta study. At 42 percent of years, patients have higher levels of miR-200c-3p expression. In COVID-19, old age appears to be one of the factors with the worst prognosis.[29]

Our study looked at the demographic and clinical characteristics of people with COVID-19 disease of varying severity. Smoking status was discovered to have a significant relationship with the severity of COVID-19 disease. Furthermore, the only cough was associated with disease severity among the clinical symptoms, and the rest were insignificant. A 2020 study by Zhou et al aimed to evaluate the clinical features associated with severe and critical coronavirus pneumonia. Eighty-three patients with COVID-19, including 25 severe and 58 mild cases, were included in the study. Their study showed that compared with mild patients, severely older patients had underlying diseases, cough, sputum, chest pain, and shortness of breath.[30] Overall, based on previous observations, we expected that increasing the expression of miR200c as a therapeutic target would eventually lead to a decrease in the expression of the ACE2 protein, thereby reducing virus entry. But the results of different studies showed differences from the results of our research. In our study, a significant decrease in ACE2 expression was associated with the severity of Covovid-19 disease. It may be better to conduct more complete and accurate analyses to design diagnostic and therapeutic methods based on these genes. MiR-146a, according to our research, also plays a role in lung disease. Future research could be considered a new factor in lung diseases. Given the role of miR-200c in the regulation of the ACE2 protein and its role in several upstream signaling pathways, it is a novel idea to study *miR-200c* and several other genes involved in respiratory disease at the same time. The concentration of ACE2 protein can also be measured using ELISA in the new research.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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