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Upregulation of MicroRNA-144 Suppresses Nrf2 Antioxidant Signaling Pathway in Patients with Severe COVID-19

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ABSTRACT

MicroRNAs (miRs) play a pivotal role in the pathogenesis of viral infections. It has been proven that the Nrf2 (NFE2 like bZIP transcription factor 2) antioxidant signaling pathway is inhibited in COVID-19 patients. Two microRNAs (*MIR144* and *MIR153-1*) have been identified as important Nrf2 regulators. The aim of this study was to analyze the *MIR144* and *MIR153-1* expression in COVID-19 patients and investigate their association with the Nrf2 signaling pathway.

The study had 82 participants with both mild and severe COVID-19 manifestations and 25 healthy as a control group. Ficoll density-gradient centrifugation was used to separate peripheral blood mononuclear cells from ethylenediaminetetraacetic acid blood tubes. *MIR144*, *MIR153-1*, and *NFE2L2* expressions were studied using real-time polymerase chain reaction. We employed the commercially available enzyme-linked immunosorbent assay to measure plasma Nrf2 protein concentration and the activity of antioxidant enzymes, superoxide dismutase, and catalase.

Compared to the control group, *MIR144* expression was significantly increased in the severe group, while *NFE2L2* expression decreased. There was no significant difference in the *MIR153-1* expression rate between COVID-19 patients and controls. Nrf2 protein and antioxidant enzyme activity significantly decreased in the severe group. A negative correlation between *MIR144* expression and Nrf2 protein concentration was observed.

Taken together, the current study's findings showed that *MIR144* upregulation probably interferes with the Nrf2 antioxidant signaling pathway in COVID-19 patients.

Keywords: COVID-19; *MIR153-1*; *MIR144*; Nrf2 Signaling pathway

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INTRODUCTION

The COVID-19 pandemic, caused by SARS-CoV-2, has caused significant global morbidity and mortality. This virus impacts lung endothelial cell morphology and function, and causes disruptions in cell connections.[1]

NFE2 like bZIP transcription factor 2 (Also known as Nrf2), a key oxidative stress defense transcription factor, binds to the antioxidant response element (ARE) in the nucleus and activates antioxidation genes. Despite being inhibited by Kelch-like ECH-associated protein 1 (KEAP1), it migrates to the nucleus and activates several antioxidation genes, when exposed to excess free radicals.[2]

Research on Nrf2 expression in respiratory tract viral infections demonstrates its protective function against the formation of oxidative stress, which can result in more severe viral respiratory infections. virus replication directly or indirectly affects Nrf2 expression.[3,4]

Nrf2 demonstrated antiviral properties, inhibiting virus growth and reducing replication of SARS-CoV-2, Zika, and Herpes simplex in cell cultures.[5]

Numerous studies have demonstrated Nrf2's significant role in reducing lung illnesses, particularly in mice lacking Nrf2, making them more susceptible to emphysema caused by cigarette smoking.[6]

The expression of Nrf2 in pulmonary macrophages has been found to decrease in both current smokers and individuals with chronic obstructive pulmonary disease.[7] The evidence suggests that Nrf2 activation may inhibit experimental emphysema progression, suggesting its potential role in pulmonary disease

The nuclear factor kappa B subunit 1 (NFKB1) maintains cytokine storm, while Nrf2 suppresses it, reducing inflammation and protecting against SARS-CoV-2 by inhibiting interleukin 6 (IL-6) synthesis.[8]

Nrf2 is a protein with anti-inflammatory and antioxidant properties, and it also regulates the expression of tight junction proteins in alveolar epithelium cells.[9]

MicroRNAs (miRs), small noncoding ribonucleic acids, regulate various physiological and pathological processes like development, homeostasis, metabolism, immunity, aging, and age-related disorders. They interact with target messenger RNAs, suppressing gene expression through translational repression or destruction.[10-13]

MicroRNAs, including miR-21, miR-27a, miR-144, miR-153, and miR-155, play a crucial role in viral infections by directly influencing the expression of Nrf2.[14-19]

Infection with COVID-19 is associated with systemic activation of the coagulation cascade and ischemic/inflammatory heart disease.[20] A growing body of evidence indicated that suppression of miR-153 exerts a cardioprotective effect.[21-24]

On the basis of in silico analysis of Nrf2 3′-UTR and previous publications, miR-144 and miR-153 have been shown to inhibit Nrf2, However, they haven't been looked at in relation to COVID-19's effects on the lung, therefore we ran a preliminary screening of these microRNAs.

This study aimed to investigate expression levels of miR-144 and miR-153 in the plasma of COVID-19 patients and their effects on the Nrf2 signaling pathway.

MATERIALS AND METHODS

Bioinformatics Development

The online MicroRNA Target Prediction Database (miRDB) (https://mirdb.org) was used for prediction of the Nrf2 mRNA target of miR-144 and miR-153 according to a score of prediction.

Subjects

This cross-sectional study included 82 patients with COVID-19 (acute and mild phases) individuals and 25 healthy individuals as the control group.

The inclusion criteria for the acute phase group in this study comprised pneumonia, a positive CT scan, acute respiratory symptoms, and a positive PCR test for COVID-19. A positive COVID-19 PCR test and the absence of acute lung symptoms were additional requirements for the inclusion of those in the moderate phase. Additionally, participants with autoimmune inflammatory disorders such as rheumatoid arthritis, other underlying problems (hypertension, diabetes, cancer, cardiovascular disease), and chronic respiratory diseases were not allowed to participate in this study. Then, a questionnaire and a premade checklist were used to gather data on the subjects' clinical, paraclinical, and demographic characteristics.

The list also contained the results of the laboratory and imaging tests, as well as the information found in the medical record that was reviewed by an expert.

Gathering of Samples and Separation of Mononuclear Cells from Peripheral Blood

Each participant provided 5 mL of peripheral blood into a sterile vacutainer tube containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMCs) were then extracted using the Ficoll-Hypaque density gradient centrifugation method (Lymphodex, Inno-Train, Germany). Next, the separated PBMCs were washed 3 times with phosphate-buffered saline (PBS from Stem Cell Technology, Iran) solution with pH 7.3±0.1.

Total RNA Isolation

In this investigation, we isolated total RNA from individual plasma and PBMC samples in cell plates. The samples were filled with about 1 mL of TRIzol solution in order to extract the RNA from the cell plate. The remaining procedures were carried out in line with the manufacturer's guidelines (GeneAll RiboEx, Seoul, Korea).

After adding 0.75 mL of TRIzol solution to 0.25 mL of plasma, the remaining procedures were carried out in accordance with the manufacturer's instructions to extract microRNAs from the plasma. Ultimately, the quality and amount of the fragments were assessed using gel electrophoresis and the nanodrop method following the extraction of RNA from the samples. Between 1.8 and 2.1 at the 260/230 and 260/280 levels. After that, the samples were kept in a freezer at −70°C until they were needed later.

Complementary DNA Synthesize

At this point, we synthesized the cDNAs from the samples using 2 different techniques: specific cDNA synthesis for PBMC samples to ascertain Nrf2 expression and specific cDNA synthesis for plasma samples. The stem-loop method with BON Stem High Sensitivity MicroRNA 1st Strand cDNA Synthesis kit (Catalog number: BON0011.41, Stem Cell Technology, Iran) was used to synthesize plasma-specific cDNA (evaluation of microRNAs). Instead of using oligo and random hexamer primers, unique primers for each miR (*MIR144*, *MIR153-1*, and *RNU6-1* (U6) as the internal control) were employed as a stem-loop for the synthesis of the required cDNA for the miRNAs.

Primer Design

The National Center for Biotechnology Information (NCBI) provided the *NFE2L2* (Nrf2) gene sequence. (Molecular Biology Insights, Inc., Cascade, CO, USA, used OLIGO7 software to create primer pairs for the *NFE2L2* mRNA sequence) in Supplementary table 1.

Real-time Quantitative PCR

Using primers and the BON-High-SpecifityQPCR master mix (from Stem cell Technology Research Center, Iran), qPCR was carried out for the analysis of the expression of the *MIR153-1* and *MIR144* genes.

The relative expression of *NFE2L2* and microRNAs was measured using a MIC instrument (BioMolecular Systems, Australia).

The final 13 μL volume was used for the real-time PCR assay for microRNAs, which included 1 μL of cDNA as template, 0.5 μL of particular forward primer, 0.5 μL of universal reverse primer, 6.5 μL of SYBR Green PCR Master Mix, and 4.5 μL of nuclease-free water. This test used a 3-step, melt-based thermal profile. The hold duration was set at 95°C for 2 minutes. Thereafter, there were 40 cycles of denaturation at 95°C for 5 seconds, annealing and extension at 60°C for 30 seconds, and melting curve analysis at temperatures between 55°C and 99°C.The expression levels of *MIR153-1* and *MIR144* were normalized to small nuclear *RNU6-1* (U6) as reference RNA and the fold change was calculated using the ΔΔCt formula.

Assessment of Plasma Nrf2 Protein Levels

Plasma concentrations of Nrf2 were measured by sandwich-based enzyme-linked immunosorbent assay (ELISA) using kits RayBio Human Nrf2 ELISA Kit (catalog number: ELH-NRF2, USA) that can be used to examine human Nrf2 in cell culture supernatants, serum, and plasma. The kit was operated in compliance with the manufacturer's guidelines.

Determination of Antioxidant Index

Plasma levels of antioxidant enzymes, including catalase and superoxide dismutase (SOD) were measured using Colorimetric Superoxide Dismutase Activity Assay Kit (ZB-SOD-96A, Zellbio, Germany), CAT (ZB-CAT-96A, Zellbio, Germany) according to the manufacturer's instructions. Antioxidant kits measured quantities of assay samples based on colorimetric methods that were read by an ELISA reader (CAT: 405 nm and SOD: 420 nm).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA). The data were examined for differences between several groups using either one-way analysis of variance (ANOVA) with Tukey's post hoc or corresponding nonparametric Kruskal-Wallis test. We employed parametric Pearson testing or nonparametric Spearman for correlation analysis. $p<0.05$ was used to indicate statistical significance.

RESULTS

Table 1 summarizes the characteristics and laboratory findings of patients with mild COVID-19 (median age, 62.25 years), severe COVID-19 (median age, 60.76 years), and control group (median age, 61.5 years).

Demographic characteristics and detailed clinical and laboratory findings of the COVID-19 patients are presented in Table 1.

Our data demonstrated that the level of *NFE2L2* mRNA expression was notably lower in the severe group than the mild ($p<0.0001$) and control groups ($p<0.01$). Interestingly, we observed the higher expression levels of *NFE2L2* in the moderate group (Figure 1C).

Our findings showed that the expression of *MIR144* was significantly increased in the severe group compared with the healthy controls ($p<0.05$). Furthermore, there was a significant increase in *MIR144* expression rate in the severe group compared to the mild group ($p<0.0001$). The lowest expression rate of *MIR144* was seen in the moderate group (Figure 1A). These results showed a consistent expression pattern with *NFE2L2* mRNA expression, and it can be hypothesized that upregulation of *MIR144* is associated with decreasing levels of *NFE2L2* gene expression and promoting oxidative stress in patients with severe COVID-19.

We also investigated the expression level of *MIR153-1* in patients with COVID-19 and the control group. Although the expression level of *MIR153-1* increased in mild and severe groups of COVID-19 patients compared to the control group, no significant difference between the 2 patient groups and the control group was observed (Figure 1B). In this case, we did not observe a consistent pattern in patients with mild and severe COVID-19, because the expression level of *MIR153-1* did not increase significantly in both groups.

We further analyzed Nrf2 protein levels in COVID-19 patients in comparison with the control group. ELISA results revealed that plasma Nrf2 protein concentration was significantly decreased in the severe COVID-19 patients in comparison with the mild and control groups ($p<0.0001$) (Figure 2A). The highest concentration of Nrf2 protein and gene expression rate were measured in the moderate group.

We then measured Nrf2-mediated antioxidant enzymes, SOD, and catalase in the plasma of COVID-19 patients and healthy people. We observed that plasma SOD enzyme was significantly lower in the severe group compared to the mild and control groups ($p<0.0001$). There was no significant difference in SOD activity between mild and control groups (Figure 2B).

The most catalase enzyme activity was seen in the mild group, and we observed a significant decrease in enzyme activity in the severe group in comparison to the control group. These results clearly indicate the suppression of the Nrf2 signaling pathway in severe COVID-19 patients.

Table 1. Patient Characteristics

Variables		Total	Control (n=25)	Mild (n=36)	Severe (n=46)	*p*
Age, years		61.6	61.5	62.25	60.76	0.69
Weight, kg		79.9	80.2	81.09	78.05	0.5
BMI, kg/m^2		26.85	27.16	27.31	26.08	0.33
Gender						
	Male	48 (44.86)	11 (10)	14 (13)	22 (20)	0.17
	Female	59 (55.14)	14 (13)	22 (22)	24 (22)	
Smoke		11 (10)	3 (2.8)	2 (1.9)	6 (5.6)	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, cells/µL		10592.58		8841.6	13016.9	0.19
RBC, cells/µL		4.23		4.23	4.21	0.91
ESR, mm/hr		50.01		53.5	45.8	0.023
CRP, mg/L		38.35		38	38.8	0.55
HCT, L/L		43.00		36.56	51.92	0.018
Hb, g/dL		12.39		12.18	12.68	0.37

* $p<0.05$. BMI: body mass index; WBC: white blood cell; RBC: red blood cell; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; HCT: hematocrit; Hb: hemoglobin.

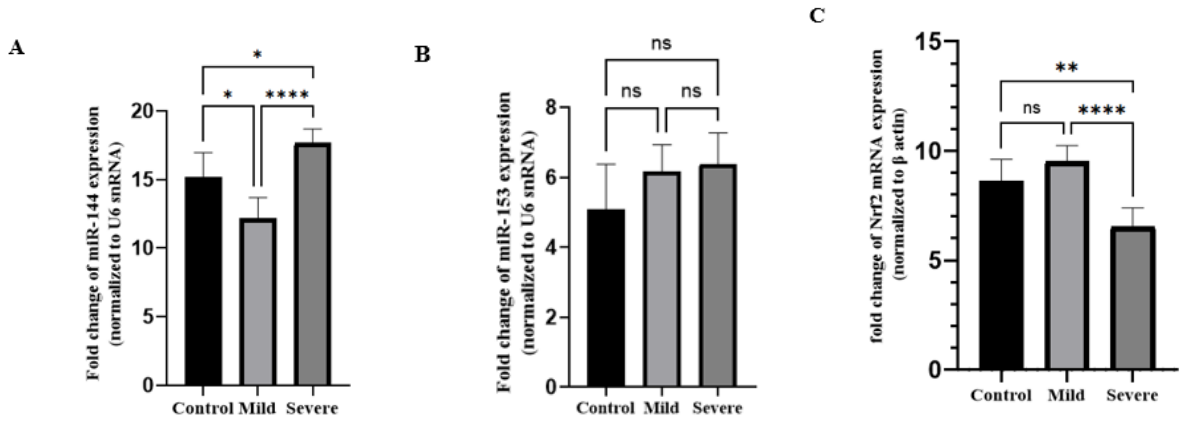

Figure 1. Relative expression of *MIR-144*, *MIR -153-1*, and *NFE2L2* (Nrf2) mRNA in different groups. (A) *MIR-144* expression was significantly upregulated in the severe group compared with the mild and control groups. (B) No significant differences in *miR-153-1* expression levels were observed among groups. (C) Significant differences in *NFE2L2* mRNA expression levels were observed in the severe group compared to the control and mild groups. Significance levels relative to the control group are indicated as follows: *p<0.05, **p<0.01, *** p<0.00 1, **** p<0.0001.

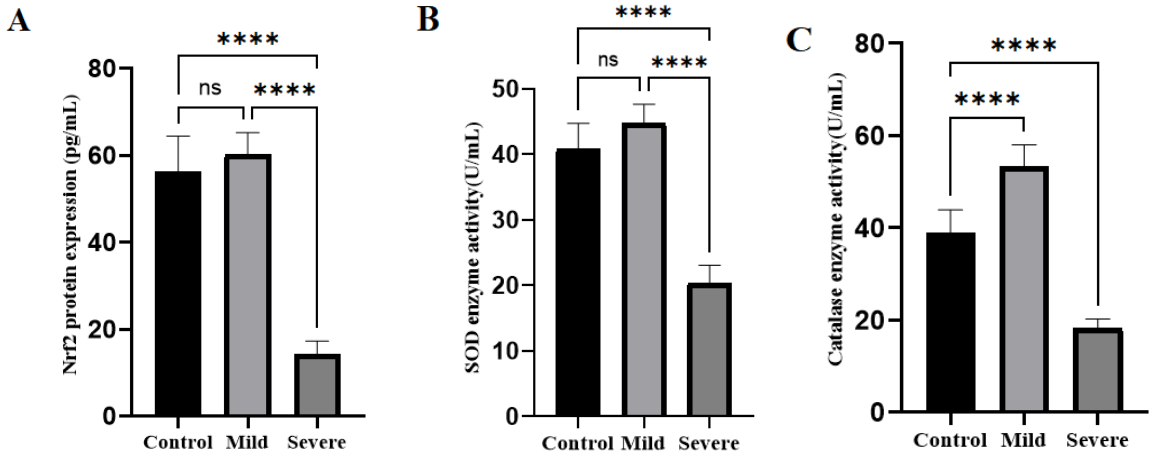

Figure 2. Nrf2 protein concentrations significantly decreased in the severe group. (A). The activity of super oxide dismutase (SOD) and catalase enzymes significantly decreased in the severe group (B, C). Catalase enzyme activity in the mild group was significantly higher than control and severe groups. For p value, each symbol is shown significance to the control group. (*p<0.05, **p<0.01, *** p<0.00 1, **** p<0.0001).

We found that the expression of *MIR144* was upregulated in the severe group but *NFE2L2* mRNA and protein concentration were downregulated in this group, therefore we detected a correlation between *MIR144* expression with *NFE2L2* mRNA and protein expression. The correlation between *MIR144* expression with *NFE2L2* gene expression was not significant (r = 0.171; p>0.05), but a significant inverse association was observed between *MIR144* expression with Nrf2 protein concentration (r = −0.349; p<0.05) (Figure 3A, B). It could be inferred that *MIR144* directly regulated the expression of Nrf2 by inhibiting the translation of *NFE2L2* mRNA, thereby decreasing antioxidant enzymes and ROS enhancement in the severe group of COVID-19 patients.

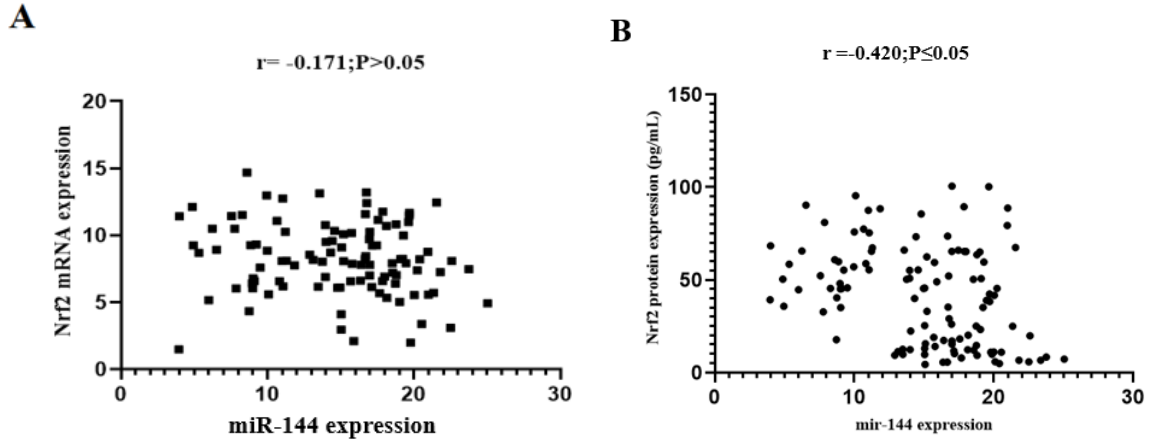

Figure 3. The scatter plots and expected linear regression of *NFE2L2* mRNA and protein expression with *MIR-144*. Pearson's correlation analysis between *MIR-144* and Nrf2 protein expression levels in COVID-19 severe group patients. (B) *MIR-144* expression was inversely correlated with Nrf2 protein expression in COVID-19 severe group patients. (A) No significant correlation was observed between *MIR-144* and *NFE2L2* mRNA expression levels (A).

DISCUSSION

Previous studies show that the Nrf2 antioxidant signaling pathway is suppressed during infection with SARS-CoV-2. Here we investigated the expression of *NFE2L2* and its dependent gene products (SOD and CAT) in samples from COVID-19 patients. We also screened the relation between *MIR-144* and *miR-153-1* expression with the Nrf2 signaling pathway in the plasma of both mild and severe groups of COVID-19 patients and healthy controls.

Based on the literature, in viral diseases, Nrf2 has a protective role in the hyperinflammatory state. Increased production of proinflammatory cytokines by the immune system, indicating a hyperinflammatory response, is one of the primary features of severe and catastrophic symptoms of COVID-19 disease. Multiple organ failure syndrome and acute respiratory distress syndrome are directly linked to this elevated output.[26-30]

Because of Nrf2 's strong cytoprotective response to stresses and possible therapeutic application in the treatment of inflammatory and oxidative diseases, Nrf2 signaling remains involved in COVID-19. In an effort to reduce the virulence of the severe acute SARS-CoV-2 by activating Nrf2 signaling, this paragraph thus outlines the key molecular mechanisms behind Nrf2 antiviral action and innovative therapeutic approaches. In biopsies taken from COVID-19 patients, Olagnier showed that the Nrf2 antioxidant gene expression pathway is inhibited. He also showed that treating cells with Nrf2 agonists such as 4-OI and DMF induce a potent antiviral program that inhibits the replication of SARS-CoV-2.[31]

The proinflammatory NFKB1–mediated pathway and antioxidant Nrf2 signaling are closely linked. Their interplay is also involved in COVID-19.[31]

In this study, we measured Nrf2 protein concentration in the plasma of COVID-19 patients and the healthy control group. Nrf2 protein concentration in the severe group was less than in other groups (p<0.0001). No significant increase was observed between the mild group compared to the control group. We also had a significant decrease in SOD (p<0.0001) and catalase (p<0.0001) antioxidant enzymes activity in the severe group compared to the other groups.

A sharp decrease in Nrf2 protein concentration and consequently in SOD and catalase antioxidant enzymes were the distinctive features of the severe group (Figures 2A–2C), therefore in the COVID-19 patients Nrf2-antioxidant response signaling pathway as a key player in the cellular defense against oxidative stress and inflammatory state was suppressed.

The induction of miR-144 within the alveolar epithelium by viral proteins associated with human immunodeficiency virus (HIV) and its inhibitory effect on Nrf2 is responsible for mediating the pathophysiological consequences of HIV in the lung and compromising the integrity of the alveolar epithelial barrier.[32].

We observed a significant increase in *MIR-144* expression in the severe group compared to mild and control groups ($p<0.0001$), but increased *miR-153-1* expression levels were not significant. *NFE2L2* expression was significantly low in the severe group compared to the control group (Figures 1A and 1C).

Previous evidence suggests a potential interplay between COVID-19 and cardiovascular disease[33] Evidence from previous studies showed that miR-153 inhibition has a cardioprotective impact.[5-8] No statistically significant difference in *miR-153-1* expression profile was observed between the control with mild and severe groups (Figure 1B).

The results of Olaginier's research on the inhibition of the Nrf2 antioxidant signaling pathway in severe COVID-19 patients were supported by our findings in this investigation.[31] The Nrf2 signaling pathway may be inhibited by SARS-CoV-2–induced microRNAs, as demonstrated by Kukoyi et al[32] they discovered that HIV-1 viral particles cause primary alveolar epithelial cells to release miR-144, which interferes with the function of the alveolar barrier by inhibiting Nrf2 production.

The results demonstrated a significantly inverse correlation between *MIR-144* and Nrf2 protein expression levels ($r = -0.349$; $p<0.05$). Taking these results into consideration, we hypothesized that infection with COVID-19 can induce Nrf2 suppression and negatively regulate the Nrf2 antioxidant signaling pathway by *MIR-144* overexpression (Supplementary figure 1).

This study investigates molecular mechanisms associated with upstream regulators of Nrf2 -ARE pathway and improves our knowledge about the pathophysiological mechanisms behind COVID-19 and its effects on lung injury. Our findings facilitate the identification and adaptation of pharmacological medicines for the treatment of COVID-19 through antagomir-144 therapy. Here, we faced some limitations that need to be addressed in future research. It was better that the western blot assay was done to compare the protein expression levels of Nrf2 to show clear up- and down-regulation. The absence of other in vitro assays that investigate the anti-miR-144 treatment effect on the Nrf2 signaling pathway failed to potentiate this study.

The findings of this study showed that overexpression of *MIR-144* during COVID-19 can be considered as a molecular mechanism caused by this virus. Therefore, antagonizing miR-144 can inhibit SARS-CoV2 replication and can be suggested as a probable therapeutic goal.

STATEMENT OF ETHICS

This investigation was carried out in accordance with Iranian national norms for conducting medical research as well as ethical guidelines and standards. Every participant gave their informed consent to participate in the study, and the Second Declaration of Helsinki was followed in conducting this survey. This research was approved by the Research Ethics Committee of the Babol University of Medical Sciences and the study's ethics number is IR. MUBABOL.REC.1402.017.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data is available upon reasonable request from the corresponding author.

AI ASSISTANCE DISCLOSURE

Not applicable.

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