



MicroRNA-296-5p Expression in COVID-19 Patients and its Relationship with Inflammatory Cytokines

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ABSTRACT

Objectives: The cytokine storm, triggered by the activation of certain pro-inflammatory genes in the second phase of COVID-19, is associated with severe acute respiratory disorder. Evidence suggests that microRNAs (miR) and cytokine levels can play pivotal roles in host cell antiviral defense mechanisms. This study aimed to investigate the changes in IFN- γ inducible protein 10 (IP-10) and miR-296-5p expression, as well as their relationship with some inflammatory cytokines and biochemical variables in COVID-19 patients.

Methods: This retrospective single-center study was conducted on 30 COVID-19 patients and 30 controls. The expression of miR-296-5p and IP-10 genes in peripheral blood mononuclear cells (PBMCs) was evaluated using real-time PCR. Serum levels of IL-6, TNF- α , and CRP were measured by ELISA.

Results: Higher expression levels of miR-296-5p and IP-10 genes were observed in COVID-19 patients compared to controls ($P=0.001$). Furthermore, IP-10, IL-6, and TNF- α levels were significantly higher ($P<0.01$) in COVID-19 patients than in controls. The results also showed positive correlations between miR-296-5p expression and serum levels of IL-6, CRP, and TNF- α in patients with COVID-19. ROC curve analysis of miR-296-5p in COVID-19 patients showed an area under the curve of 0.830, 95% CI (0.658-0.815), $P<0.001$, with an optimal cut-off point of 0.32965.

Conclusion: Our results suggest a regulatory role for miR-296-5p in cytokine secretion in COVID-19. The results indicate that the expression of miR-296-5p and IP-10 genes in PBMCs might serve as convenient novel biomarkers for the prognosis of COVID-19.

Keywords: SARS-COV-2; COVID-19; miRNA; Inflammation; Cytokine; IP-10

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Introduction

Coronavirus disease 2019 (COVID-19) was first identified in December 2019 in Wuhan, China, as a respiratory disease (1). This transmissible infectious disease spread worldwide, leading to a pandemic that affects billions of people. COVID-19 spread extensively due to its intrinsic infectious and contagious properties, leading to severe respiratory disease (2). In addition to the lungs, which are the primary target, the virus also affects other organs. The harmful effects of COVID-19 are accompanied by hypertension, cardiovascular diseases, and lung diseases (3). Infected patients exhibit symptoms ranging from mild to severe, including respiratory and other organ failures, during the first phase of the disease (4).

The second phase of COVID-19 disease is a profound inflammatory state, known as the cytokine storm, which is caused by the activation of pro-inflammatory genes such as signal transducer and activator of transcription-3 (STAT-3), nuclear factor kappa B (NF- κ B), interleukin 18 (IL-18), IL-6, and granulocyte colony-stimulating factor (G-CSF) (5). It has been reported that in COVID-19 patients with severe diseases, plasma concentrations of pro-inflammatory cytokines such as G-CSF, monocyte chemo-attractant protein 1 (MCP1), and macrophage inflammatory protein (MIP-1 α) also increase (6). The increased level of these cytokines following SARS-CoV-2 infection is associated with poor outcomes in patients with COVID-19 (7). Interferon gamma (IFN- γ) inducible protein 10 (IP-10 or CXCL10) is an inflammatory chemokine with a low molecular weight (10 kDa). IP-10 binds to CXCR3 (a G protein-coupled receptor) and activates different types of leukocytes. Previous studies have established the innate properties of IP-10 in immunologic responses, where IP-10 $^{-/-}$ mice showed extensive impairment in T-cell immunologic responses. Researchers have revealed that IP-10 $^{-/-}$ mice might have other distributive immunologic responses, including a reduced contact hypersensitivity response. Moreover, it has been demonstrated that the host's immune response against the mouse hepatitis virus is related to IP-10 and is impaired in IP-10 $^{-/-}$ knockout mice (8). Experimental studies have revealed that some miRNAs have the potential to intercede as an intracellular defense mechanism against some RNA viruses, including the SARS-CoV-2 genome, through their targeting (9). An in-vitro study showed that SARS-CoV-2 infection could induce the expression of six out of 128 human miRNAs (10). Our previous study showed that the serum IP-10 concentration and peripheral blood mononuclear cells (PBMCs) gene expression of IP-10 and miR-296-5p in coronary artery disease (CAD) patients were significantly higher than in controls (11). Recent studies have shown that the targeting of coronavirus by miRNAs could be an important strategy in understanding their role as an intracellular defense mechanism against pathogenic

coronavirus infections. The present study aimed to evaluate the expression of miR-296-5p in COVID-19 patients and controls. Moreover, the relationship of miR-296-5p expression with inflammatory cytokines was assessed for the first time.

Materials and Methods

Population

The current retrospective single-center study consists of 30 moderate COVID-19 adult patients with positive COVID-19 test results and 30 controls referred to Imam Khomeini Hospital, Tehran, Iran. The diagnosis of COVID-19 was based on a PCR test.

Inclusion and exclusion criteria

To meet our inclusion criteria, patients with COVID-19 had to exhibit at least one of the following clinical manifestations: (1) respiratory distress, defined as a respiratory rate of more than 25 times per minute, (2) an oxygen saturation percentage of $\leq 92\%$ at rest in room air, and (3) fever. According to the patients' initial sorting screening, the exclusion criteria were: (1) pregnancy and lactation, (2) a history of kidney, liver, and/or heart failure, (3) occurrence of cancer, (4) admission to the intensive care unit (ICU), (5) inability to receive oral medication, and (6) participation in two or more clinical studies simultaneously.

Ethics statement

All participants provided written consent that was entirely in accordance with the Declaration of Helsinki. The consent script was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (SBMU); Reference number: IR.SBMU.MSP.REC.1399.311, dated 2020-10-13.

Sampling

Blood samples were collected after a 12-hour fast. The serum was isolated and stored at $-80\text{ }^{\circ}\text{C}$. Levels of IL-6, IP-10, TNF- α , C-reactive protein (CRP), and ferritin were measured using the ELISA method. The serum concentration of CPK, LDL-C, ALT, and AST was measured using a commercially available Pars Azmon kit from Iran. The erythrocyte sedimentation rate (ESR) was evaluated using the Westergren method, and D-dimer (a fibrin degradation product) was measured by immunofluorescence. The neutrophil concentration, lymphocyte count, WBC count, duration of hospitalization, O₂ saturation, body temperature, heartbeat, cough, olfactory and taste senses, headache, body pain, and shortness of breath were also recorded.

Table 1: Anthropometric and laboratory assessment of COVID-19 patients

Parameter*	Ranges	Mean±SD	N
Oxygen saturation (%)	85-95	91.26±1.98	30
Body Temperature (C)	35.6-38.5	37.23±0.79	30
Heartbeat (n)	69-93	80.86±6.77	30
WBC count (x1000/uL)	3-15.2	7.31±3.12	30
Neutrophile (%)	41.67-92	74.69±12.30	30
Lymphocyte (%)	5-47.04	19.31±10.10	30
ESR (mm/H)	14-144	60.23±30.62	30
LDH (U/L)	132-1019	544.53±275.47	30
D-Dimer (ng/ml)	0.19-8.4	1.16±1.70	23
Ferritin (ng/ml)	41-331	190.65±82.71	28
CRP (mg/L)	16-206	80.96±54.69	30
CPK (U/L)	26-220	95.06±60.07	30
ALT (U/L)	10-80	34.10±19.08	30
AST (U/L)	14-92	40.53±21.12	30
Creatinine (mg/dl)	0.6-6.8	1.08±1.10	30

* WBC (white blood cell), ESR (Erythrocyte sedimentation rate), LDH (lactate dehydrogenase), CRP (C-reactive protein), CPK (creatin phosphokinase), ALT (alanine aminotransferase)

PBMCs isolation and RNA extraction

PBMCs isolation was based on a method described by Fadaei et al. (12). Total RNA was extracted from the PBMCs lysates using a miRNAs mini kit (QIAGEN, USA) according to the manufacturer's instructions. Furthermore, the purity and concentration of the extracted RNA were determined by a Nanodrop spectrophotometer (Thermo Scientific, US) and 2% agarose gel electrophoresis (Bio-Rad, US).

IP-10 genes expression

The synthesis of the first-strand cDNA was performed using 250 ng of total RNA by means of a cDNA synthesis kit (Takara, Japan). Real-time PCR was conducted using SYBR Premix Ex Taq II (Takara, Japan) and specific primers (Table 1 of the supplementary file) for IP-10 and β -actin (QIAGEN, USA) in duplicate, according to the standard program on ABI-Step One (Applied Biosystems, USA). The $2^{-\Delta\Delta C_t}$ method was used to compare the expression of IP-10 in the study groups.

miR-296-5p expression

The first-strand cDNA was synthesized using 250 ng of total RNA by means of the miScript II RT Kit (QIAGEN, Germany). Quantitative Real-Time PCR was performed using the miScript SYBR® Green PCR Kit (QIAGEN, Germany) and specific primers for miR296 and U6 (QIAGEN, USA). PCR amplification was carried out using ABI-Step-One (Applied Biosystems, USA). The expression level of miRNA was determined using the $2^{-\Delta\Delta C_t}$ method, normalized relative to U6 snRNA.

Statistical analysis

IBM SPSS Statistics 18.0 statistical software (SPSS, Inc., Chicago, IL, USA) was utilized for statistical

analyses. The normal distribution was evaluated by the Shapiro-Wilk test. Categorical data in this study were displayed as frequencies, and continuous data were presented as mean \pm standard deviation (SD). Depending on the normality of the data, the Student's t-test and Mann-Whitney U test were used for comparison. The correlation between variables was evaluated by Pearson analysis.

Results

The anthropometric and laboratory measurements of patients with COVID-19 are presented in Table 1. As depicted in Table 2 and Figure 1, a significant difference exists between the TNF- α (Fig. 1a) and IL-6 (Fig. 1b) levels of COVID-19 patients and controls ($P < 0.001$ and $P = 0.033$, respectively). The serum and PBMCs gene expression levels of IP-10 (Fig. 1c and 1d, respectively) in patients with COVID-19 were significantly higher ($P < 0.001$) than those in controls. The PBMCs miR-296-5p expression (Fig. 1e) of COVID-19 patients was significantly higher ($P < 0.001$) than that of the controls.

As illustrated in Fig. 2a and Tables 3 and 4, a significant positive correlation exists between the serum concentration of TNF- α and miR-296-5p expression in COVID-19 patients and controls. Figures 2b and 2c, along with Table 3, highlight positive correlations between IL-6 and CRP with the miR-296-5p expression of COVID-19 patients.

Figure 2d and Table 3 also demonstrate a significant negative correlation between heartbeat and IP-10 serum concentration in COVID-19 patients. It's worth noting that no significant correlation exists between other variables in COVID-19 patients.

There is a significant positive correlation exists between the IP-10 serum concentration and IP-10 gene expression in controls. Notably, the correlation between the IP-10 serum concentration and IP-10 gene expression in COVID-19 patients was not significant. Additionally, no

Table 2: Anthropometric and laboratory assessment of COVID-19 and control groups

Parameter	COVID-19 group (n=30)	Control group (n=30)	P value*
BMI (kg/m ²)	26.87±3.38	25.19±4.31	0.100
TNF- α (pg/ml)	87.55±20.22	40.80±24.02	0.000
IL-6 (pg/ml)	7.99±5.89	5.32±3.18	0.033
Serum IP-10 (pg/ml)	160.84±50.40	86.48±25.29	0.000
IP-10 gene expression level	185.57±80.19	100.00±28.50	0.000
miR-296-5p expression level	225.36±94.15	100.00±71.53	0.000

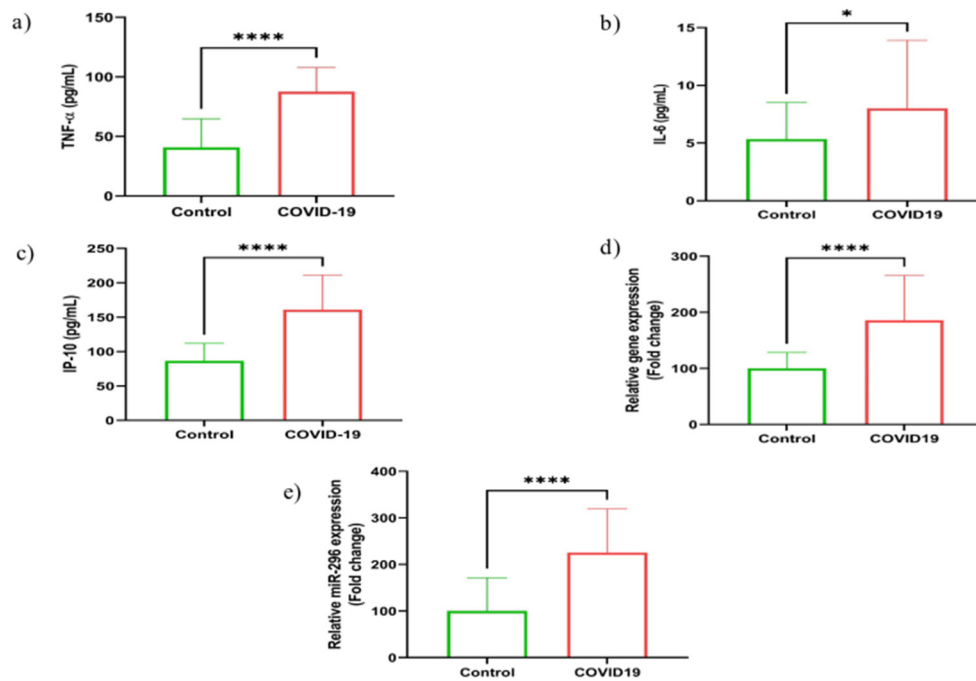
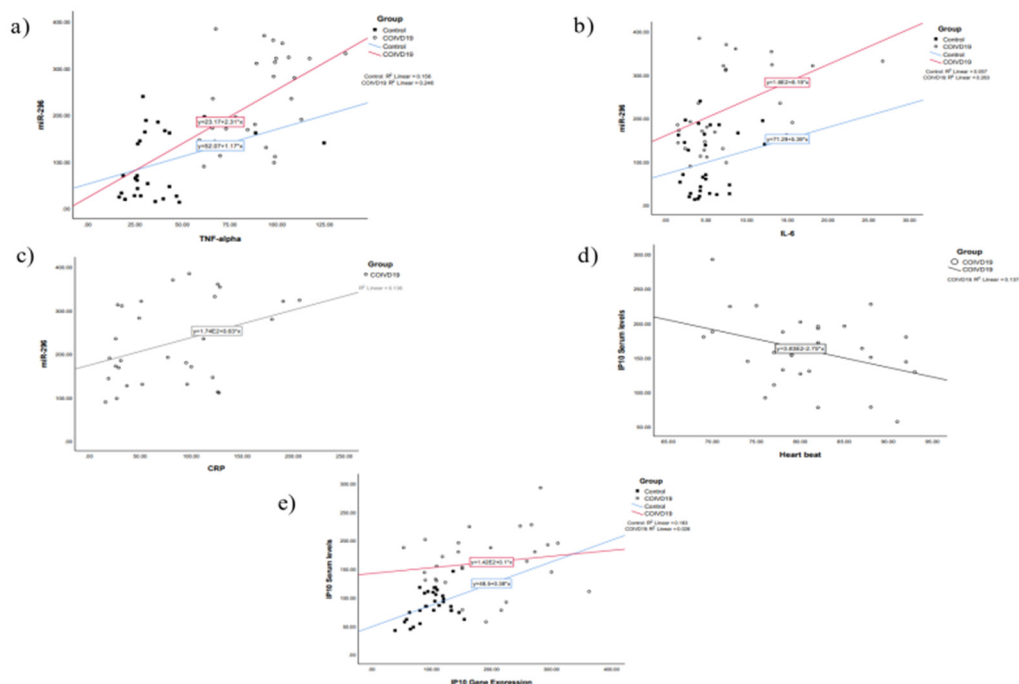
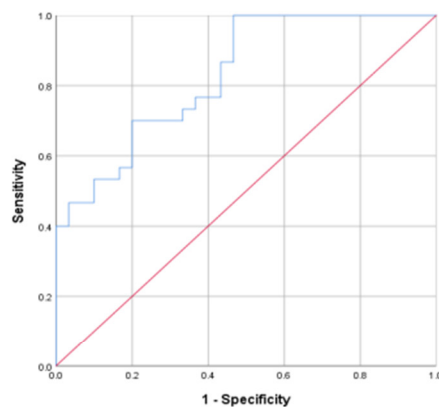
**Figure 1:** Comparison between serum levels of (a) TNF- α , (b) IL-6, (c) IP-10, and PBMC gene expression levels of (d) IP-10 and (e) miR-296-5p in COVID-19 patients and controls (* P <0.05, **** P <0.001)**Figure 2:** Correlation between (a) TNF- α and miR-296-5p expression, (b) IL-6 and miR-296-5p expression, (c) CRP and miR-296-5p expression, (d) heartbeat and IP-10 serum level, and (e) IP-10 serum level and IP-10 gene expression in COVID-19 patients and controls

Table 3: Pearson analysis of the correlation between the variables in the COVID-19 group

	IP-10 serum concentration	IP-10 gene expression level	miR-296-5p expression level
	Pearson Correlation		
Serum concentration of IP-10	1	0.161	0.121
IP-10 gene expression level	0.161	1	-0.069
miR-296-5p expression level	0.121	-0.069	1
BMI	0.291	0.261	0.052
Age	0.007	0.056	-0.058
Hospitalization	-0.223	-0.119	0.027
Oxygen saturation	0.094	-0.012	-0.218
Body temperature	-0.039	0.018	0.254
Heartbeat	-0.370*	-0.237	0.191
WBC	0.135	-0.051	0.237
Neutrophil	-0.150	-0.039	0.338
Lymphocyte	0.049	0.037	-0.343
IL-6	-0.013	-0.032	0.513**
TNF- α	-0.002	0.062	0.496**
ESR	-0.215	-0.175	0.278
LDH	-0.101	0.070	0.332
Ferritin	0.107	0.106	0.311
CRP	-0.094	0.048	0.368*
CPK	0.054	0.144	0.257
ALT	0.064	0.193	0.152
AST	-0.025	0.044	0.200
Cr	-0.104	-0.116	0.044

Table 4: Pearson analysis of the correlation between the variables in the Control group

	IP-10 serum concentration	IP-10 gene expression level	miR-296-5p expression level
	Pearson Correlation		
IP-10 serum concentration	1	0.428*	0.183
IP-10 Gene expression	0.428*	1	0.010
miR-296-5p expression level	0.183	0.010	1
BMI	0.040	0.018	0.280
Age	-0.259	-0.151	-0.069
IL-6	0.259	0.189	0.240
TNF- α	0.137	0.117	0.395*

**Figure 3:** ROC curve analysis of PBMC expression of miR-296-5p in COVID-19 patients

significant correlation was found between other variables (Fig. 2e and Table 4).

The ROC curve analysis for PBMCs expression level of miR-296-5p in COVID-19 patients showed an area under the curve of 0.830, with a 95% CI (0.658–0.815), and a p-value of less than 0.001. The optimal cut-off point was

0.32965 (Fig. 3). Therefore, based on the obtained results, miR-296-5p can be considered a reliable biomarker for the prognosis and progression of COVID-19 infection.

Discussion

Medical experience indicates that a short-term immune

system response could be sufficient to eliminate the virus from the lungs in most patients with severe SARS-CoV-2 (13). However, a dysregulated immune response with a hyper-inflammatory state, which might occur in less than 15% of patients with COVID-19, can lead to lung injury, multiple organ dysfunction, and mortality (14).

The present study investigated the serum concentration and PBMC expression of IP-10 and miR-296-5p genes in patients with moderate SARS-CoV-2, as well as in controls. We found that the levels of pro-inflammatory cytokines such as TNF- α , IL-6, IP-10, and PBMC gene expression of IP-10 and miR-296-5p in moderate COVID-19 patients were significantly higher than those in the control group. A significant positive correlation of TNF- α , IL-6, and CRP serum concentration with miR-296-5p expression in COVID-19 patients suggests that these cytokines might be potential targets for miR-296-5p. This correlation could also be a possible underlying mechanism for the association of miR-296-5p with COVID-19. The increase in miR-296-5p expression in COVID-19 patients can be attributed to a probable protective mechanism against the inflammatory cytokine storm. Despite a significant positive correlation between IP-10 levels and PBMC gene expression of IP-10 in controls, no significant correlation was observed in COVID-19 patients. This discrepancy might be related to the dysregulation of IP-10 levels and PBMC IP-10 gene expression in the patient group.

The study by Hung *et al.* revealed that plasma concentrations of IL-2, IL-6, IL-7, IL-10, G-CSF, IP-10, MCP-1, MIP-1 α , and TNF- α were higher in ICU patients than in non-ICU COVID-19 patients. It was suggested that a cytokine storm could be correlated with disease severity (15). Therefore, scholars evaluated the role of inflammatory cytokines in expressing the prognosis of COVID-19 and recognized IL-6 as a severity-related cytokine in the disease [13]. It has been reported that the IL-6 level is considerably increased as an important activator of the JAK/STAT signaling pathway [11].

The results of a cohort study on 52 COVID-19 patients showed that IP-10 levels of 12 intensive care unit (ICU) hospitalized patients were correlated with the patient's prognosis, COVID-19 severity, and duration of the admission period in the ICU (10). In addition, the IP-10 level has been defined as a promising biomarker for predicting COVID-19 progression. It could also be associated with the prognosis and reflect patient mortality (15, 16).

The results of *in vitro* experiments reported that 128 miRNAs could have potential properties to target the SARS-CoV-2 genome. They also revealed that only six miRNAs are known to be differentially expressed in studies that stimulated SARS-CoV-2 *in vitro* models. This result was demonstrated on the SARS-CoV genome targeted by 28 microRNAs and also the 23 microRNAs that target the genomic content of MERS-

CoV (17). A study on Osteoarthritis model cells showed that the molecular cascade of miR-296-5p within the cells may inactivate a signaling pathway consisting of TGF- β 1, p38, and MAPK. This property of miR-296-5p is based on the direct targeting of the TGF- β 1 mRNA 3'- untranslated region (3'-UTR).

During the statistical analysis, the ROC curve, which calculates the miR-296-5p expression statistical model performance, was also plotted to evaluate the preference of PBMCs miR-296-5p expression in COVID-19 patients. Our results suggest that PBMCs expression of miR-296-5p could be a convenient novel biomarker for the prognosis and monitoring of COVID-19 disease. This study had some limitations. The first limitation is a small sample size, and our observations need to be confirmed in a larger sample of patients. The impact of social and economic conditions, as well as health care statutes of the studied groups, are not negligible and should be considered in future studies. In summary, serum levels of IL-6, TNF- α , IP-10, and PBMCs expression of IP-10 and miR-296-5p genes were significantly increased in COVID-19 patients compared with controls. Our results also suggest that IL-6, TNF- α , and CRP could be targeted for miR-296-5p. Consequently, miR-296-5p could be suggested as an underlying therapeutic target in COVID-19 that acts by targeting TNF- α , IL-6, and CRP pro-inflammatory cytokines.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Shahid Beheshti University of Medical Sciences (SBMU); Reference number: IR.SBMU.MSP.REC.1399.311, dated 2020-10-13.

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