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EXPRESSION PROFILE OF MIR-9, MIR-138, MIR-424, MIR-155 AND *CTLA-4* IN THE BLOOD MONONUCLEAR CELLS OF PATIENTS WITH SARS COV-2 INFECTION

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Abstract

Background: The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the pathogen that causes COVID-19, a highly infectious illness. More research is being done on the potential of host biomarkers as diagnostic and prognostic tools for COVID-19. MicroRNAs have been shown to be essential for both the pathogenicity of coronavirus and the host's antiviral responses. Our aim was to assess the blood mononuclear cells expression profiles of mir-9, mir-138, mir-424, mir-155 and *CTLA-4*, and their association with the clinicopathological features of the patients with SARS CoV-2 infection.

Method: This case-control study included 66 SARS-CoV-2 positive patients in the chronic and active phase of the disease and a healthy group of 41 people matched for age and sex. Blood samples were taken from the subjects and total RNA was purified from the peripheral blood mononuclear cells. The qRT-PCR was used to reveal the expression profile of miR-9, miR-138, miR-424, miR-155, and *CTLA-4* genes, and compared between the patient and control groups. The diagnostic potential of *CTLA-4* and miRNAs was assessed using ROC curve analysis.

Results: This investigation demonstrated a significant increase in the expression levels of miR-9, miR-138, miR-424, miR-155, and *CTLA-4* ($P < 0.0001$) in SARS-CoV-2 patients compared to the healthy controls. We found that the expression levels of miR-9, miR-138, and miR-424 were positively correlated with CRP (p value=0.000) in the patients group. Also, a significant negative correlation was obtained between the expression of miR-9 and ESR (p-value = 0.040) in the patients group. Furthermore, the results of the ROC curve analysis indicated that the expression levels of miR-

9, miR-138, miR-424, miR-155, and *CTLA-4* in the blood mononuclear cells could distinguish the SARS-CoV-2 patients from healthy controls.

Conclusion: Expressions of miR-9, miR-138, miR-424, miR-155, and *CTLA-4* were upregulated in COVID-19 which might be considered as potential molecular biomarkers for SARS-CoV-2 diagnosis.

Keywords: miR-9, miR-138, miR-424, miR-155, CTLA-4, SARS Cov-2, Biomarker, Gene expression

Introduction

SARS-CoV-2 outbreak was deemed a pandemic by the World Health Organization (WHO) on March 11, 2020. The virus responsible for the disease, which is typified by severe respiratory disease and cardiovascular illness, is SARS-CoV-2 (1, 2). Many efforts have been made to produce vaccinations and therapies since the epidemic was declared. The extensive research on the effectiveness of vaccinations, therapies and molecular pathology of the disease revealed that the future antiviral prevention and treatment techniques may be guided by knowledge of an individual's sensitivity to SARS-CoV-2 infection and other viral assaults by identifying particular vital biomarkers (3).

During the early pre-symptomatic phase, the patient has relatively high levels of host biomarkers like microRNAs (miRNAs), in contrast to viral RNA molecules (4). These small non-coding RNAs control various biological functions, such as the host's immune response against viruses. In the initial stages of viral infection, before symptoms appear and virions become noticeable, the pathogen initiates signaling pathways in the host immune system's innate effectors. Like myeloid cells, these first-line responders act quickly and release circulated expressed miRNAs (4, 5).

A member of the miRNA family, microRNA-9 (miR-9), has been linked to various processes, including neural development, immunological response, variable malignancies, and post-traumatic stress disorder (6). It has been discovered that miR-9 contributes to the host immune response by establishing a feedback control of the immune response dependent on the peroxisome proliferator-activated receptor δ or nuclear factor kappa-B (NF-κB) (7). Research has indicated that the coronavirus's N protein is essential for the virus to replicate and attach to genomic RNA to form helical capsids. By binding to miR-9, the OC43 N protein activates NF-kB. Therefore, the OC43 nucleocapsid protein can modify NF-kB expression by a direct interaction with miR-9 (8).

Recent research has demonstrated that miR-138 may directly interact with immunological checkpoints and control their expression. The miR-138 has been found to influence various biological processes, including cell differentiation and inflammatory processes (9).

Additionally, research has demonstrated that miR-138 can target and control the expression of the genes encoding programmed cell death ligand-1 (PD-1) and cytotoxic T lymphocyte-associated molecule 4 (CTLA-4), which may have an impact on how well the immune system functions in the body (10). On the other hand, miR-424 possesses an immunosuppressive potential and plays role in the control of monocyte and macrophage development (11, 12). Researchers found that the miR-424 could predict thromboembolic events in COVID-19 patients and was linked to hypercoagulability in these patients (13, 14).

The miR-155 gene encodes a crucial innate and adaptive immune response modulator. It plays essential roles in viral and parasite infections and in developing ancestral mammalian host defensive systems against pathogen challenges (15). Thus, certain diseases like inflammation are linked to the

miR-155. The most promising target gene for miR-155 is SOCS1, a negative regulator that inhibits the JAK2/STAT3 and NF-kB signaling pathways to reduce inflammation (15).

The cytotoxic T lymphocyte-associated molecule 4 (CTLA-4), often called CD152, is an essential protein receptor in controlling immunological responses. It plays a role in immune reaction downregulation and serves as an immunological checkpoint.

Although CTLA-4 is elevated in conventional T cells only after activation, especially in cancer, it is produced constitutively in regulatory T cells (Tregs). The protein attaches to antigen-presenting cells' surface antigen-presenting receptors, CD80 or CD86, and functions as an "off" switch (16, 17).

The aim of the current study was to compare the expression levels of miR-9, miR-138, miR-424, miR-155, and CTLA-4 in periphery blood mononuclear cells of SARS-Cov2 patients and healthy controls. Additionally, the correlation between the expression levels of miR-9, miR-138, miR-424, miR-155, and CTLA-4 and patient clinical parameters such as age, gender, BMI, fever, oxygen saturation, respiratory rate, C-reactive protein (CRP), white blood cell (WBC), and erythrocyte sedimentation rate (ESR) were investigated.

Material and methods

Patients and controls

This case-control study was carried out to examine the expression levels of miR-9, miR-138, miR-424, miR-155, and CTLA-4 in 66 SARS-CoV-2 positive patients in the chronic and active phase of the disease and healthy control group of 41 people matched for age and sex who referred to Imam Reza and 22 Bahman Hospital in Mashhad. All participants gave their informed consent to participate in the investigation. A four mL blood sample was taken from each participant and used for subsequent analysis. Also, clinicopathological data were obtained from the patients' hospital records.

RNA extraction, cDNA synthesis and qRT-PCR

The RNX puls RNA extraction kit (Sinagen, Iran) was used to extract total RNA from peripheral blood mononuclear cells (PBMC) following the kit's instructions. The RNA concentration and purity was measured using a Nanodrop spectrophotometer. About two µg from each sample RNA was treated with DNase I to remove any genomic DNA contamination and then used for cDNA synthesis. The cDNA was synthesized using DyNAmo-cDNA kit following the kit instructions and used in qRT-PCR. The Gene Runner and Primer blast programs were used for designing the oligonucleotide primers (Table 1).

The BLAST program was utilized to validate the primers to avoid producing non-specific PCR results. The housekeeping genes *U6* and *GAPDH* were used to normalize the expression data. Table 1 presents the primer sequences of miR-9, miR-138, miR-424, miR-155, *CTLA-4*, *U6* and *GAPDH*. For qRT-PCR, we used Thermo Scientific Maxima SYBR-Green qRT-PCR master mix and a CORBETT-6000 real-time PCR instrument for expression analysis.

The amplification protocol was; polymerase activation step 3 min at 95 °C, 40 cycles of denaturation 15 sec at 94 ºC, primer annealing and elongation 30 sec at 60 °C. The delta Ct method was employed to determine the expression levels of the studied genes.

Table 1: Sequences of primers

Gene name	Primer sequence
miR-9	Forward: 5′-AGGCATCTTTGGTTATCT-3′
	Reverse:
miR-138	Forward: 5′-GCTGGTGTTGTGAATC-3′
	Reverse:
miR-424	Forward: 5′-GCAGCAATTCATGTTT-3′
	Reverse:
miR-155	Forward: 5′-CCGTTAATGCTAATC-3′
	Reverse:
CTLA-4	Forward: 5′-ACTACCTGGGCATAGGCAAC-3′
	Reverse: 5′-CCGAACTAACTGCTGCTGCAAGGA-3′
U6	Forward: 5′-GCTTCGGCAGCACATATACTAAAAT-3′
	Reverse: 5′-CGCTTCACGAATTTGCGTGTCAT-3′
GAPDH	Forward: 5′- ATGGGGAAGGTGAAGGTCG -3′
	Reverse: 5′- GGGGTCATTGATGGCAACAATA -3′

Statistical analysis

Statistical analyses were carried out using SPSS version 26 and GraphPad Prism. The Shapiro-Wilk test was initially used in data analysis to determine whether the data were normal.

The Mann-Whitney test was employed for non-normal data. The One Way ANOVA was used to compare expression levels between the groups. The association between the qualitative factors was investigated using the chi-square test. Regression analysis and the Pearson correlation coefficient were used to examine the variables' relationship. The ROC curve analysis was used to assess the biomarker potential of the miRNAs and *CTLA-4* levels for COVID-19. The acceptable level for statistical significance was $P \leq 0.05$.

Results

Description and comparison of the clinicopathological characteristics

In this case-control study, 66 SARS-CoV-2 positive patients who were in the chronic and active phase of the illness, and 41 healthy individuals matched for age and gender of the patients, were included in the study. About 59% of the patients' group was male. The mean age of the total subjects was 42.06±16.21 years. The average BMI was also 27±4.49. There were no significant differences between the case and control groups regarding the age (P-Value=0.611) and sex (P-Value=0.955) parameters.

However, differences between the case and control groups regarding the BMI (P-Value=0.0074), fever (P-Value=0.0001), oxygen saturation (P-Value=0.0001), respiratory rate (P-Value=0.0001), CRP (P-Value=0.0001), WBC (P-Value=0.0001), and ESR (P-Value=0.0001) were all statistically significant (Figure 1). The mean of BMI and oxygen saturation parameters were significantly lower in the cases than controls, however, the CRP, WBC, fever, respiratory rate, and ESR parameters were significantly higher in the patients than the control group.

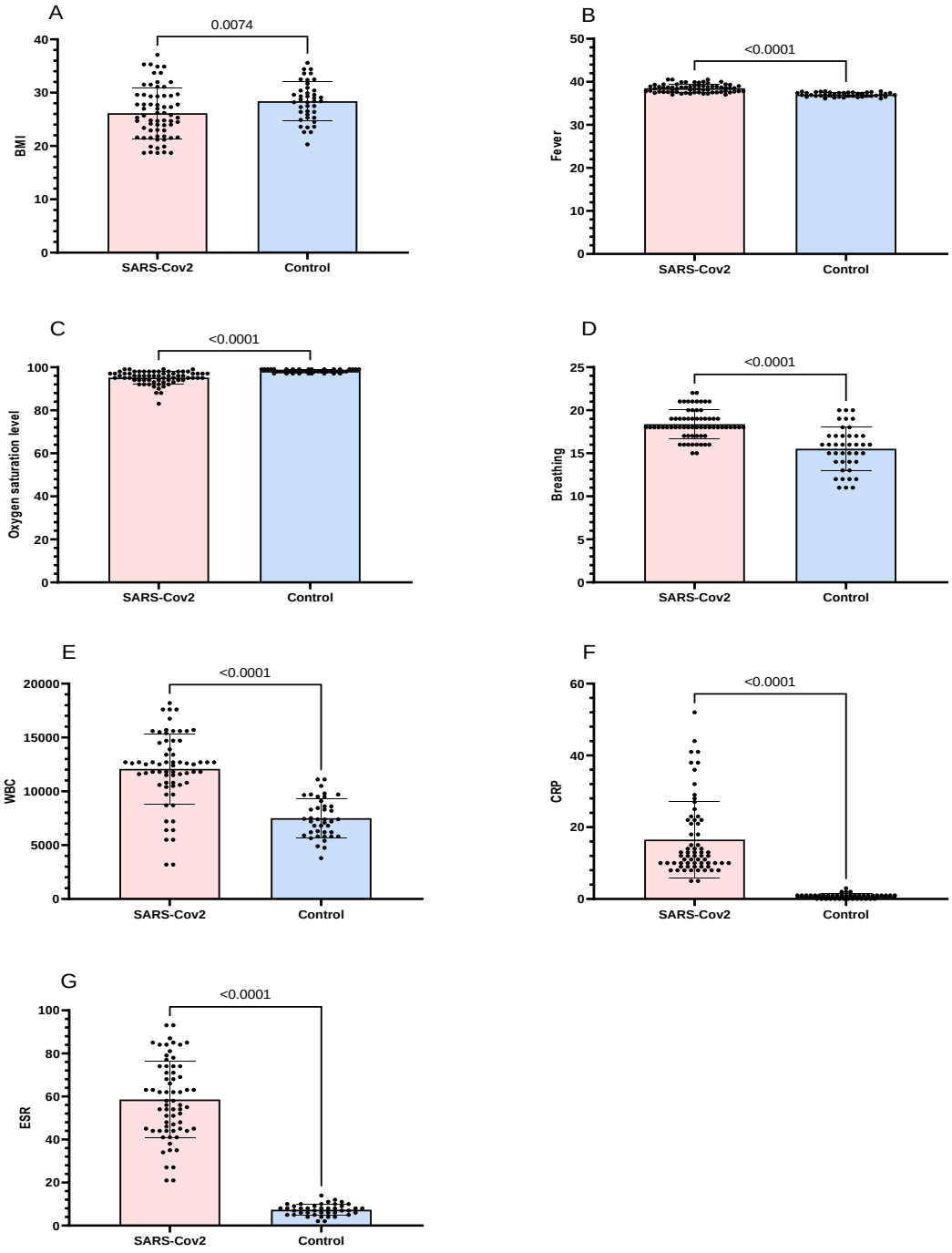

Figure 1: Comparison of the clinicopathologic characteristics of the case and control groups; (A) BMI, (B) fever, (C) oxygen saturation, (D) respiratory rate, (E) WBC, (F) CRP, and (G) ESR. WBC: White Blood Cell, CRP: C-reactive protein, ESR: Erythrocyte Sedimentation Rate.

Expression profile of the miRNAs and *CTLA-4*

The qRT-PCR was used to measure the relative expression of the miR-9, miR-138, miR-424, miR-155, and *CTLA-4* genes, and their relative expressions were compared between the patients and healthy controls. As indicated in the Figure 2, we observed significant rises in the relative expressions of the studied microRNAs and CTLA-4 gene in the cases compared to the control samples ($p < 0.0001$) (Figure 2).

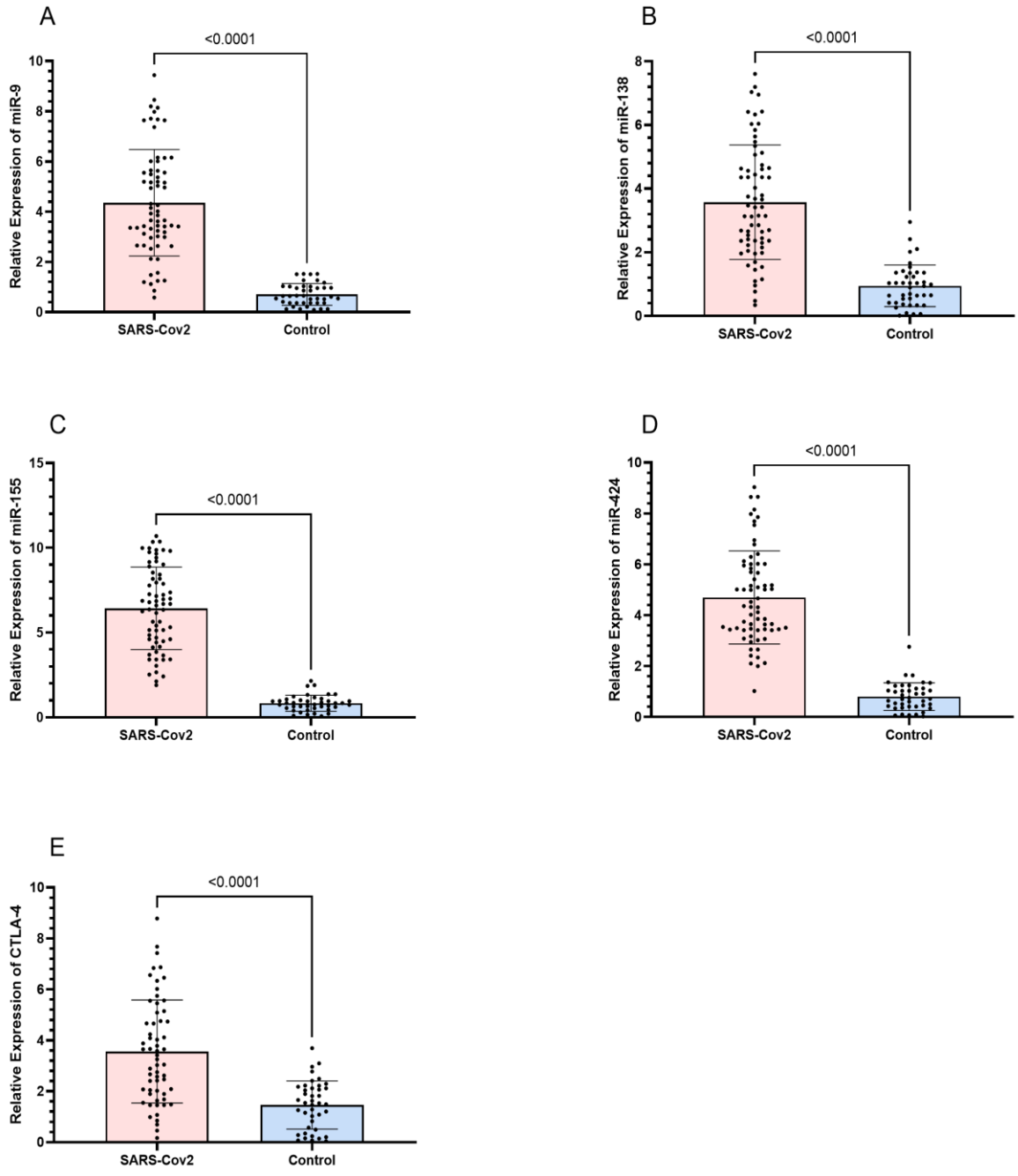

Figure 2: Relative expressions of the studied genes. The relative expression levels of A) miR-9, B) miR-138, C) miR-424, D) miR-155, and E) CTLA-4 were compared between the case and control groups.

Association of miRNAs and CTLA-4 expression levels with the clinicopathological characteristics

To understand whether the relative expressions of the studied genes are correlated with the clinical parameters of the patients, correlation analysis was done. The results showed that the expression levels of miR-9, miR-138, and miR-424 all were positively correlated with the CRP (p value=0.000) in the patients but not in the control group. Furthermore, the expression level of miR-9 was also negatively correlated with the ESR (p value=0.040).

Table 2. The Relationship between different indices and relative expression of miR-9, miR-138, miR-424, miR-155, and CTLA-4 and clinical variables

	miR-9		miR-138		miR-424		miR-155		CTLA-4	
	patients	control	patients	control	patients	control	patients	control	patients	control
Age	r = 0.113 p= 0.368	r=-0.324 p= 0.039	r=0.105 p=0.401	r=-0.091 p=0.569	r=0.097 p=0.437	r=0.067 p=0.676	r=-0.089 p=0.478	r=-0.103 p=0.521	r=-0.021 p=0.869	r=-0.071 p=0.660
Duration	r = -0.171 p= 0.169	r=0 p=0	r=-0.044 p=0.723	r=0 p=0	r=-0.043 p=0.729	r=0 p=0	r=0.102 p=0.415	r=0 p=0	r=-0.082 p=0.513	r=0 p=0
BMI	r= -0.037 p=0.769	r= 0.288 p=0.068	r=-0.040 p=0.750	r=0.077 p=0.634	r=0.047 p=0.705	r=0.126 p=0.432	r=0.006 p=0.964	r=0.075 p=0.639	r=0.149 p=0.234	r=-0.260 p=0.101
Fever	r=-0.050 p=0.693	r=0.261 p=0.099	r=-0.012 p=0.921	r=0.115 p=0.474	r=0.046 p=0.716	r=-0.047 p=0.772	r=-0.145 p=0.246	r=0.050 p=0.755	r=0.168 p=0.179	r=-0.060 p=0.711
O2 sat	r= -0.056 p= 0.655	r=-0.084 p=0.603	r=-0.102 p=0.416	r=0.098 p=0.541	r=-0.155 p=0.214	r=-0.007 p=0.964	r=0.010 p=0.934	r=-0.137 p=0.393	r=0.129 p=0.302	r=0.191 p=0.232
RR	r=-0.072 p=0.566	r=0.226 p=0.155	r=-0.077 p=0.539	r=0.066 p=0.681	r=0.013 p=0.918	r=0.043 p=0.789	r=0.039 p=0.759	r=0.145 p=0.367	r=-0.038 p=0.764	r=-0.145 p=0.366
WBC	r=0.103 p=0.412	r=0.066 p=0680	r=0.127 p=0.308	r=-0.146 p=0.362	r=0.087 p=0.485	r=-0.030 p=0.852	r=0.090 p=0.473	r=0.204 p=0.201	r=-0.067 p=0.594	r=0.046 p=0.775
CRP	r=0.549 p=0.000	r=0.222 p=0.163	r=0.541 p=0.000	r=0.052 p=0.745	r=0.609 p=0.000	r=-0.021 p=0.897	r=0.105 p=0.400	r=0.169 p=0.292	r=-0.197 p=0.113	r=0.172 p=0.284
ESR	r=-0.254 p=0.040	r=-0.053 p=0.740	r=-0.166 p=0.182	r=-0.175 p=0.275	r=-0.171 p=0.170	r=0.071 p=0.658	r=-0.124 p=0.321	r=0.125 p=0.435	r=0.003 p=0.978	r=0.113 p=0.483

Potential of the miRNAs and CTLA-4 as biomarkers for COVID-19

The receiver operating characteristic (ROC) curve analyses were carried out to assess the miR-9, miR-138, miR-424, miR-155, and CTLA-4 diagnostic values for SARS-CoV-2 infection. The miR-9 had an AUC of 0.86 (95% confidence interval (CI) 0.793–0.929; sensitivity = 82.93%, specificity = 75.76%), miR-138 had an AUC of 0.88 (95% CI; 0.808–0.958; sensitivity = 90.24%, specificity = 82.35%), miR-424 had an AUC of 0.84 (95% CI; 0.771–0.919; sensitivity = 80.49%, specificity = 77.27%), miR-155 had an AUC of 0.80 (95% CI; 0.767–0.913; sensitivity = 87.80%, specificity = 69.70%), CTLA-4 had an AUC of 0.81(95% CI; 0.735–0.896; sensitivity = 85.37%, specificity = 68.33%). These results showed that miR-9, miR-138, miR-424, miR-155, and CTLA-4 might be considered as potential biomarkers for SARS-CoV-2 diagnosis (Figure 3).

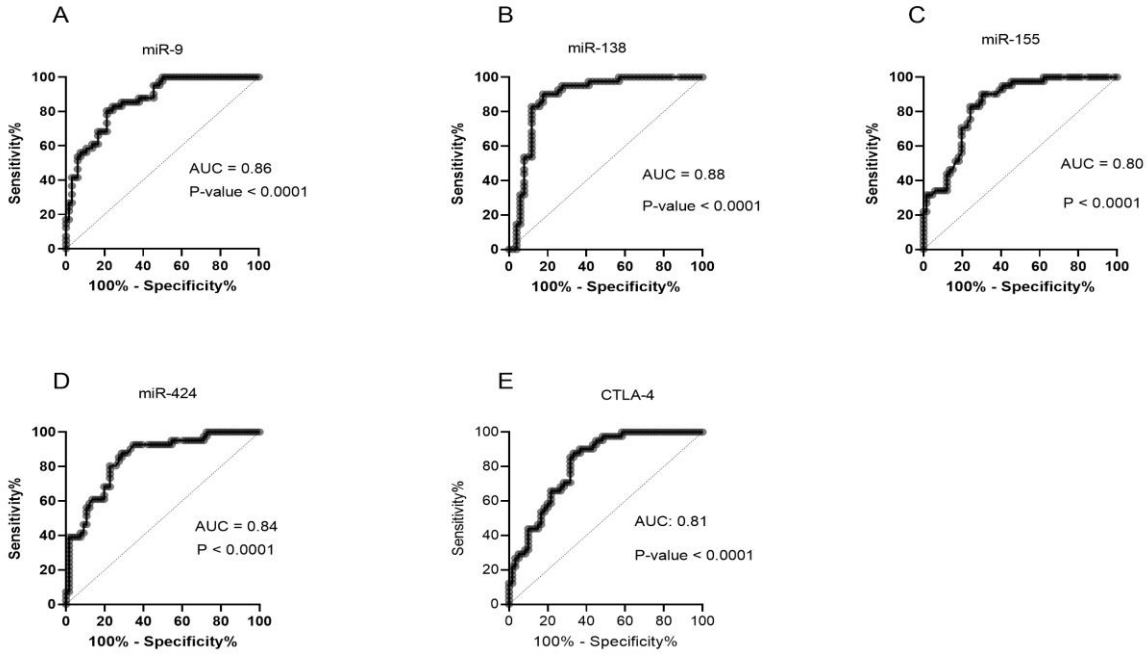

Figure 3: ROC curve analysis for (A) miR-9, (B) miR-138, (C) miR-155, (D) miR-424, , and (D) CTLA-4 expression levels revealed significant biomarker potential for SARS-CoV-2 infection.

Correlation between the trelative expressions of the studied genes in COVID-19 patients

Spearman correlation analysis showed that in patients with COVID-19 expression level of miR-424 were positively correlated with those of miR-138 (r = 0.842, P value = 0.0001) and miR-9 (r = 0.828, P value = 0) (Figure 4A and B). Furthermore, as indicated in the Figure 4C, the expression levels of miR-9 and miR-138 (r = 0.741, P value = 0) were also positively correlated. However, we observed a significant negative correlation between the expression levels of miR-155 and *CTLA-4* gene (r = -0.842, P value = 0.0001) (Figure 4D). Correlations between the expression levels of others were not statistically significant. As outlined in Table 2, this analysis revealed no correlation between the relative expressions of the studied genes.

Table 3: Expression correlation analysis of the studied genes both in the control and patient groups

			miR-424	miR-138	miR-9	miR-155	CTLA-4
Control group	miR-424	Pearson Correlation	1	-0.043	0.136	-0.075	0.108
		Sig. (2-tailed)		0.789	0.395	0.641	0.503
	miR-138	Pearson Correlation	-.043	1	0.013	0.212	-0.138
		Sig. (2-tailed)	0.789		0.937	0.182	0.389
	miR-9	Pearson Correlation	0.136	0.013	1	0.033	0.105
		Sig. (2-tailed)	0.395	0.937		0.837	0.514
	miR-155	Pearson Correlation	-0.075	0.212	0.033	1	-0.044
		Sig. (2-tailed)	0.641	0.182	0.837		0.784
	CTLA-4	Pearson Correlation	0.108	-0.138	0.105	-0.044	1
		Sig. (2-tailed)	0.503	0.389	0.514	0.784	
Patient group	miR-424	Pearson Correlation	1	0.842**	0.828**	0.178	-0.180
		Sig. (2-tailed)		0.000	0.000	0.153	0.149
	miR-138	Pearson Correlation	0.842**	1	0.741**	0.176	-0.201
		Sig. (2-tailed)	0.000		0.000	0.159	0.105
	miR-9	Pearson Correlation	0.828**	0.741**	1	0.166	-0.201
		Sig. (2-tailed)	0.000	0.000		0.184	0.106
	miR-155	Pearson Correlation	0.178	0.176	0.166	1	-0.801**
		Sig. (2-tailed)	0.153	0.159	0.184		0.000
	CTLA-4	Pearson Correlation	-0.180	-0.201	-0.201	-0.801**	1
		Sig. (2-tailed)	0.149	0.105	0.106	0.000	

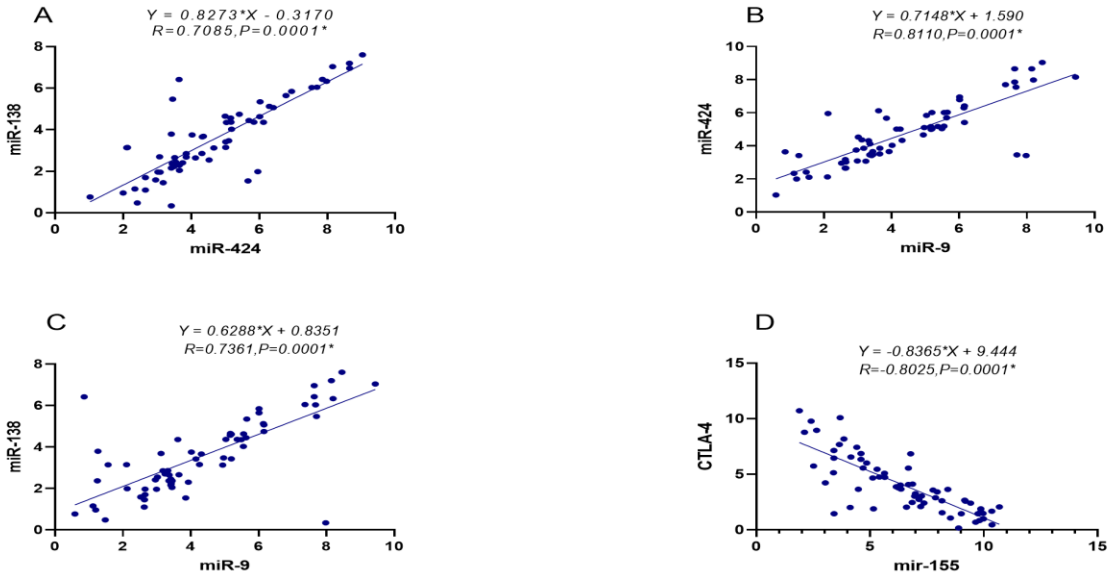

Figure 4: Regression plots indicating correlations between the relative expressions of the A) miR-424 and miR-138, B) miR-424 and miR-9, C) miR-9 and miR-138, and D) miR-155 and CTLA-4

Discussion

The ability of SARS-CoV-2 to maintain pre- and asymptomatic human-to-human transmission is one of its more perilous characteristics. The U.S. CDC estimates that 40% of transmission happens before symptoms appear (18, 19). Additionally, about 35 percent of COVID-19 infections do not show any symptoms at all during the illness (20, 21). These characteristics of COVID-19 have contributed to the virus's quick dissemination and the catastrophic worldwide pandemic (22). This indicates the need for innovations to close gaps in the SARS-CoV-2 diagnostic landscape (19, 23).

In this regard, miRNAs have attracted much attention and are believed to be helpful in narrowing down the gap (24, 25). Much research is being done on non-coding RNAs, including miRNAs as potential innovative biomarkers and therapies for different diseases (26, 27) especially to prevent viral infections such as SARS-CoV-2 (28). The miRNAs can target receptors, structural or nonstructural proteins of SARS-CoV-2, or impede the translation of the virus after it attaches to the 3′-UTR of the viral genome without changing the expression of human genes (29, 30).

According to recent research, miR-9 appears to be closely related to immunity and inflammatory disorders. During immunological reactions triggered by cytokines or lipopolysaccharides (LPS), monocytes and neutrophils are stimulated to produce miR-9 (31). Moreover, miR-9 is upregulated by activated CD4+ T cells, repressing Blimp-1 and increasing IL-2 and IFN-c production (32). Immune responses are triggered in inflammatory illnesses such as multiple sclerosis and bronchial asthma by increased release of these pro-inflammatory cytokines. Consistent with these reports, we found that the expression of miR-9 was upregulated in the patients with COVID-19 compared to the healthy controls. Other clinical conditions, such as brain inflammation, can also stimulate miR-9. Two independent investigations have demonstrated that inflammatory stimuli (LPS) can elevate miR-9 in monocytes and microglia. Accordingly, the NF-kB pathway is how miR-9 regulates microglial activation and inflammatory response, and targeting the miR-9 has explicitly been proposed as a potential therapeutic approach for treating neuroinflammatory disorders (8, 33, 34).

We also assessed the expression level of the miR-138 and compared its levels between the patients with COVID-19 and healthy controls. We found significant upregulation of the miR-138 in the patients group. According to Liu. et al., miR-138 expression levels were inversely correlated with hepatitis B virus (HBV) viral load, indicating a potential role for miR-138 in HBV-related diseases. MiR-138 was also found to be downregulated in patients with liver cirrhosis, HBV-associated hepatocellular carcinoma, and chronic hepatitis B. They discovered that by specifically targeting the 3'-UTR of PD-1, miR-138 can directly contribute to the production of PD-1. Furthermore, miR-138 overexpression may induce the production of antivirus cytokines such as IFN-γ and TNF-α, suggesting that miR-138 may have antiviral effects on HBV infection (35).

We also observed significant upregulation of the miR-424 in the COVID-19 patients. Consistent with our finding, in a study on SARS-CoV-2 infection, Gambardella. et al. found that patients in the high D-dimer subgroup had considerably higher levels of exosomal miR-424 than individuals in the low D-dimer subgroup. A blood test can identify D-dimer, a fragment of fibrin degradation (two D fragments of the fibrin protein) that is present in the blood following the breakdown of a blood clot and helps diagnose thrombosis (36).

According to research by Haroun. et al., miR-155 is essential for the pathophysiology and severity of COVID-19 (8). It may also be a useful clinical biomarker for diagnosing the disease and assessing the extent of infection. In line with these results, we also observed that miR-155 expression level was significantly higher in the patients group compared to the healthy controls. Besides, ROC curve analysis pointed out that the miR-155 expression level may distinguish between the COVI-19 patients and the healthy controls, highlighting its potential as a biomarker for the disease diagnosis. Consistent with these findings, miR-155 was reported by Lerner. et al. to be a reliable indicator of COVID-19 mortality. They propose that testing for miR-155 in patients' blood at hospital admission will improve care for COVID-19 patients (37). Another research has also reported that COVID-19 patients had a substantial overexpression of miR-155 compared to controls (38).

Jebbawi. et al. showed that miR-9 and miR-155 directly control CTLA-4. They found out that the downregulation of miR-9 and miR-155 resulted in the reduced expression levels of CTLA-4 (39). Opposite to this report, when we analyzed the correlations between the expression levels of the studied genes, a significant negative correlation was obtained between the expression levels of miR-155 and CTLA-4. Another study revealed that in the severe group of SARS-CoV-2 patients compared to the mild group of the SARS-CoV-2 patients, there was an increase in CD8+ T cells expressing high levels of CTLA-4 (40). Consistently, the expression level of CTLA-4 in the COVID-19 patients was significantly higher than that of controls in our study.

Conclusion:

In conclusion, the results indicated that miR-9, miR-138, miR-424, miR-155, and CTLA-4 were upregulated in the peripheral blood mononuclear cells of the patients with COVID-19 and their expression levels might be considered as potential biomarkers for COVID-19. Besides, expressions of miR-424, miR-138 and miR-9 were positively correlated in the patients group. However, the expressions of miR-155 and CTLA-4 were negatively correlated.

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