ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2023; 22(1):82-90.
DOI: 10.18502/ijaai.v22i1.12009

Investigating the Relationship between the Levels of IL18, RANKL Gene Expression, MicroRNA-146a and Inflammatory Factors with the Severity of COVID-19

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Received: 13 September 2022; Received in revised form: 10 December 2022; Accepted: 17 December 2022

ABSTRACT

COVID-19 can induce lung inflammation, and inflammatory factors play an essential role in its pathogenesis. This inflammation can be controlled to a great extent by microRNAs (miRs). This study evaluated miR-146a-5p expression levels in the serum of patients with COVID-19 and their association with the expression of interleukin (IL)-18 and receptor activator of nuclear factor kappa-B ligand (*RANKL*) genes, and lung damage.

Eighty-Five patients with COVID-19 were divided into two groups: mild and severe phases. The severe phase is defined as having a positive polymerase chain reaction (PCR) for SARS-CoV2, and acute pulmonary symptoms. The subjects' demographic, clinical, and paraclinical characteristics were collected according to a pre-prepared checklist. Total RNA was isolated from all samples using the Trizol kit to assess gene expression. The extracted product was then evaluated for the expression of miR-146a and the target genes (i.e., IL-18 and RANKL) using real-time PCR.

The miR-146a gene's mean expression in mild and severe patients was 0.73 and 1.89, respectively, and this difference was statistically significant between the two groups. Also, the mean Expression of the IL-18 gene, 1.37±0.38 in the mild and 2.83±0.58 in the severe groups of the disease, demonstrated a significant difference between the two groups. In contrast, the expression levels of the RANKL gene did not show a significant difference between the two groups.

Therefore, it may be hypothesized that altered levels of miR-146a may contribute to the severe COVID-19 that is more commonly observed in smokers, but further research is required.

Keywords: COVID-19; Interleukin-18; MicroRNA-146a-5p; Respiratory disease; Receptor activator of nuclear factor kappa-B ligand

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INTRODUCTION

COVID-19 is caused by the genus Betacoronavirus, which belongs to the family Coronaviridae and subfamily Coronavirinae. In late 2019, this virus was identified as the source of a pneumonia outbreak in Wuhan, China.[1–3] World Health Organization (WHO) declared COVID-19 a pandemic in March 2020.[4] Globally, 6.4 million deaths from COVID-19 have been reported, along with more than 570 million confirmed cases. The COVID-19 pandemic has affected 230 nations.[5] SARS-CoV-2 is a significant RNA strand of the virus's envelope that measures between 85 - 160 nm in size and has an approximate genome size of 27–35 kb that causes and leads to COVID-19.[6,7]

Epigenetics is characterized as heritable alterations in cellular phenotype or gene expression without affecting the underlying DNA sequence. It includes chromatin variations, DNA methylation, and noncoding RNAs (particularly microRNAs).[8–10] MicroRNAs, which contain 18–24 nucleotides, regulate gene expression post-transcriptionally. By attaching to the untranslated regions of mRNA, microRNAs function as an RNA interference machinery and can cause mRNA to degrade or prevent protein translation.[11] It is estimated that microRNAs target more than 30% of human genes , implying their widespread impact on eukaryotic transcriptomes and proteomes.[12,13] Essential enzymes involved in epigenetic events, such as histone methyltransferases, DNA methyltransferases, and histone deacetylases, are the targets of epigenetic modulators like microRNAs.[14–16]

As one of the epigenetic mediators, microRNAs create different regulatory pathways in cells. Finally, microRNAs are used for intracellular regulation such as apoptosis, its biological function,[17] differentiation,[18] and cell proliferation.[19]

The expression pattern of microRNAs may vary between the mild and severe stages of the disease depending on how viruses interact with cellular components. Due to the high inflammatory state in COVID-19 patients,[20–22] it has been proposed that the expression pattern of microRNAs associated with inflammation (e.g., miR-146a, miR-155, Let-7, and miR-29),[23–25] could differ between mild and severe phases of the disease. miR-146a is evaluated as a biomarker to detect disease progression in several cancer-related mechanisms. It is used for diagnostic and therapeutic purposes in tissues and bodily fluids, including blood and urine.[26] Additionally, research on the SARS-CoV-2 virus may benefit from understanding the role of miR-146a-5p in acute respiratory distress syndrome.[27]

Cytokines play an essential role in the immunopathology of viral infections. Cytokine production is a rapid and well-coordinated immune response that is the initial line of defense against viral infection. However, an overactive immune response can also cause damage. Cytokine storm syndrome refers to the immune system overactivation in reaction to both non-infectious and infectious stimuli, characterized by the production of proinflammatory cytokines and multiple organ failures.[28–31] IL-18 is one of the 11 members of the IL-1 family, and like IL-1, it responds promptly to initiate proinflammatory responses. IL-18 overexpression can occur in the airways of patients with chronic obstructive pulmonary disease, emphysema, blood vessel fibrosis, and pulmonary hypertension.[32,33] Due to its helper T cell (T_H)2-inducing properties, IL-18 can also contribute to the hyperreactivity and inflammation of the respiratory tract in asthma and encourage the recruitment of eosinophils to the lungs.[34] Both T_H1 and T_H2 responses are regulated by IL-18.

Moreover, many T-cell populations are thought to be activated and differentiated by IL-18, and IL-12 binding to the Th1 lymphocyte,[35] natural killer cells, T and B cells, and a propensity for interferon production.[36] The receptor activator of nuclear factor kappa-B ligand (RANKL) is found on the surface of activated T cells and is thought to function as an adaptive immunity regulator.[37] Furthermore, RANKL-expressing T cells can influence osteoclastogenesis, a disease-related bone loss explained by chronically active inflammatory responses.[38]

The identification of novel biomarkers for the diagnosis and prognosis surveillance of COVID-19 may be aided by all studies Our research aims to assess the pattern of mir-146a-5p expression along with IL-18 and RANKL in the serum of COVID-19 patients during both the mild and severe forms of the illness.

MATERIALS AND METHODS

Subjects

From October 2020 to December 2020, 85 COVID-19 patients admitted to health centers affiliated with

Urmia University of Medical Sciences in West Azerbaijan Province, Iran, were recruited, of whom 45 had the severe form of the disease and 40 had the mild form. The collected samples were randomly chosen for the sampling. This study was designed as a prospective case-control study. We calculated the appropriate sample size with respect to the findings of a study by Liu et al,[39] where the association between values in patients with COVID-19 was about 68% and α=0.05.

The inclusion criteria for the severe group included pneumonia, acute respiratory symptoms, and a positive COVID-19 polymerase chain reaction (PCR) test.[40] The mild group had positive COVID-19 PCR results but an absence of acute respiratory symptoms. This study did not include people with chronic respiratory conditions or other underlying illnesses, including hypertension, diabetes, cancer, cardiovascular disease, or autoimmune inflammatory diseases.

Demographic, Clinical, and Paraclinical Information

A questionnaire and checklist were used to collect the patients' demographic, clinical, and paraclinical data. The checklist also contained the laboratory results and imaging findings and a specialist's assessment. Samples were taken from persons and patients with severe stages by healthcare staff relating to medical training facilities connected to Urmia University of Medical Sciences. Molecular tests were performed at the University's Molecular Laboratory.

Total RNA Extraction and RT-PCR

We first separated peripheral blood mononuclear cells (PBMCs) from the serum using Ficoll (Lymphodex, inno-train, Germany). Then, we isolated RNA from the samples using the TriZol kit. In this technique, according to the existing protocol (GeneALL Biotechnology, Seoul, Korea), we used about 1 mL of the TriZol solution for cell plate samples and 0.75 mL for serum samples. Finally, we used electrophoresis and nanodrop techniques to evaluate the purity and amount of isolated RNAs. The 260/230 and 260/280 values were 1.8 to 2.1 for all isolated samples.

This stage involved the synthesis of the sample cDNA using two distinct procedures (synthesis of specific cDNA for PBMC samples and specific cDNA for serum samples), both of which required the same quantity of RNA (1 g) for cDNA synthesis in PBMC samples. The manufacturer's protocol for the kit was then followed when using various real time-PCR stages (Thermo Fisher Scientific, USA). We created serum-specific cDNA using the stem-loop technique (evaluation of microRNAs). The cDNA synthesis step was exclusively used in this procedure to develop the specific MiR-146a and U6 sequences using the specified primers coated inside the vial. After that, the expression of target genes was evaluated using the samples. Specific primers were used to produce the desired cDNA. The expression of the target genes was measured using the available samples. Instead of oligo and random hexamer primers, customized primers for each microRNA (miR-146a-5p, U6) were employed as a stem-loop for synthesizing the necessary microRNAs' particular cDNA.

Primer Design

The National Center for Biotechnology Information (NCBI) and mirVana were used to obtain the information on IL-18, RANKL gene sequence, and MiR-146a-5p. Primer pairs were created for the IL-18, RANKL, and mRNA sequences using the Oligo 7 primer analysis software (Molecular Biology Insights, Inc., Cascade, CO., USA). Additionally, utilizing the mirVana website, MiR-146a-5p was designed (Supplementary Table).

Expression of *MiR-146a,* IL-18 and RANKL Genes

Using a real-time PCR instrument (BioMolecular Systems, AUSTRALIA, MIC), the relative expressions of IL-18 and RANKL were determined. The following primers are listed in Supplementary Table and were used to measure the expression of β-actin and U6, which were chosen as internal references for identifying expression and copy number changes. The $2^{-\Delta\Delta Ct}$ formula was used to determine the relative expression levels of IL-18, RANKL, and MiR-146a. Three biological replicates were used for each test.

Statistical Analysis

SPSS statistical software, version 23, was used to analyze data. The mean and standard deviation (SD) were used to report the quantitative data. GraphPad Prism Version 7 was used to create the diagrams. The frequency of qualitative data was reported (percent). The Kolmogorov-Smirnov test was used to assess data distribution. The Chi-squared test, paired-sample t-test, independent-sample t-test, and one-sample t-test were

used for qualitative data to compare quantitative variables between groups, compare quantitative variables within groups, compare gene expression to a fixed value, and a significance level was considered as p value <0.05.

RESULTS

Demographic characteristics are provided as mean (SD); qualitative results are expressed as percentages. Table 1 describes the clinical features, demographics, outcomes, and comparisons for each variable.

The mean age in the group with mild COVID-19 was 61.37 years, compared to 59.49 years in the group with severe COVID-19. women comprised 35 patients (56.5%), compared with 27 (43.5%) men. The mean age and sex did not significantly differ between groups. The mean body mass index was 27.31 kg/m^2 in the mild group and 26.08 kg/m^2 in the severe group, which did not significantly differ. Table 1 displays a statistically significant difference between the two patient groups in the mild and severe stages for erythrocyte sedimentation rate (ESR), smoking status, presence of cough, and hematocrit (HCT) variables (p=0.05).

Fluorescence radiation graphs, cycle threshold (CT)number, and melting curve diagrams were taken by the output of the real-time PCR device. At the conclusion, we calculated the data on the patient features and the level of gene expression. The mean expression of the miR-146a gene differed significantly between groups, roughly 0.73±0.3 and 1.89±0.52 in the case of mild and severe patients, respectively (p=0.01) (Figure 1). Additionally, there was a significant difference between groups (p=0.05) in the mean expression of the IL-18 gene, which was roughly 1.37±0.38 and 2.83 ± 0.58 in the mild and severe groups, respectively (Figure 1).

In this regard, after dividing the patients into two age groups of <60 and >60, we found no correlation between age and disease severity in patients with coronary artery disease. Age, sex, IL-18, and miR-146a gene expression were not correlated with one another (p>0.05); however, miR-146a gene expression was significantly different in cough, and smoking was associated with both miR-146a and IL-18 gene expression.

HCT (p=0.018) and ESR (p=0.023) were statistically different between groups. Among IL-18, RANKL, and miR-146a genes, only the miR-146a gene expression were statistically different.

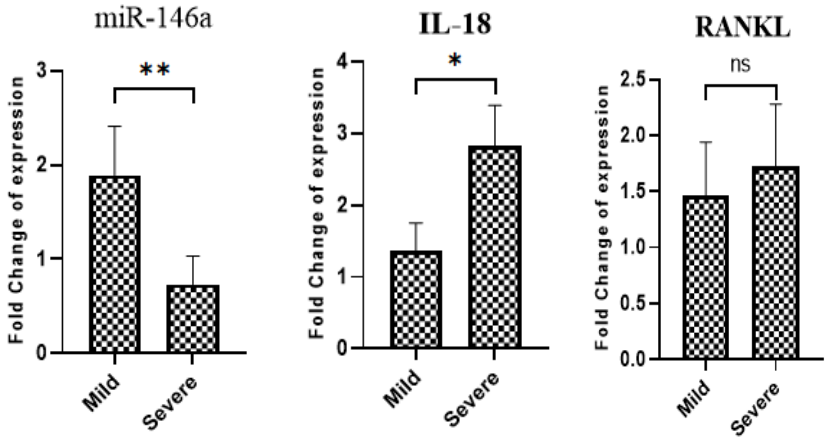

Figure 1. MiR-146a expression in the mild and severe disease groups Altered Expression of *MiR-146a* genes in the severe and mild disease groups. As shown in the figure, *MiR-146a* expression significantly increased significantly in the mild group compared with the severe group (p<0.01). Also, IL-18 expression in the mild and severe disease groups, as shown in the figure, *IL-18* gene expression showed a significant decrease compared with the severe disease groups. In contrast, the expression levels of the RANKL gene did not show a significant difference between the two group .

*** p<0.05, ** p<0.01. Ns: non-significant**

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Table 1. Demographic, clinical, and paraclinical characteristics of patients

Variables	Severe (n=32)	Mild (n=25)	Total	*p*
Age in Years	59.49	61.37	60.4	0.54
Weight (kg)	78.05	81.09	79.9	0.5
Body Mass Index (kg/m^2)	26.08	27.31	26.85	0.33
Gender				
Male	18 (32%)	8 (14%)	26 (46.5)	0.17
Female	14 (25%)	17 (30)	31 (53.5)	
Smoking Status	6 (9.8)	2 (3.3)	8 (13.1)	**0.002**
Clinical features				
Oxygen Saturation	84.88	85.55	85.27	0.24
Cough	20 (32.3)	30 (48.4)	50 (80.7)	**0.001**
Dry Cough	5 (29.4)	2 (11.8)	7 (41.2)	
Cough with Sputum	7 (41.2)	3 (17.6)	10 (58.8)	
Fever	16 (25.8)	14 (22.6)	30 (48.4)	0.31
Sore Throat	3 (4.8)	2 (3.2)	1 (1.6)	0.37
Headaches	9 (14.5)	8 (12.9)	17 (27.4)	0.2
Gastrointestinal Symptoms	11 (17.7)	8 (12.9)	19 (30.6)	0.6
Breast graph	34(54.3)	25(40.3)	59(95.2)	0.6
Percentage of Pulmonary involvement				
0-20 %	0	1 (5)	1 (5)	
20-40 %	3 (15)	2 (10)	5 (25)	
40-60 %	2 (10)	3 (15)	5 (25)	
60-80 %	3 (15)	3 (15)	6 (30)	
80-100 %	3 (15)	0	3 (15)	
Paraclinical Profile				
WBC (x103/mm3)	13016.9	8841.6	10592.58	0.19
RBC (x106/ µL3)	4.21	4.23	4.23	0.91
ESR(mm/h)	45.8	53.5	50.01	**0.023**
CRP(mg/L)	38.8	38	38.35	0.55
HCT (%)	51.92	36.56	43.00	**0.018**
Hb(g/dL)	12.68	12.18	12.39	0.38

Subjects are divided into the mild and severe groups.

WBC: white blood cell; RBC: red blood cell; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; HCT: hematocrit; Hb: hemoglobin.

DISCUSSION

In the current study, the expression of mir-146a and IL-18, and RANKL were compared in blood samples of patients with mild and severe COVID-19. The levels of MiR-146a predicts the development of the disease, diagnosis, and treatment response in many cancer-related pathways, both in tissues and body fluids.[40-43] In addition, miR-146a-5p is important for acute respiratory distress syndromeand may play a role in the development and exacerbation of its related symptoms.[44] The prevention of infections is significantly aided by IL-18.[45] Few observational data support IL-18 function in lowering the risk of COVID-19.[46,47] It is difficult to understand because it involves patient observational studies where IL-18 may be a defense mechanism, a symptom, or a contributor to consequences. The immune system and bone are tightly connected and share many regulatory components, including cytokines and receptors, according to research by the receptor activator

of nuclear factor kappa B ligand (RANKL) in recent years. RANKL is crucial for controlling bone metabolism and developing lymphoid organs, including the thymus and lymph nodes.[48]

According to this study, there was a considerably smaller expression level of mir-146a in the severe group than in the mild group. Age and illness severity, as well as age and mir-146a expression levels in COVID-19 patients, did not correlate with one another. When compared to the female group, the male group showed a substantial increase in the expression rate of the mir-146a gene.

Keikha et al,[49] found that the relative expression of anti-neuroinflammatory miRNAs, is inversely correlated with all microRNAs, including mir-21, mir-124, and mir-146a. It's interesting to note that the COVID-19 severity increased the relative expression of human mRNA targets of anti-neuroinflammatory miRNAs, including IL-12, p53, STAT3, and TRAF6 (p=0.05). The expression of (mir-21 and IL-12p53 mRNA), (mir-124 and Stat3 mRNA), and (mir-146a and TRAF6 mRNA) in COVID-19 patients at all grades showed a significantly negative correlation ($p=0$.05), and the results in those studies were consistent with those of our research, even though they used different genes. According to a recent study, it (levels of microRNAs) is crucial in inflammation and age-related illnesses.[50-54] They recently suggested that the current pandemic's features strongly recommend inflammation's role in worse COVID-19 outcomes, which predominantly affects the elderly.[55]

Dysregulation of miR-21-5p, miR-146a, miR-126-3p, miR-144, and miR-155 are the most significant microRNAs that can be involved in the pathogenesis of COVID-19. Therefore, these microRNAs could be used as potential diagnostic biomarkers. miR-146a is a potential biomarker for disease severity. Due to their involvement in viral replication, immune response, inflammation, cardiovascular dysfunction, hyperactivation of human microglia, and bone remodeling, microRNAs may also serve as predictors of mortality, disease stage, and therapeutic targets for COVID-19. More primary research is needed to show how miRNAs function as COVID-19 biomarkers.[56-57]

IL-18 may offer a defense against COVID-19.[45] The function of IL-18 is when infectious illnesses are present.In animal models, it has been determined that interleukin-18 plays a role in cytokine storm and leads to endotoxin shock and lung mucosal damage.. In this study, the expression levels of IL-18 in the severe group increased significantly compared to the mild group. Moreover, the expression levels of IL-18 in the older age group (>60 years old) increased significantly compared to the younger group (≤60 years old). The expression of IL-18 and RANKL genes differed significantly among gender groups. However, there was no discernible difference in the two groups RANKL gene expression levels (Between mild and severe group).

Yasuda et al,[58] showed an involvement of IL-18 in the development of COVID-19 cytokine storm. This could offer an explanation on some of the illness's potentially fatal consequences. And how IL-18 inhibition could potentially treat COVID-19. In the early stages of infections, IL-18 appears protective, triggering a proper response against the pathogen. However, IL-18 inhibition may be a treatment strategy for COVID-19 despite the lack of information on the involvement of IL-18 in SARS-CoV-2.[58]

In this study, we compared the demographic and clinical characteristics of patients in relation with the severity of COVID-19. Smoking history showed a strong correlation with COVID-19 severity. The other elements were also unimportant regarding clinical symptoms, and the cough was only related to the severity of the illness.[59-60] Li et al, studied how clinical manifestations were connected to a more severe and critical coronavirus pneumonia: there were 83 COVID-19 patients, of which 58 had moderate symptoms, and 25 had severe ones. Their findings showed that older people had underlying diseases, sputum, cough, shortness of breath, and chest pain compared to those with milder symptoms.[61]

According to the results of different studies, we hypothesized that increasing the expression of miR-146a as a therapeutic target might eventually cause or decrease the production of IL-18 and RANKL. So, as we had anticipated, the results of numerous research confirmed what our study had found, but for RANKL, they were different. In this study, the severity of COVID-19 was correlated with a considerable decline in miR-146a expression. It could be preferable to undertake more thorough and precise analyses to develop diagnostic and therapeutic procedures based on these genes. Our study's focus gene, MiR-155, is also involved in respiratory illnesses. The findings lead us to recommend further research into additional microRNAs, such as mir-155, mir-21, mir-326 that are involved in the lung and the development of inflammation.

Possible new factors in respiratory disorders could be seen in future research. The study of miR-146a and many other genes involved in lung diseases has been further enhanced by the discovery that miR-146a-5p regulates IL-18 and acts as a critical component of multiple upstream signaling pathways.[59]

STATEMENT OF ETHICS

This study has been approved by ethics committee of Urmia University of Medical Sciences(IR.UMSU.REC.1400.384).

FUNDING

This study was scientifically (not financially) approved by the Urmia University of Medical Sciences (grant number 11180) and was done at the Department of Clinical Biochemistry.

CONFLICT OF INTEREST

The authors affirm that they have no known financial or interpersonal conflicts that may have looked to have influenced the research presented in this study.

ACKNOWLEDGEMENTS

The authors wish to thank Urmia University of medical sciences and all Shahid Taleghani Hospital staff members in Urmia for helping in our research. Additionally, the authors wish to thank the patients and healthy volunteers who willingly participated in the study.

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