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Association between MicroRNA-155 Expression and Pro inflammatory Cytokines in Severe Covid-19 Patients

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ABSTRACT
Covid-19 is a respiratory disease similar to pneumonia that results from an infection with SARS-CoV-2, a recently identified virus that became a global pandemic in 2020. The severe cases of the disease show a cytokine storm, which is excessive, uncontrolled production of pro inflammatory cytokines. MicroRNA-155 is an epigenetic microRNA that has the ability to control pro-inflammatory responses in many diseases. We aim to determine the relationship between microRNA-155 expression and some cytokines (interleukin-6, interleukin-8, and interleukin-1β) in severe covid-19 cases. A case-control study of 235 samples was collected from 120 patients with severe covid-19 and 115 of mild covid-19 cases and healthy individuals of different sexes and ages. After RNA extraction and conversion to cDNA, RT-PCR was performed on both studied groups. The levels of studied interleukins were determined for 50 severe covid-19 patients and 40 control individuals (mild and healthy). A substantial elevation in the expression of microRNA-155 was seen in severe COVID-19 patients compared to its expression in the control group consisting of mild cases and healthy individuals. Interleukin-6, interleukin-8, and interleukin-1β also show elevated levels in severe covid-19 serum than control individuals. This investigation revealed a strong and statistically significant correlation between the expression of microRNA-155 and interleukin-6, as well as a substantial correlation between the expression of microRNA-155 and interleukin-1β. MicroRNA-155 might have a role in inducing some pro-inflammatory interleukins that leads to cytokine storm.

Keywords: microRNA-155, cytokine storm, interleukin, covid-19, SARS-CoV-2

INTRODUCTION

One of the most important viral diseases in the last five years is covid-19. The disease progressed through SARS-Cov-2 infection [1]. The virus has considered as a severe pandemic since 2019, when it was first discovered [2]. This Coronavirus infect the upper and lower respiratory system in humans. The clinical manifestations of the host are mostly determined by its immunological responses. The symptoms can range from mild to moderate, including fever, cough, impairment of smell and taste, and gastrointestinal complaints. In some cases, these symptoms may advance to more severe complications such as pneumonia and even death [3,4].

The severity of SARS-Cov-2 depend on a cytokine storm progression that takes place as an aftermath [5]. Cytokine storm was marked by the uncontrolled, massive yield of several pro inflammatory markers, both locally and systemically. These markers, which include interleukin 6, interleukin-1β, Interleukin-17, interleukin-8, and TNF-α, have a prominent role in increasing the severity of the disorder and the risk of morbidity [6-8].

Among more than ten thousand human microRNAs that have been identified, microRNA 155 was quite interested. It is an example of a typical multifunctional microRNA. According to a research, microRNA-155 regulates Several physiological and pathological processes, including as haematopoiesis, heart disease, inflammatory processes, immunology, and cancer, are involved. [9]. Growing research has also suggested that microRNA-155 has a role in a number of viral illnesses, including respiratory, circulatory, and neuroviral infections [10].

Recent studies begin to explore the role of microRNA-155 during immune response [11]. MicroRNA-155 may control the immune response and inflammation through inducing the synthesis of many pro-inflammatory interleukins [12,13]. During the viral infection, microRNa 155 affects both innate and adaptive responses. It is associated with altered expression during infections caused by viruses like human rhinovirus (hRV), influenza virus (IV), human metapneumovirus (hMPV), human coronavirus (HcoV), and respiratory syncytial virus

(RSV). Specifically, microRNA-155 contributes to maintaining the epithelial cell barrier in the respiratory tract and regulates anti-viral host defense [14,15].
Accordingly, the main goal of the study is to demonstrate the association between microRNA-155 expression and excessive release of cytokines that promote inflammation occurs in severe cases of COVID-19 and lead to cytokine storm and eventually death.

MATERIALS AND METHODS

Study Design

A case-control study was worked during October 2022 to June 2023 to assess SARS-CoV 2 infections collected from Ibn Al-Khatteeb Hospital in Baghdad, Iraq. The study proposal and protocol (Reference: CSEC/0922/0083) were approved by the Ethical Committee of the Department of Biology in the University of Baghdad and the Iraqi Ministry of Health. All participants provided written informed consent. The research was conducted in compliance with the Code of Ethics of the World Medical Association, namely the Declaration of Helsinki. The present study comprised a sample of 120 hospitalized persons with severe cases of covid-19, as well as 115 individuals with mild cases of covid-19 and apparently healthy individuals of varying genders and ages, who were used for the control group. The mild cases were chosen depending on mild symptoms of covid-19 and normal SPO2 levels.

Sampling

This study included two types of samples (blood and sputum). Five milliliters of blood taken from vein was drawn under hygienic conditions from both severe covid-19 patients and control group (mild cases of covid-19 and healthy individuals). The blood was used for immunological assays (five milliliters aliquot was dispensed in a gel tube for serum separation). Nasopharyngeal swabs were also collected from both groups by swabbing the area of the pharynx. Swabs were repeated and agitated to confirm the presence of epithelial cells in the sample. Then, samples were centrifuged at 5000 g/min for 5 min. The supernatant was omitted. The solution used for microRNA-155 expression determination; swabs were placed in Trizol reagent to protect the RNA in the sample before applying qPCR procedure.

Molecular Assays

After sample collection, the ReliaPrep Viral Total Nucleic Acid Purification Kit (Promega) was used to extract and purify RNA from sputum and blood samples. An aliquot of sample 300 µl was added to 600 µl of TRIZOL reagent in a 1.5 ml micro-centrifuge tube, the mixture homogenized, and incubated for 10 minutes at room temperature for complete dissociation of the nucleoprotein complex. Proteinase K Solution (20 µl) and Cell Lysis Buffer (200 µl) were added, respectively, to each 1.5 ml tube. Samples were incubated at 56°C for 10 minutes using a heat block device. Then, 250µl of 100% isopropanol was added and placed in a vortex for ten seconds. Then, transferred to the ReliaPrep Binding Column. The centrifuge take a place for 1 minute at maximum speed (1200 rpm). A volume of 500µl wash solution was transferred and centrifuged for three minutes (1200 rpm). The remains are discarded. The last step was repeated two times for three washes achievement. The elution step requires adding 60 µl of nuclease-free water to the column, followed by centrifugation for 1 minute (1200 rpm). The silica tube (ReliaPrepTM Binding Column) was discarded, and the elutes were saved in 1.5-ml tubes for processing. After the extraction of RNA from the samples, the concentration of RNA obtained is calculated using a quantum fluorometer device. A volume of 1–20µl of unknown sample was added to 200µl of QuantiFluor RNA Dye working solution in 0.5 ml PCR tubes. Then, the tube was placed in a vortex and protected from light. The fluorescence of the unknown sample was measured using the Quantum Fluorometer. The number displayed represents the concentration of the original sample.
In the present study, microRNA-155was targeted by itself but not the gene that expresses it. The sequence of microRNA-155 was obtained and targeted using three types of primers. All the primers for micoRNA-155 expression were delivered from Macrogen, Korea. They include: forward primer, adaptor primer, and universal reverse primer. The design of the primers presented in table 1. The endogenous control that was used in the present study was the U6 reference gene (table 2).

Table (1): The sequences of microRNA-155 primers

Primer	Sequence
Stemloop primer (adaptor)	3′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACAACCCC -5′
Forward primer	3′- CGCGCGTTAATGCTAATC-5′

Universal reverse primer	5′-CCA GTG CAG GGT CCG AGG TA-3′

Table (2): The sequences of U6 reference gene primers [16]

Primer	Sequence
Forward Primer	5'-GCTTCGGCAGCACATATACTAAAAT-3'
Reverse Primer	5'- CGCTTCACGAATTTGCGTGTCAT-3'

Luna-Script RT Super-Mix Kit (Biolabs, England) was used for the conversion of extracted RNA to cDNA. The lyophilized primers were liquefied by adding 200 µl of free nuclease water to prepare the stock solution. The working solution was prepared at a ratio of 1:10 of stock solution to free nuclease water. The reaction mixture was placed on ice. After dispensing aliquots of this mixture into the microtubes, the RNA sample was added. The reaction mixture was incubated at 25 °C for 2 minutes (primary annealing), 55 °C for 15 minutes (reverse transcriptase, cDNA synthesis), followed by 90 °C for 1 minute (heat inactivation) for one cycle only.
Sacycler, Saccac, Italy: Quantitative RT-PCR (qPCR) technology was carried out using the BioLabs, England-based LunaScript RT Master Mix Kit (5X). The RT-PCR mixture included 0.5 µl of each forward primer and reverse primer and 10 µl of the master mix. After adding 5 µl of cDNA from each sample, primers and reverse primers, 4 µl of nuclease-free water was also added to bring the volume up to 20 µl. The endogenous control was the U6 gene. A qPCR was run for one minute at 95°C to activate the polymerase. This was followed by 45 cycles of denaturing the double-stranded cDNA for 15 seconds at 95°C and annealing for 20 seconds at 60°C with channel scanning. Melting curve analysis was used to examine the separation properties of double-stranded cDNA during cycles with increasing denaturing TM. The Ct value of microRNA-155 was normalized to the U6 reference gene, and the expression of microRNA-155 was evaluated using the relative quantitative technique, employing the comparative Ct formula and the 2^-ΔΔCT analysis.

Serological Assays
Serological assays include the determination of interleukin-6, interleukin-8, and interleukin 1β in blood samples included in this study using the Human Interleukin-6 (IL-6) ELISA kit (96 test)/ (BT LAB Bioassay Technology Laboratory, China), the Human Interleukin-8 (IL-8) and the Human Interleukin-1β (IL-1β) ELISA kit (96 test)/ (YL Biont, Shanghai). This sandwich ELISA kit is for the accurate quantitative detection of human interleukins in serum. Obligations listed by the manufacturers were applied.

Statistical analysis
The raw data of the present study were input in the computer and analyzed using SPSS program of the version 23. It includes some known statistical parameters like mean, St. deviation, St. error, and T-test. The significance of the differences depends on equals or less than 0.05. Less than 0.01 was written as highly significant. In addition, the correlation coefficient (r) was determined between microRNA-155 expression and the levels of pro-inflammatory cytokines. These analyses were carried out through the statistical package SPSS version 23.

RESULTS
The individuals included in this research were selected randomly. Severe covid-19 patients' group was 56.4% males and 43.6% females with a mean age of 40.3 years, while the control group of healthy and mild cases of covid-19 were 52.2% males and 47.8% females with a mean age of 37.8 years. The ages of the studied individuals were divided into three groups (group1: from 20 to 40 years, group 2: from 41to 60 years, and group 3: older than 60 years). Non-significant differences ($P > 0.05$) between the sexes and ages of the studied groups ($P = 0.497$; $P = 0.067$), respectively

MicroRNA-155 Expression
A real-time PCR procedure was applied to nasopharyngeal samples from patients and control group to evaluate the expression of microrna-155 in both groups. U6 gene was used in the procedure for standardization of Ct value (endogenous control). The level of microRNA-155 was determined using the relative quantitative approach, which involved calculating the expression using the comparative Ct calculation and analyzing the fold change using the 2^-ΔΔCT method. The results of RT-PCR revealed that microRNA-155 expression in severe covid-19 patients were higher than its expression in control individuals of mild covid-19 cases and healthy individuals. The mean fold number of microRNA-155 in covid-19 patients was 4.167970, while in control group

was only 1.631186. Highly significant differences between the expression of microRNA 155 in severe covid-19 patients and control individuals (P = 0.00, P <0.01) (table 3).

Table 3: MicroRNA-155 expression in severe covid-19 cases and mild and healthy control group

Studied groups		N	Mean	Std. Deviation	Std. Error	P - value
microRNA-155	Controls	115	31.991	1.6709	.1558	P = 0.006 Highly significant (P<0.01)
	Severe patients	120	30.692	4.7642	.4349	
	Total	235				
	Severe patients	120	18.496	3.3351	.3045	
	Total	235				
	Severe patients	120	-.483	3.8381	.3504	
	Total	235				
Fold number	Controls	115	1.631186	1.6556037	.1543858	P = 0.00 Highly significant (P<0.01)
	Severe patients	120	4.167970	5.7691001	.5266444	
	Total	235				

Pro-inflammatory cytokines levels

An enzyme-linked immunosorbent assay (ELISA) was applied to 90 randomly selected serum samples present in our study, these included fifty (50) severe covid-19 group and forty (40) individuals from control group of mild and healthy individuals. Results of serum levels show that interleukin-6 was expressed in severe covid-19 patients higher than the control group. The mean of interleukin-6 serum levels in severe covid-19 patients was 34.6 (ng/l) while the mean of control group was 4.6 (ng/l). Results from the ELISA technique showed highly significant differences between the interluekine-6 levels of severe covid-19 patients and the control group (P <0.01) (figure 3).

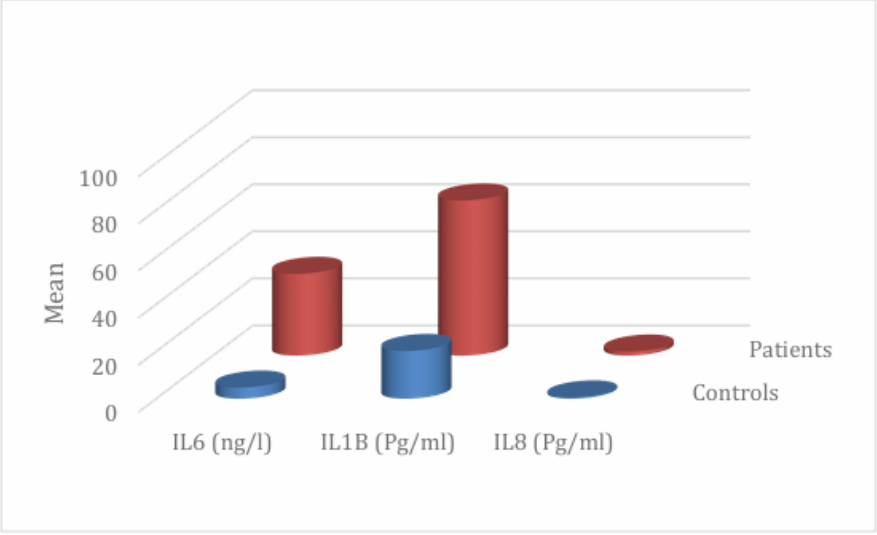

Figure 1: Interleukins' levels in severe covid-19 patients and control group

The correlation between microrna-155 expression and pro-inflammatory cytokines

Pearson Correlation test (r) was performed between the expression of microRNA-155 in severe covid-19 patients and levels of cytokines that promote inflammation (interleukin-6, interleukin-8, and interleukin-1β). Results revealed a positive correlation between microRNA-155 expression in severe covid-19 patients and interleukin-6 (r=0.578; P–value=0.00, P <0.01) (figure 3). Furthermore, there was a direct relationship observed between the expression of microRNA- 155 and the levels of interleukin-1β. in severe covid-19 patients (r=0.394; P-vlaue= 0.005, P<0.01. The results also revealed a negative non-significant relationship between

microRNA-155 expression in severe covid-19 patients and interleukin-8 levels (0.247; P-vlaue= 0.081), (P>0.05) (table 4).

Table 4: Pearson Correlation between the studied parameters

Pro-inflammatory cytokines	Pearson Correlation	microRNA-155 expression
Interleukin-6 (ng/l)	r	0.578
	P - Value	0.00
	Significance	Highly significant
Interleukin-8 (Pg/ml)	r	0.247
	P - Value	0.081
	Significance	Non-significant
Interleukin-1β (Pg/ml)	r	0.394
	P - Value	0.005
	Significance	Significant

DISCUSSION

In March 2020, the global outbreak of covid-19 was officially classified as a pandemic. The disease's clinical manifestations might be asymptomatic, minor symptoms, or severe and urgent [17]. Many studies confirmed that people of higher ages show more hospitalized cases than those of lower ages [18,19]. These studies are consistent with our findings, which declare that the mean age of male severe cases was 40.3 and that of females was 37.8.

This study additionally examines the levels of three significant pro-inflammatory interleukins (interleukin-6, interleukin-8, and interleukin-1β) in individuals with severe instances of covid-19 and a group of control subjects. Elevated amounts of interleukin-6, interleukin-8, and interleukin-1β were seen in severe instances of COVID-19 compared to both mild cases and healthy individuals. Prior articles have indicated elevated levels of interleukins in severe cases of covid-19 [20,21].

Articles reported that interleukin-1β was highly expressed during auto-inflammatory diseases when the control of cytokine expression was abused [22]. Interleukin-1β is considered as the leader of pro-inflammatory activity during lung injury, which promote cytokine storms [23]. In critical cases of covid-19, interleukin-6 gene activation was documented to be elevated in SARS-Cov-2-infected blood [24]. In addition, the level of interleukin-6 quantity was confirmed to be raised during the SARS infection and related with the severity of the disease [25]. It is considered as the main player in the induction of cytokine storm through many signaling pathways in critically ill patients [26]. A huge cohort study research revealed that scoring high interleukin 6 levels was connected to death and clinical satisfaction of the persons [27,28]. In November 2022, a researcher suggested that interleukin-6 control could be invested as a promising therapy for covid-19 and rescue patients from hyper cytokine expression [29].

Many other researches demonstrated that patients with a critical or severe SARS-CoV-2 infection show considerably larger expression of inflammatory cytokines than mild covid-19 cases, especially interleukin-8, interleukin-6, and interleukin-1β [30,31]. Researches of patients with critical lung infections reveal that the acute-response cytokine is interleukin-1β. Interleukin 8 which is considered as chemotactic chemokine appears in the early minutes of infection, followed by elevation in interleukin-6, which is induced by interleukin-1β [32].

We observed a notable increase in the expression of microRNA-155 in severe instances of covid-19 compared to mild cases and healthy individuals. This conclusion agrees with many findings dealing with this topic. Garg etal. (2022) confirmed that microRNA-155 was highly expressed in critically ill covid-19 cases. The control group shows significantly lower expression than the severe cases [33]. Another study dealing with several microRNAs in SARS-Cov-2 infected persons demonstrates that micoRNA-155 can be used for the diagnosis of the disease using ROC analysis because of its high sensitivity and specificity which agrees with our findings [34].

Studies examined the levels of microRNA-155 linked to lung injury, which is a crucial state of covid-19, and inflammation, given the potential of microRNAs as clinical indicators and potential therapy [35,36]. Previous studies demonstrated that microRNA-155 controls the expression of genes linked to inflammation. These genes include SHIP1 and SOCS. Macrophages rely on them to regulate the inflammatory response. These two genes have been found to be targeted by microRNA-155 [37]. MicroRNA-155 was highly expressed in severe covid-19 cases. The pro-inflammatory microRNA-155 might have an impact on inducing pro-inflammation.

Severe cases of covid-19 had heightened levels of pro-inflammatory cytokines, specifically interleukin-6, interleukin-1β, and interleukin-8. Furthermore, a direct association was observed between individuals with

severe cases of covid-19 and microRNA-155. The expression of microRNA-155 shown a strong correlation with interleukin-6 and interleukin-1β, which are pivotal cytokines in driving the advancement of cytokine storm in severe instances of covid-19. MicroRNA-155 potentially influences the cytokine storm by regulating the amounts of interleukins.

CONCLUSION

Pro-inflammatory cytokines that lead to cytokine storm (interleukin-1β and interleukin-6 and interleukin-8) show considerably higher quantities in severe diseased patients of covid-19 than mild and healthy individuals. In addition, microRNA-155 was overexpressed in severe cases of covid-19. MicroRNA-155 demonstrates a positive relationship with the pro-inflammatory cytokines (interleukin-6 and interleukin-1β). So, microRNA-155 may regulates the progression of cytokine storm.

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