Original Article

Gene Expression of TNF-α among Iraqi COVID-19 Patients with a Different Severity Status

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Abstract

Background: Coronavirus disease 2019 (COVID-19) individuals with varied severity group are affected by the cytokine storm brought on by SARS-CoV2 infection, which is a significant cause of acute respiratory distress syndrome. **Objective:** The goal of the current study was to examine tumor necrosis factor (TNF-α) gene expression in COVID-19 at various severity levels. **Materials and Methods:** The study includes 140 divided into 105 COVID-19-positive patients (35 for each mild, moderate, and severe group) and 35 COVID-19-negative healthy people as control. COVID-19 positive patients had 46 males and 59 females, while COVID-19-negative healthy people included 16 males and 19 females. The separation of peripheral blood mononuclear cells (PBMC) was achieved using Ficoll, and then Ribonucleic acid was extracted and converted to cDNA and the gene expression using glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene. **Results:** The results revealed non-significant differences at $P < 0.05$ in age among different COVID-19 groups and control (*F*-ratio value is 0.54257 and *P*-value is 0.65397). The results revealed over-expression of TNF-α gene among COVID-19 patients and the relative quantification (fold change) (mean ± standard deviation) values were 6.542 ± 7.29, 5.740 ± 6.41, 7.306 ± 8.85, and 6.580 ± 6.47 for all, mild, moderate, and severe COVID-19 patients, respectively. One-way analysis of variance test relative quantification (fold change) TNF-α (mean ± standard deviation) for mild, moderate, and severe groups revealed non-significant at $P < 0.05$, the *F*-ratio value is 0.39889 and the *P*-value is 0.672109. **Conclusion:** The present study concludes upregulation of TNF-α gene in PBMC of COVID-19-positive patients without significant differences among different severity groups.

Keywords: COVID-19, gene expression, severity, TNF-α, upregulation

Introduction

Coronavirus disease 2019 (COVID-19) is the third coronavirus to cause severe respiratory illness in humans, also known as SARS-CoV-2.[1,2] The World Health Organization declared this to be a pandemic in March 2020, and it has had a significant negative impact on both health impacts and economic conditions in the world.[3,4] The COVID-19 outbreak has presented significant difficulties for the fields of public health, research, and medicine.[5] The capacity of the SARS-causing CoV-2 to enter cells depends on both the priming of S proteins by the host membrane serine protease TMPRSS2 and the binding of S proteins covering the virion's surface to the cellular ACE2 receptor. SARS-CoV-2 causes the generation of inflammatory cytokines after infecting respiratory epithelial cells.[6,7] One of the most significant researches to determine the most effective treatment modalities is the COVID-19 cytokine trial. Because saving patients with severe COVID-19 may depend on preventing and reducing the cytokine storm.[8,9] Acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndromes may also arise in COVID-19 individuals with severe respiratory disease.[10,11]

The term "cytokine storm" describes the excessive production of inflammatory cytokines from a number

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of organs and cell types (mainly immune cells). It is, in essence, an ongoing, extremely severe activation and attack of the immune system.[12] tumor necrosis factor (TNF-α) is a COVID-19 cytokine storm pro-inflammatory cytokine that is crucial for multiple organ failure, systemic inflammation, and ARDS.[13] The severity of the disease was found to be correlated with cytokine storm or cytokine release syndrome, which is indicated by higher TNF-α.[14,15]

The aim of this study was to examine TNF-α gene expression in COVID-19 at various severity levels.

Materials and Methods

Sample collection

The 140 blood samples were collected including 105 patients (COVID-19 positive) and 35 healthy control (COVID-19 negative) from COVID-19 healing center at medical city department and nursing home private hospital of Baghdad, Iraq during a period from November 2021 to June 2022. All COVID-19 positive patients were positive for real-time polymerase chain reaction (PCR) for SARS-CoV2. The Blood samples were taken to separate peripheral blood mononuclear cell (PBMC) and transferred to –20°C. Project No: M220501, Date: 18/5/2022.

PBMC separation

Ficoll density gradient method was used to separate PBMC according to Grievink *et al.*[16] Briefly, 10 mL of peripheral blood was collected with the anticoagulant. The blood was added to the conical Ficoll tube after being diluted to a Phosphate buffer saline-equivalent ratio. The two layers were then separated and centrifuged at 2500 rpm for approximately 25–20 min. Ribonucleic acid (RNA) was extracted from isolated mononuclear cells after they had been washed with Phosphate buffer saline.

RNA extraction

EX6101-RNX plus solution for total RNA isolation (SinaClon, Iran) was used to extract the total RNA from PBMC in accordance with the manufacturer's instructions. Briefly, at 4°C, samples that had been prepared in the preceding stage were centrifuged at 1000 rpm for 10 min. The samples were mixed with 1 mL of a cold RNX-Plus solution, vortexed for 30 s, and then left to sit at room temperature for 5 min. Each sample was mixed with 200 mL of chloroform and stirred for 15 s before samples were incubated for 5 min on ice. Following that, the samples were centrifuged at 4°C for 4 min at 12,000 rpm. Each sample's liquid phase was transferred to an RNase-free tube, and an equivalent volume of this phase was then mixed with isopropanol. The samples were incubated for 15 min on ice. The samples were centrifuged once again for 15 min at 4°C using 12,000 rpm. After discarding the supernatant, each sample received 1 mL of 70% ethanol, which was then a vortex. The supernatant was entirely removed after 8 min of centrifugation at 7500 rpm and 4°C, and the precipitate was then dissolved in 50 L of RNase/DNase-free water for cDNA synthesis.

Quantitative analysis of extracted RNA

After RNA extraction, NanoDrop (NP80 Implen, Germany) device was used to quantify it. In this way, 1–2 µL of the sample was placed in the mentioned device and the samples were read at 260/280 and 260/230 wavelengths.[17]

cDNA synthesis SYBR green real-time PCR reaction

According to earlier investigations, specific primers for the genes encoding TNF-, interferon, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were chosen.[18] Following that, the primer combination was validated using the Blast tool.[19,20] Table 1 lists the primers that were used in the current study. The following ingredients were added to a mixture: 1 µL of oligo dT, 1 µL of dNTP, 3 µL of nuclease-free water, and 5 µL of total RNA. Following a 5-minute incubation period at 65 °C, the mixture was chilled for 2 min and then centrifuged. The following elements were added to the previously described combination: 7 µL of nuclease-free water, 1 µL of M-MulV RT enzyme, and 2 µL of M-Mul V buffer. Next, the mixture was incubated for 5 min at 85°C after 60 min at 42°C. The completed product was stored at –70°C after the real-time PCR experiment was complete.

Gene expression by SYBR green real-time PCR

Using the AMPLIQON kit, the device (Rotor-Gene 6000 Corbett) carried out a real-time RT PCR reaction. The final volume of each reaction was calculated to be 25 µL after adding 12 µL of Masterpix AMPLIQON, 2 µL of cDNA (about 100 ng), 1 µL of particular gene primers (200 nM), and 7.5 µL of water without RNase and DNase. Then the device was subjected to a Real-time PCR thermal program in two stages, the first stage (denaturation) lasting 15 min for one cycle and the second stage (annealing and polymerization) lasting 40 cycles, with each cycle lasting 40 cycles at 95°C for 15 s, 60°C for 25 s, and 72°C for 20 s.[21,22]

Real-time PCR data analysis

After the reaction is completed, the device displays information; the most important of which is the reaction

Table 1: Primer used for this study

Gene	Sequence 5′-3′	References
GAPDH	F: TTCACCACCATGGAGAAGGC	[18]
	R: GGCATGGACTGTGGTCATGA	
TNF-α	F: CAGACCCTCACACTCACAAAC	[18]
	R: CAGCCTTGTCCCTTGAAGAGA	

TNF-α, tumor necrosis factor

progression curve and the crossing of the threshold. The gene expression ratio in both groups was determined using the delta-delta Ct (ct) computational approach after all Cts for the TNF-α gene and GAPDH for COVID-19 positive and healthy controls were obtained. The relative rate of increase in the expression of the desired gene in COVID-19 patients and healthy individuals is then computed using the equation relative quantification (RQ) = 2-Ct.[23,24]

Statistical analysis

The Livak and Schmittgen equation (RQ = 2-Ct) was used to calculate gene expression.[25] analysis of variance analysis was performed to compare groups using one (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, CA).

Ethical approval

The research related to human use has been complied with all the relevant national regulations and institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. The project no. M220501 was approved at 18/05/2022.

Results

Thirty-five healthy controls and 105 COVID-19 patients (35 each for the mild, moderate, and severe categories) were included in the study (COVID-19 negative). The research indicates no age differences between the various COVID-19 groups and the control group at *P* 0.05 (*F*-ratio value is 0.54257 and *P*-value is 0.65397) [Tables 2 and 3]. The results of the melting curve for TNF-α and GAPDH primers show that all primers act specifically and only one peak with a specific melting temperature confirms that the primers used are single products [Figures 1 and 2].

Table 2: Age of COVID-19 groups

Group	*N*	Age (years) Mean ± standard deviation
Mild COVID-19	35	58.742±14.382
Moderate COVID-19	35	58.857±20.045
Sever COVID-19	35	54.542±17.324
Control (non-COVID-19)	35	56.171±15.010

Table 3: One way analysis of variance test age (mean ± standard deviation) for COVID-19 groups

Source	SS	Df	MS	*F*-ratio	*P*-value
Between-groups	461.5071	3	153.8357	F = 0.54257	0.65397
Within-groups	38560.6286	136	283.534		
Total	39022.1357	139			

The *F*-ratio value is 0.54257. The *P*-value is 0.65397. The result is not significant at $P < 0.05$

Figure 3 shows the distribution of ΔCt TNF-α and RQ value among COVID-19 groups and control. When compared to healthy controls (non-COVID-19), the results of gene expression show that TNF- genes are overexpressed in COVID-19 patients. The relative quantification (fold change) (mean ± standard deviation) was 6.5427.29, 5.7406.41, 7.3068.85, and 6.5806.47 for all, mild, moderate, and severe COVID-19 patients, respectively [Table 4]. The results of the one-way analysis of variance test RQ

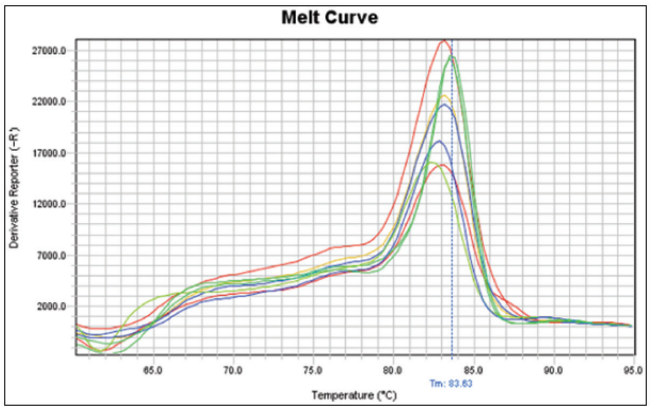

Figure 1: Melt curve for GAPDH showing one product has been amplified

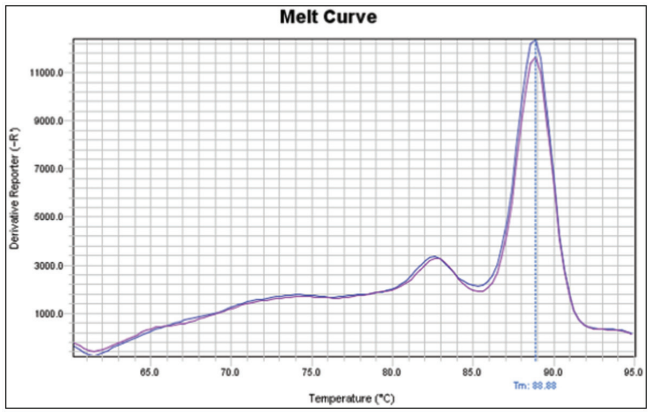

Figure 2: Melt curve for tumor necrosis factor-α showing one product has been amplified

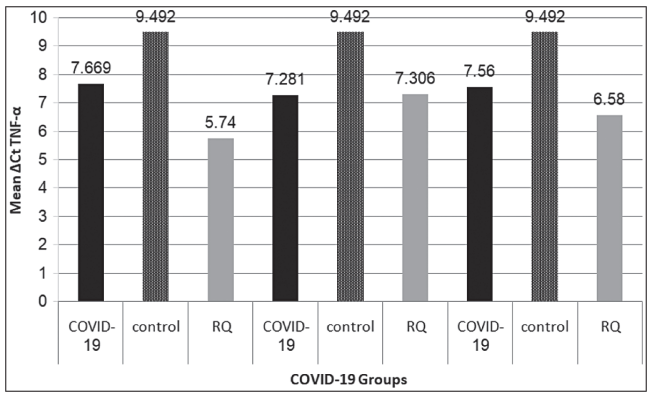

Figure 3: Distribution of ΔCt tumor necrosis factor-α and relative quantification value among COVID-19 groups

Table 4: Relative quantification (fold) change for tumor necrosis factor-α among different severity groups

Patient group	Relative quantification (fold change) Tumor necrosis factor-α (Mean ± standard deviation)
All COVID-19 (n = 105)	6.542 ± 7.29
Mild COVID-19 (n = 35)	5.740 ± 6.41
Moderate COVID-19 (n = 35)	7.306 ± 8.85
Sever COVID-19 (n = 35)	6.580 ± 6.47

Table 5: One way analysis of variance test relative quantification (fold change) tumor necrosis factor-α (mean ± standard deviation) for mild, moderate and sever groups

Source	SS	Df	MS	*F*-ratio	*P*-value
Between-groups	42.9824	2	21.4912	F = 0.39889	0. 672109
Within-groups	5495.5639	102	53.8781		
Total	5538.5463	104			

The *F*-ratio value is 0.39889. The *P*-value is 0.672109. The result is not significant at $P < 0.05$

(fold change) TNF-α (mean ± standard deviation) for mild, moderate and severe groups revealed non-significant at $P < 0.05$, the *F*-ratio value is 0.39889 and the *P*-value is 0.672109 [Table 5].

Discussion

The current study shows that COVID-19 patients have higher levels of TNF- than healthy controls. The findings were in line with other investigations that discovered patients with COVID-19 had higher levels of TNF-α than adults without the disease.[26,27] Immune cells such as macrophages, NK cells, T cells, and dendritic cells are thought to become activated as a result of SARS-CoV-2 infection. Due to the activation of many inflammatory pathways, including the TNF-, JAK/STAT signaling, and toll-like receptor pathways, pro-inflammatory cytokines were produced.[28,29] TNF-α is a key pleiotropic facilitator of both acute and chronic systemic inflammatory responses. It also plays a role in a number of physiological processes, including the immune system's homeostasis, anti-tumor responses, and inflammation management.[30,31]

TNF-α expression, which is markedly higher in intensive care unit patients, is crucial in determining how severe the COVID-19 cytokine storm will be. Ang-II has the capacity to stimulate ADAM-17's enzymatic activity, which raises TNF levels and causes the release of numerous other epidermal growth factors, increasing NF-B activity.[32,33] During lung injury, it has been discovered that TNF-α expression is elevated, and this causes a variety of biological reactions to be triggered in an effort to control the anomaly by increasing the expression of various additional inflammatory mediators.[34] Hyaluronan-synthase-2 is induced by TNF-α in the lungs of COVID-19 patients, specifically in EpCAM+ lung alveolar epithelium, CD31+ lung alveolar endothelium, and fibroblasts. A major contributor to the fluid inflow in the lung alveoli that leads to deoxygenation and ventilator admission is hyaluronan.[35]

The results of the present study were in agreement with those of Merza *et al*. from Erbil, Iraq, discovered that the concentrations of IL-1 and TNF- were not differ significantly among various severity groups of COVID-19 patients. The present study revealed non-significant differences in the expression of TNF- among mild, moderate, and severe COVID-19 groups of patients.[36,37] Additionally, Chen *et al*. discovered that among patients with severe symptoms, the concentration of TNF-, IL-1, IL-8, and IL-10 was not changed.[38] Given that elevated TNF- was dynamically correlated with disease severity as reported by numerous studies, it is possible that differences in TNF- expression between mild, moderate, and severe groups of COVID-19 patients did not reach statistical significance. Alternatively, the small sample size or inaccurate clinical classification of severity groups may also contribute to this situation.[39,40]

Conclusion

The present study concludes upregulation of TNF-α gene in PBMC of COVID-19 positive patients without significant differences among different severity groups.

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Conflicts of interest

There are no conflicts of interest.

Informed consent statement

Informed consent has been obtained from all individuals included in this study.

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