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Assessment of Alterations in the Expression of *P53* and *Cyclin-D* Genes in COVID-19 Patients Before and After Remdesivir Treatment

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ABSTRACT

Coronavirus disease-19 (COVID-19), caused by the new coronavirus severe acute respiratory syndrome coronavirus 2 **(**SARS-CoV-2), has become a worldwide pandemic. The disease primarily spreads through respiratory droplets and manifests with a wide range of symptoms, from mild respiratory illness to severe pneumonia, acute respiratory distress syndrome (ARDS), and multi-organ failure. The virus manipulates the host cell cycle to create a favorable environment for its replication and propagation. One of the key regulators of the cell cycle is cyclin-D, a protein essential for the G1 to S phase transition in the cell cycle, and P53, a critical tumor suppressor and regulator of cell cycle arrest and apoptosis. Therapeutic strategies, including antiviral drugs like Remdesivir, have shown varying efficacy in managing symptoms and reducing mortality. This study obtained blood samples from 30 COVID-19 patients before and after Remdesivir treatment and 20 healthy individuals. RNA was isolated, and cDNA was subsequently synthesized. The expression levels of the *p53* and *cyclin-D* genes were then assessed using Real-time PCR. The results demonstrate that c*yclin-D* expression increased 9 times in COVID-19 patients compared to the control group ($P< 0.001$), which remained unaffected by Remdesivir treatment. Conversely, *p53* gene expression was reduced by 50% in the patient group compared to the control group ($P< 0.05$). Treatment with Remdesivir increased *P53* gene expression twofold compared to the control group ($P< 0.001$). Furthermore, *P53* gene expression positively correlated with CRP (C Reactive Protein) levels in both the control and patient groups ($P< 0.01$). The study's findings indicate that certain symptoms of COVID-19 may be linked to the virus's impact on crucial cell cycle genes, such as *cyclin-D* and *p53*. Remdesivir, by reducing inflammation and inhibiting viral replication, can help restore normal expression levels of these genes. This may support the therapeutic benefits of using Remdesivir in treating COVID-19.

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Introduction

COVID-19, a novel coronavirus disease in early 2020, emerged in Wuhan, China, where an outbreak of pneumonia cases of unknown origin was reported in December 2019 (Li *et al*., 2021; Muralidar *et al*., 2020). The causative agent was identified as an enveloped RNA beta coronavirus, later designated as SARS-CoV-2. This viral infection rapidly escalated into a global pandemic, profoundly impacting public health systems, economies, and daily life worldwide (Arthur-Mensah and Kyei, 2021; Malande, 2020; Jalali and Khoramipour, 2022).
The disease is primarily transmitted through respiratory droplets and presents a broad

spectrum of clinical symptoms (Lamers and Haagmans, 2022; Peiris *et al*., 2003). As of late 2020, millions of confirmed cases and fatalities have been documented globally, highlighting the virus's significant morbidity and mortality rates (Malande, 2020; Wang *et al*., 2020).

The most reported symptoms include fever, cough, fatigue, body aches, headache, and dyspnea. Common long-term effects include difficulty concentrating, memory loss, chest pain, hair loss, and ongoing respiratory issues (Carfì *et al*., 2020; Cirulli *et al*., 2020; Richard *et al*., 2023). Since the emergence of SARS-CoV-2 in late 2019, extensive research has been conducted on therapeutic interventions and markers of disease severity (Brodin, 2021). One of the complications of COVID-19 is hair loss and changes in the number of white blood cells, which can be related to the virus's effects on the cell proliferation cycle.

Studies have revealed enriched cell cycle-associated gene co-expression modules and differentially expressed proteins in COVID-19 patients, which correlate with disease severity (Prado *et al*., 2023). Cyclin D is a critical component of the cell cycle, particularly involved in regulating the transition from the G1 phase to the S phase. Cyclin-D proteins interact with cyclin-dependent kinases (CDKs), primarily CDK4 and CDK6, to form active complexes facilitating cell cycle progression into the S phase (Montalto and De Amicis, 2020). Research indicates that during infection, cyclin D1 and cyclin D3 are redistributed from the nucleus to the cytoplasm, followed by proteasomal degradation (Gupta and Mlcochova, 2022). Studies showed that increasing *cyclin-D* gene expression in COVID-19 patients can promote cell cycle progression, which may be advantageous for viral replication.

The p53 protein functions primarily as a transcription factor that regulates the expression of various genes involved in critical cellular processes, particularly in response to stress signals and tumor suppression. *p53* can induce cell cycle arrest, allowing time for DNA repair before a cell proceeds to divide (Chen, 2016). P53 activates the gene *p21*, which inhibits the cyclin-dependent kinases (CDKs) necessary for cell cycle progression (H. Wang *et al*., 2023). If DNA damage is irreparable, p53 triggers programmed cell death (apoptosis) to eliminate potentially cancerous cells. The expression of P53, a critical tumor suppressor, is significantly decreased during COVID-19 infection (Ma-Lauer *et al*., 2016; X. Wang *et al*., 2023). The p53 can influence the levels of pro-inflammatory cytokines and help keep inflammation in check, acting as a kind of brake on the immune response (Gudkov *et al*., 2011).

Antiviral drugs play a crucial role in treating COVID-19, with Remdesivir being the only antiviral approved by the EMA and FDA (Moreno *et al*., 2022). Other antivirals used include Molnupiravir, Ribavirin, Favipiravir, and lopinavir/ritonavir (Sydorenko, 2023). These drugs target various viral mechanisms, such as RNA polymerase inhibition and protease inhibition (Şimşek Yavuz and Ünal, 2020).

Remdesivir is a prodrug that, once inside the cell, is metabolized to its active form, remdesivir triphosphate (RTP). RTP acts as an adenosine triphosphate (ATP) analog, allowing it to be incorporated into the growing RNA chain during viral replication (Kokic *et al*., 2021). Remdesivir demonstrates a higher selectivity for incorporation into the viral RNA compared to natural nucleotides, making it a potent inhibitor of SARS-CoV-2 replication (Blair, 2023). Remdesivir has been approved for emergency use in treating COVID-19 and has effectively reduced recovery time for hospitalized patients. Clinical trials have shown that it can significantly benefit patients with moderate to severe symptoms, particularly when administered early in the course of infection (Blair, 2023). This study investigates gene expression changes of *cyclin-D* and *p53* in COVID-19 patients compared to healthy people. Furthermore, the correlation between these genes and blood factors will be assessed.

Materials and Methods

Ethical statement

This study followed medical ethical standards and received approval from the Ethics Committee of Masih Daneshgari Hospital. Informed consent was obtained in writing from all participants who met the eligibility criteria. The Ethical number is IR.SBMU.NRITLD.REC.1399.087.

Blood sample collection

Blood samples were collected from 30 patients aged 20 to 70 hospitalized with acute COVID-19 caused by the Omicron variant, which was diagnosed by PCR kit at Masih Daneshvari Hospital. Additionally, samples were taken from 20 healthy controls aged 20 to 70 who had not been infected with COVID-19 until then. All participants had no underlying health conditions such as diabetes, cancer, hypertension, or a history of heart disease or stroke. The COVID-19 patients received an initial dose of Remdesivir at 5 mg/kg on the first day, followed by a daily dose of 2.5 mg/kg from the second to the fifth day. Blood samples were collected before the first dose and again after the last dose of Remdesivir. Control samples were collected from healthy individuals. Some blood factors, such as white blood cell count (WBC) and C-reactive protein (CRP), were measured.

RNA extraction and cDNA synthesis

Total RNA was extracted from leukocytes in the blood samples using RiboEX (No. cat RiboEX302-001, GeneALL, Korea) according to the manufacturer's instructions. The integrity and size of the RNA were assessed using a 1% agarose gel, and RNA concentrations were measured spectrophotometrically with a BioPhotometer (Eppendorf, Hamburg, Germany). A 0.5 µg sample of RNA was reverse transcribed using oligo(dT) and random hexamer primers along with SuperScript II reverse transcriptase (Easy cDNA synthesis kit, cat. No. A101161, Pars Toos, Iran). The resulting cDNA was stored at -20°C.

Real-time quantitative PCR

Primer sequences were designed using Oligo 7 software. The specificity of the primers for the target genes was confirmed using the Basic Local Alignment Search Tool (BLASTn) available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The primer sequences can be found in Table 1. Primer efficacy was validated by generating a single product of the correct size, which was confirmed through agarose gel analysis. Real-time PCR was conducted by the YTA SYBR Green QPCR Master Mix 2X kit (Pars Tos Company) and Rotor-Gene 6000 device (Qagen Company). The tube was filled with 10 µL of master mix, 0.5 µL each of forward and reverse primers, and 3 µL of cDNA. Finally, it was topped off with 6 µL of RNase-free distilled water to reach a total volume of 20 µL. The thermal cycle of real-time PCR was 95ºC (4min), 94ºC (30sec), 57ºC(30sec), 72ºC (30sec), and 72 ºC (5min). GAPDH was utilized as the housekeeping gene. Real-time PCR results were analyzed using the formula $2^{-\Delta\Delta Ct}$.

Table 1. Primer Sequences of Cyclin-D, P53, and GAPDH Genes.

Genes	Oligomer (5'→3') as a forward primer	Oligomer (5'→3') as a reverse primer
Cyclin-D	5'-ATCAAGTGTGACCCGGACTG-3'	5'-CCTTTGGGTCCAIGTCTGCG-3'
p53	5'-GCGAGACTGCCAAACAACAC-3'	5'-TCACGCCCACGGATCTGAAGG-3'
GAPDH	5'-CTCCAAAATCAAGTGGGGCG-3'	5'-TGITTICACCCCATGACGAA-3'

Statistical analysis

A Sample K-S test was employed to assess the normality of the data distribution. The quantitative analysis of the data was performed using central dispersion indices, including the mean and standard deviation, based on the information obtained from real-time PCR. The ANOVA and Tukey post-hoc tests were utilized to examine the relationships among quantitative variables across different groups. The Spearman/Pearson correlation method was also applied to explore the relationships between quantitative variables. Linear regression was conducted to evaluate the simultaneous effects of variables on gene expression. All statistical analyses in this study were performed using SPSS version 20 software, and a significant level of $P < 0.05$ was maintained for all calculations.

Results

Cyclin-D and *p53* gene expression level

The *cyclin-D* gene's expression level in COVID-19 patients' blood cells increased about 9 times compared to the control sample (8.8 in COVID-19 *vs.* 1 in control) ($P < 0.001$). Furthermore, the expression level of *Cyclin-D* increased about 12

times in the treatment group concerning the control group (11.8 in treatment *vs.* control) ($P < 0.001$). However, there was a slight increase in the expression of the *cyclin-D* gene in the patient group after treatment with Remdesivir, which did not show a significant difference when compared to the patient group (Fig. 1).

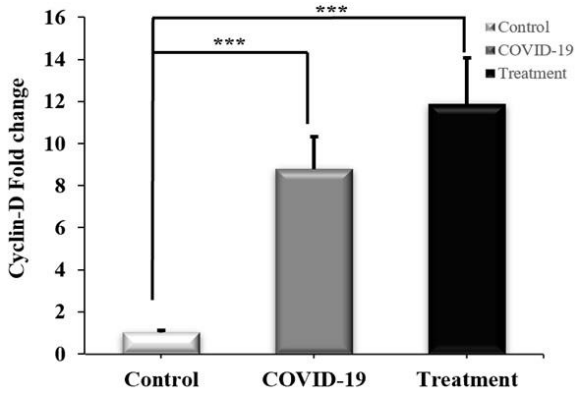

Fig. 1. Changes of *cyclin-D* gene expression between control, patients, and treatment groups (**= P<0.001).

The expression level of the *P53* gene decreased by 50% in COVID-19 patients compared to the control (0.5 in COVID-19 *vs.* 1 in the control) ($P < 0.05$). Additionally, treatment with Remdesivir doubled the expression level of this cell cycle-related gene compared to the control group (2 in the treatment group vs. 1 in the control) ($P < 0.001$). The difference in the *P53* expression between the patient and treatment groups was statistically significant ($P < 0.001$) (Fig. 2). The Spearman test was separately applied in patient and treatment groups to assess the connection between gene cycle expressions. The examination revealed a negative correlation between the gene expressions of *p53* and *cyclin-D* in the patient and treatment groups, but these correlations were not statistically significant.

Correlation of blood factors with *p53* gene expression level

The Spearman correlation test was utilized in the three groups to evaluate the relationship between blood factors and *p53* gene expression. A positive and significant correlation was found between *p53* gene expression and CRP in the control group ($r = 0.552$, $P = 0.012$) and the COVID-19 group ($r = 0.634$, $P = 0.002$), which is shown in Figures 3A and 3B. CRP is a protein produced by the liver during inflammatory responses and is often utilized as an indicator of systemic inflammation. The correlation between White blood cell counts and the gene expression was not statistically significant.

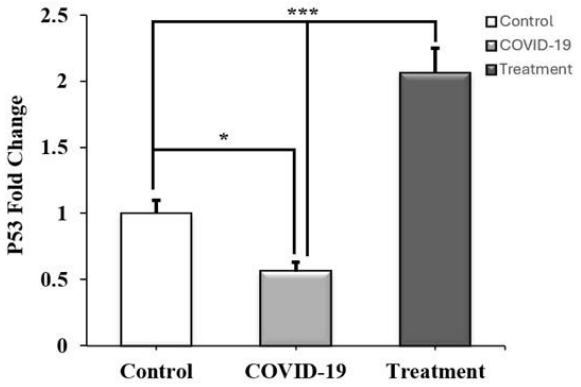

Fig. 2. Changes of *p53* gene expression between control, patients, and treatment groups (***= P<0.001).

Simultaneous examination of all factors' effects on the *cyclin-D* and *p53* gene expression

A linear regression analysis was conducted to control the interdependent effects of variables on gene expression. The results indicated that CRP strongly influenced *p53* gene expression among the factors investigated in COVID-19 patients ($P < 0.000$).

Discussion

The SARS-CoV-2 virus causes the COVID-19 pandemic, and its symptoms can range from mild issues like fatigue, fever, and muscle pain to more severe complications such as lung damage and respiratory distress (Malande, 2020). In addition to affecting the respiratory system, SARS-CoV-2 can significantly disrupt the normal cell cycle in the body. The virus impacts crucial regulators of cell division, including *cyclin-D*, which can lead to irregularities in how cells divide (Harrison *et al*., 2007). Understanding the changes in cell cycle genes among COVID-19 patients offers valuable insights into the disease's molecular mechanisms and effects on cellular functions.

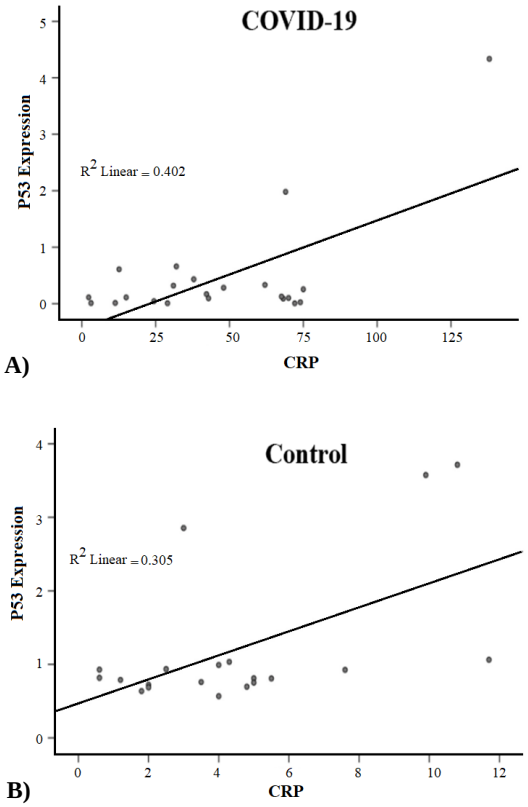

Fig. 3. The correlation between *p53* expression and CRP levels: A) Correlation of P53 expression with CRP levels in COVID-19 patients; B) Correlation of P53 expression with CRP levels in the Control group.

Research shows that several genes involved in the cell cycle are notably affected during COVID-19 infection. Genes like Aurora kinase B (AURKB) and cyclin-dependent kinase inhibitor 1A (CDKN1A or p21) were consistently elevated in patients with COVID-19, while others, such as CDKN1C (p57), were downregulated. This pattern indicates a complicated interaction between how the virus causes disease and how it affects the host's cell cycle. It appears that SARS-CoV-2 may hijack the host's cellular mechanisms to boost its replication while also triggering stress responses that disrupt the normal cell cycle process (Prado *et al*., 2023; Välikangas *et al*., 2022).

Studies have shown that many upregulated genes are associated with more severe cases of COVID-19, indicating that the extent of dysregulation may reflect the host's response to viral infection (Chellapandian *et al*., 2024). For example, increased expression of pro-proliferative genes and decreased expression of cell cycle inhibitors could contribute to uncontrolled cellular proliferation and inflammation, exacerbating lung injury and other complications associated with severe COVID-19 (Prado *et al*., 2023; Välikangas *et al*., 2022).

SARS-CoV-2 has been shown to interact with host cell machinery in ways that can alter cell cycle regulation. Research indicates that during infection, cyclin D1 and cyclin D3 are redistributed from the nucleus to the cytoplasm, followed by proteasomal degradation (Gupta and Mlcochova, 2022). Cyclins are crucial molecules for regulating the cell cycle, particularly the transition from the G1 phase to the S phase (Montalto and De Amicis, 2020; Yuan *et al*., 2007). An increase in *cyclin-D* expression can promote cell cycle progression, which may be advantageous for viral replication. When a virus boosts cell growth, it can set the stage for its replication and spread throughout the body. In a recent study, researchers used advanced systems immunology techniques to examine gene activity in COVID-19 patients. They discovered that certain genes tied to cell division, particularly those involving cyclin-D, behaved differently in patients with severe symptoms than those with milder cases. These finding hints that higher levels of *cyclin-D* might play a role in worsening COVID-19 symptoms (Prado *et al*., 2023).

The immune response in inflammation conditions can also influence cyclin-D expression (Zhang *et al*., 2021). As we know, COVID-19 is accompanied by a cytokine storm (Zanza *et al*., 2022). The inflammatory cytokines released during infection may lead to changes in gene expression patterns, including those of cell cycle regulators like *cyclin-D*. Elevated levels of pro-inflammatory cytokines can stimulate pathways that promote cell cycle progression as part of a broader immune response. Interestingly, studies have shown that the depletion of cyclin-D3 can enhance viral titers in infected cells (Gupta and Mlcochova, 2022). This suggests that cyclin-D3 may play a role in restricting viral replication. The transcription of numerous proinflammatory genes is enhanced during the G1 phase of the cell cycle in a manner dependent on cyclin-dependent kinases (CDKs). This mechanism entails the recruitment of CDK6 to the nuclear chromatin by cytokines, interacting with transcription factors from the NF-κB,

STAT, and AP-1 families (Schmitz and Kracht, 2016). Therefore, an increase in *cyclin-D* expression could represent a compensatory mechanism by the host cells attempting to counteract viral replication by promoting cell division and immune responses. SARS-CoV-2 also reorganizes the host cytoskeleton for efficient cell entry and controls host transcriptional processes to support viral protein translation (Suryawanshi *et al*., 2021). The virus dysregulates innate cellular defenses, resulting in delayed hyperinflammation and weakened interferon response (Suryawanshi *et al*., 2021). Multiple signaling pathways are involved in the host response, including those leading to cytokine storms and various forms of cell death (Farahani *et al*., 2022). SARS-CoV-2 proteins exploit the host's genetic and epigenetic mediators to hijack key host signaling pathways for viral pathogenesis (Khan and Islam, 2021). In agreement with existing evidence, we showed an increase in *cyclin-D* expression in patients with COVID-19 compared to the control group.

Remdesivir doesn't directly interact with cyclins or their related pathways, so it doesn't cause changes in cyclin D expression levels. Its main job as an antiviral drug is to stop viruses from replicating rather than affecting host cell cycle genes like cyclin D (Kokic *et al*., 2021). However, because nucleoside analogs (the class of drugs Remdesivir belongs to) are known to potentially harm mitochondria, one study found that even short-term exposure to Remdesivir (24 hours) harmed cell health by slowing down cell growth, as shown by a significant drop in 3H-thymidine uptake. Additionally, Remdesivir caused mitochondrial damage in heart cells, leading to reduced oxygen use, a breakdown in mitochondrial membrane potential, and increased lactate production after 24-48 hours of treatment (Merches *et al*., 2022). Remdesivir impacts gene expression by upregulating RNA polymerase and nutrient stress response pathways, specifically those driven by ATF3 and ATF4. Genes involved in synthesizing the purine precursor inosine monophosphate (IMP), such as ATIC, GART, and PFAS, are among the most depleted after remdesivir treatment. This likely restricts cellular concentrations of adenosine, increasing the relative abundance of remdesivir (Akinci *et al*., 2020).

The changes observed in *cyclin-D* expression during COVID-19 largely result from the viral infection itself and its interactions with host cellular processes. Therefore, while Remdesivir effectively reduces viral loads and mitigates some aspects of COVID-19 pathology, it does not directly influence *cyclin-D* gene expression in infected patients. In our study, remdesivir did not lead to any notable alterations in cyclin gene expression compared to the patient group.

Another goal of this study is to investigate the possible effects of the SARS-CoV-2 virus on *P53* gene expression and its related pathways. The *P53* gene, as one of the most important regulatory factors of cell cycle and apoptosis, plays a vital role in maintaining the stability of the genome and preventing the growth of cancer cells (Ozaki and Nakagawara, 2011).

The expression of *p53*, a critical tumor suppressor, is significantly decreased during COVID-19 infection (Ma-Lauer *et al*., 2016; X. Wang *et al*., 2023). Research indicates that the spike protein of SARS-CoV-2 can inhibit the transcriptional activity of P53 in cancer cells. This inhibition disrupts the interaction between *p53* and MDM2, an E3 ligase responsible for *p53* degradation (Zhang and El-Deiry, 2024). Another research found that SARS-CoV-2 induces cellular senescence in retinal pigment epithelial (RPE) cells via the ROS/p53/p21 pathway. This suggests that the virus may trigger stress responses that alter normal cellular functions and promote senescence, further implicating *p53* in the cellular response to COVID-19 (Zhang *et al*., 2023). Gene expression profiling has shown that COVID-19 patients exhibit changes in inflammatory pathways (Li *et al*., 2021). For example, certain genes that control the production of cytokines and immune responses often don't work as they should. This can cause the immune system to overreact, leading to a dangerous condition called a cytokine storm, which has been tied to severe cases of COVID-19. Adding to the complexity, P53 can influence the levels of pro-inflammatory cytokines. Research is increasingly showing that P53 helps keep inflammation in check, acting as a kind of brake on the immune response (Gudkov *et al*., 2011). Studies showed that the severity of COVID-19 infection was associated with decreased levels of eosinophils,

neutrophils, lymphocytes, and monocytes and higher levels of CRP (Azarfar *et al.*, 2023). The results of our study indicate a reduction in *P53* gene expression among COVID-19 patients, consistent with earlier studies. Additionally, *P53* expression positively correlated with CRP levels in these patients. Therapeutic approaches aimed at regulating *p53* activity might offer a way to control the inflammatory responses associated with severe COVID-19. Restoring a balanced *p53* function or preventing overactivity could help alleviate the impact of cytokine storms. The absence of a significant relationship between the level of *p53* gene expression and other variables, such as the number of white blood cells, can be due to the small number of samples or the sampling time. In this study, blood samples were collected on the first day of diagnosis and the fifth day after receiving the last dose of Remdesivir. Changes in other blood factors, as well as the expression of studied genes, may be observed after the completion of the treatment period. Therefore, to complete the results obtained, it is better to investigate the long-term complications of COVID-19.

In this study, it was found that Remdesivir treatment elevates *P53* gene expression in COVID-19 patients. The P53 level was elevated 2 times after Remdesivir treatment in comparison to the control group. However, other Studies indicate that Remdesivir can increase the expression of the *p53* gene (de Sousa Pinto *et al*., 2024). This upregulation appears to be a response to genetic damage potentially caused by the drug itself. The activation of *p53* is often associated with DNA repair mechanisms and cellular stress responses, suggesting that Remdesivir may induce a form of genotoxicity that triggers P53 activation.

Studies show that P53 can dial down the production of another protein, cyclin D1. It does this by interfering with a process called the NF-κB signaling pathway. When P53 is activated, it reduces the activity of the cyclin D1 gene, leading to lower levels of the cyclin D1 protein and its genetic instructions (mRNA). This tells us that *p53* is important for keeping cyclin D1 in check, especially when cells are under stress, like when DNA gets damaged (Pera *et al*., 2001; Rocha *et al*., 2003). The results showed a negative correlation between these two genes. The correlation between *cyclin-D* and *P53* gene expression is complex and context-dependent, involving direct and indirect regulatory mechanisms.

In summary, the findings revealed elevated *cyclin-D* levels in COVID-19 patients, which remained unaffected by Remdesivir treatment. Conversely, *p53* gene expression was notably reduced in the patient group. Treatment with Remdesivir increased *p53* gene expression twofold compared to the control group. Furthermore, *p53* gene expression positively correlated with CRP levels in both the control and patient groups. The results of this study reported Remdesivir as an effective drug in improving patients' conditions. Remdesivir will help improve and balance patients' condition by returning to normal levels of *cyclin-D* and *p53* gene expression. However, more research is needed to confirm the possible effects of Remdesivir on the cell cycle.

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Conflicts of Interest

The authors declare no conflict of interest.

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