

Research Article

A Comparative study to Evaluate the Relative Gene Expression of the *PML-II* Gene in SARS-COV-2 Infected and Uninfected People

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Abstract

Background: Promyelocytic Leukaemia -Nuclear Bodies (PML-NBs) represent a key component of the innate immune defense against pathogens, including viruses such as Coronavirus 2 (SARS-CoV-2). **Aim:** Evaluate the relative expression of the *PML-II* gene in blood samples from individuals confirmed to be infected with SARS-CoV-2 and compare it with its expression in healthy individuals serving as a control group. **Methodology:** 112 Blood samples were collected from individuals infected with SARS-CoV-2 at various laboratories in Iraq, following confirmation of infection via RT-PCR by accredited laboratories, 73 samples from healthy individuals were also collected to be as a control group. After extracting RNA from these samples, the material was used to assess the gene expression of the *PML-II* protein using qPCR technology. **Conclusion:** Demonstrating increased gene expression of the *PML-II* protein upon SARS-CoV-2 infection reinforces the understanding that viruses elicit distinct immune responses from the body's first line of defense: the intrinsic innate immune system.

1. Introduction

Innate immunity is characterized by unique features, specifically the presence of cellular restriction factors constitutively expressed proteins that limit the progression and spread of numerous viral pathogens, acting as the first line of immune defence against infection [1–3]. The Promyelocytic Leukemia protein (PML) or Tripartite Motif (TRIM) is a member of a large family comprising over 70 human proteins, it functions as both a SUMO E3 ligase and a scaffold protein, organizing spherical intranuclear compartments known as PML nuclear bodies (PML-NBs) [4, 5].

These bodies play pivotal roles in cellular stress responses, the PML protein forms the outer shell of these nuclear bodies, recruiting various target proteins (SUMO-conjugated) including Speckled Protein of 100 Kilo Dalton (SP100), Dead-Domain associated Death (Daxx) and Alpha-Thalassemia Mental Retardation X-Linked syndrome (ATR-X), transcription factors, and DNA repair proteins into their interior [6, 7]. The PML protein product of interferon stimulated genes (ISGs) exists in seven major isoforms generated through alternative mRNA splicing and post-translational modifications [8, 9]. These isoforms share a common structural motif at the N-terminus known as RBCC, the RING motif is essential for the formation of PML nuclear bodies. However, the isoforms differ functionally based on their distinct C-terminal domains, which recruit different interaction partners and mediate isoform-specific interactions with nuclear components [10–12]. The PML protein is a key structural component of these nuclear bodies, essential for their formation; it coordinates a complex network of protein interactions based on sequences spanning its tripartite RBCC (RING, B-box, and Coiled-Coil) motif [13]. The PML-I and *PML-II* isoforms are considered the most abundant [14]. These small proteins modified by ubiquitin-like molecules (SUMOylation) perform a variety

of important functions, including antiviral defence, maintenance of genome integrity [15], protein degradation, cellular senescence, and the interferon response [16].

These nuclear bodies have been observed near sites of DNA damage, viral DNA, and regions of nascent RNA transcription [16]. These proteins undergo radical reorganization during cell division, and their morphology may change throughout the cell cycle [17]. To limit viral activity, the proteins constituting PML nuclear bodies (PML-NBs) play a mediating role in innate immunity by suppressing viral replication through mechanisms dependent on SUMO signalling [3]. PML proteins facilitate the induction of antiviral cellular genes such as those encoding cytokines and PML-NBs components which are regulated by interferon signalling. Furthermore, viral infection can trigger the production of antiviral proteins that act either directly on PML-NBs or on the overall cellular architecture. Despite the diverse activities of these nuclear proteins across various cellular pathways, substantial evidence documents their link to the interferon system and their contribution to antiviral defence, as well as their status as preferred targets for viral infection [18].

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) caused a global pandemic in 2019, infecting over 500 million people and resulting in more than 6 million deaths worldwide; it is the third coronavirus to emerge, following SARS-CoV in 2002 and Middle East Respiratory Syndrome-associated coronavirus (MERS-CoV) in 2012 [19]. SARS-CoV-2 belongs to the order Nidovirales and the genus Beta coronavirus [20]. Infections in mammals are associated with beta coronaviruses (β -COVs) a group that includes SARS-CoV-2 [21], which is an enveloped, spherical virus characterized by the presence of spike proteins [22]. Its genome consists of a single-stranded, positive-sense RNA (+ssRNA) molecule, with a determined nucleotide sequence length of 30 kilobases (kb); this sequence was the first to be isolated from patients infected with the Wuhan virus. The mechanisms of gene regulation and expression in this virus are like those found in all coronaviruses [23].

Although SARS-CoV-2 infection has generated considerable interest in the mechanisms of innate immunity, the molecular response of several viral restriction factors remains unclear, particularly the role of promyelocytic leukemia protein nuclear bodies, known as PML-NBs. The problem lies in the limited information regarding changes in *PML-II* isoform expression among infected individuals compared with uninfected individuals, as well as the scarcity of local studies addressing this molecular marker. Therefore, the present study aimed to evaluate the relative expression of the *PML-II* gene in blood samples from individuals confirmed to be infected with SARS-CoV-2 and compare it with its expression in healthy individuals serving as a control group. This was achieved using reverse transcription quantitative polymerase chain reaction, with *GAPDH* employed as an internal reference gene. The findings may clarify the potential role of *PML-II* as a marker of innate immune response and provide a basis for future investigations into molecular defense mechanisms and infection-related diagnostic or therapeutic biomarkers.

2. Methodology

2.1. Samples collection

A collected of (112) blood samples from patients, all of them confirmed for infection with SARS-COV-2, at the same time a healthy (73) persons treated as a control, all aged between (15-70) years, the diagnoses carried out with RT-PCR technique, a random collection of the total (185) samples have been followed from a different area from Basrah Governorate in Iraq Country during November and December of 2021, at -20°C, the collected blood samples were kept in EDTA plain, even molecular use for the purpose of genetic evaluation analysis. Those samples have been collected for the purpose of the evaluation of gene expressions for the related genes *PML-II*, and Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*) as per sequences mentioned in Table 1. The laboratory works taken place in Central Lab. for Medicine College in Basrah University.

Table 1: Primers were used to evaluate gene expression

Primers		Sequence 5' - 3'	Reference
<i>PML-II</i>	Forward	AGGCAGAGGAACGCGTTGT	[24]
	Reverse	GGCTCCATGCACGAGTTTTTC	
<i>GAPDH</i>	Forward	GGTCGGAGTCAACGGATTT	[25]
	Reverse	CCAGCATCGCCCCACTTG	

2.2. Molecular Examination

2.2.1. RNA extraction

The RNA extracted from blood samples has been taken to analyze the *PML-II* gene expression in the total (185) samples using Solarbio Cat # R1200 Kit from BEIJING SOLARBIO SCIENCE & TECHNOLOGY CO., LTD.

2.2.2. Reverse transcription to synthesize complementary DNA

By using Solarbio Universal RT-PCR Kit (M-MLV, free Taq Polymerase) Cat # RP1105 from BEIJING SOLARBIO SCIENCE & TECHNOLOGY CO., LTD., the cDNA obtained from the extracted RNA samples, under the mentioned conditions in the enclosed leaflet the reaction was carried out, and the RNA was mixed with the reaction components.

2.2.3. Gene expression assay (Relative quantification)

By using (Intron) Kit (CA: K-6210) and as per enclosed instruction the reaction of qPCR have been prepared. The reaction mix was prepared by mixing 10 μ L of SYBR green and adding 3 μ L of cDNA template, 100 Pmol of each forward and reverse primers of *PML-II* and *GAPDH*, and 5 μ L of free (DEPEC-D. W) to make a final volume of 20 μ L [26]. The qPCR conditions were set as follow: the reaction was submitted

to a first step of denaturation at 94°C for 5 min followed by 45 cycles of 15 seconds of denaturation at 95°C, annealing step was set at 58°C for 30 sec, an extension step at 72°C for 45 sec with a final extension conducted at 72°C for 5 min [27].

2.3. Statistical analysis

For all samples the standard deviation analysis has been applied using Microsoft excel software for both *PML-II* and charts with three replicates, the results were normalized to the level detected in control samples. *GAPDH* considered as an internal control. Relative expression assay was achieved using SYBR green as intercalating dye. Obtained data were analyzed by $\Delta\Delta$ CTs, and the analysis considered for assessment and the average value of *GAPDH* for each sample used as a reference for calculation, the calculation considered and applied as per Livak and Schmittgen [28].

3. Results

3.1. qPCR examination

To evaluate the relative expression of *PML-II* genes associated with SARS-COV-2 infection. The (185) blood samples underwent to qPCR examination. The synthesized cDNA from the extracted RNAs was used as a template and mixed with *PML-II* specific primers, and using qPCR relative expression assay (SYBR green) to measure *PML-II* gene expression.

3.2. *PML-II* relative expression and response in SARS-COV-2 infected samples

The finding shows that more than 10.2-folds detected for *PML-II* as a relative expression for negative patients with SARS-COV-2 compared with the positive infected samples Figure 1, (A, B and C). The expression of *PML-II* gene was assessed in both positive and negative samples.

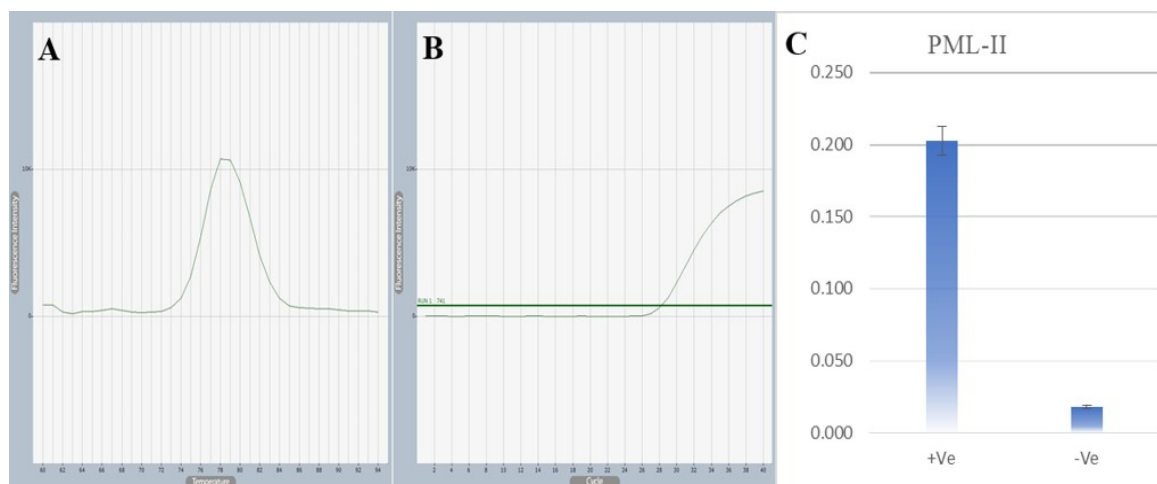


Figure 1: (A): Melting curve analysis of *PML-II* gene expression, degree of plot specificity was displayed in one peak at 78°C, (B): Amplification curve analysis of *PML-II* gene expression for one replicating of one sample, the signal was detected at the CTs range, (C): *PML-II* relative expression. *PML* mRNA was detected by qPCR; the results were normalized to the level detected in control samples and the averages and standard deviation of three technical replicates. *GAPDH* was employed as an internal control. Relative expression assay was achieved using SYBR green as intercalating dye. Obtained data were analyzed by $\Delta\Delta$ CTs

4. Discussion

Given the importance of PML nuclear bodies (PML-NBs) in antiviral defence, we conducted a comparative study to evaluate the expression of the *PML-II* isoform (it is one of the nuclear PML isoforms resulting from alternative splicing) in individuals infected with SARS-CoV-2 and uninfected controls. To understand the relationship between viral infection and the host defense response, it is essential to focus on the behavior of the genes responsible for this defense starting with the first line of defense mediated by the innate immune system. Consequently, it is crucial to identify the mechanisms employed by specialized antiviral proteins, such as the various forms of PML nuclear bodies. This study specifically highlights the *PML-II* isoform and its role during SARS-CoV-2 infection by evaluating its gene expression in infected individuals compared to uninfected ones examining whether the gene is upregulated or downregulated during the course of the infection.

The results showed an increase in the relative gene expression of the *PML-II* isoform in SARS-CoV-2-infected samples; specifically, mRNA expression for this gene was 10.2-folds higher in infected patients compared to uninfected ones. This increase should not be considered as indicative of the increase of *PML-II* proteins activity but rather as the transcriptional reaction of SARS-CoV-2 infection. PML nuclear bodies are dynamic nuclear structures and their biological functions are dependent on association with multiple proteins and on their sumoylation. Hence, upregulation of *PML-II* mRNA might be a sign of activation of antiviral response, but it does not necessarily reflect an proportionate rise in the amount of *PML-II* protein or a direct action on the virus [17].

Variations in the gene expression of nuclear PML proteins can be attributed to the specific viral infection involved; expression levels may be elevated with one virus and reduced with another. These nuclear bodies exhibit differential gene expression patterns depending on the virus, often resulting from viral alteration of the pre-mRNA splicing process for PML. This virus-dependent variation could be due to the structural and functional differences between the PML isoforms. The presence of PML is present in the common domains that are

essential for the nuclear body formation of PML, but the differences within the C termini domains of the isoforms may influence their cellular location and interactions with other nuclear proteins. Thus, the observed expression pattern of *PML-II* in SARS-CoV2 infection may not be a true reflection of the response of all PML isoforms. The current identification should therefore be taken in the light of an edited expression of *PML-II* transcript and not as an increase of the expression of all PML proteins [8]. For instance, in Herpes Simplex Virus type 1 (HSV-1) infections, specific PML exons such as exons 5 and 6 are deleted, thereby inhibiting the antiviral function of the resulting cytoplasmic isoform [29]. This variation in expression was evident in the present study, which demonstrated a marked increase in expression levels among SARS-CoV-2 patients compared to healthy individuals. This comparison is based on the normalization of *PML-II* expression by loading it relative to *GAPDH* reference gene expression, and on the use of the $2^{-\Delta\Delta CT}$ comparative method. Normalized transcript levels were then calculated for both infected and control groups, and a 10.2-fold change in normalized transcript level is reported. But this fold change magnitude must be interpreted with caution since the abundance of RNA transcripts can be affected by RNA quality, the efficiency of the reverse-transcription process, and by natural variations between blood samples [28].

These findings contrast with a previous study we conducted Al-Asadi, evaluating *PML-II* gene expression in HPV-infected patients, where expression was found to be lower in positive samples compared to negative ones [30]. It's scientifically established that PML nuclear bodies perform various cellular functions, including antiviral responses, that vary depending on the type of viral infection. Numerous studies have extensively investigated the factors influencing the enhancement or suppression of the response associated with specific PML genes by evaluating their gene expression during viral infection, offering valuable insights in this regard. However, as noted by Fairchild et al., the molecular basis underlying the functions performed by these nuclear bodies remains unclear [16]. This association of upregulation of *PML-II* to the broad anti-viral response is also not fully understood. Presumably, PML-NB-associated proteins are not necessarily acting as antiviral factors but rather as a closely interacting network of defense molecules. DAXX is for instance one of the cellular restriction factors linked to the SARS-CoV-2 infection, and SARS-CoV-2-induced *PML-II* response might be correlated with coordinated changes of other parts of the nuclear antiviral apparatus. However the present study did not analyze the other factors such as DAXX, SP100, ATRX, interferon related genes, viral load and viral replication thus cannot rule out whether the seen *PML-II* rise is indeed an activation of the whole PML-NB pathway or not [31].

Limitations

There are several limitations that should be taken into account when considering these findings. It should be noted that this is a cross sectional study and more so a correlation to the changes in the expression of *PML-II* and SARS-CoV-2 infection cannot be established. Moreover, samples were obtained between November and December 2021 in Basrah Governorate, and results and conclusions of the study might not be transferable to addressing other patient populations, seasons, viral variants, or clinical scenarios. The mRNA level of only one PML iso-form was also studied and there were no protein-expression or functional antiviral assays. Larger and scientifically recruited patients cohorts to explore the role of *PML-II* and its association with viral load, illness severity, span of infection and prognosis should be studied in Future.

5. Conclusion

The results of this study demonstrating increased gene expression of the *PML-II* protein upon SARS-CoV-2 infection reinforces the understanding that viruses elicit distinct immune responses from the body's first line of defense: the intrinsic innate immune system. PML nuclear bodies (PML-NBs) play a crucial role in this system, utilizing various cellular signaling pathways to defend against viral threats. Depending on viral behavior and the host's defensive genetic response to contain the infection, the expression of these components may either increase-as observed with the *PML-II* protein in this study-or decrease. Consequently, it is essential for researchers to expand their studies to better understand the genetic mechanisms involved in resisting, limiting, or eliminating infections and pathogens, thereby facilitating the identification of optimal therapeutic strategies.

Article Information

Ethical approval: Ethical approval was obtained from Basra University/ Medical college/ Central Lab, and Basra education Directorate (Number 30619 on July 6th, 2021) before starting the study.

Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest: The authors declare that they have no conflict of interest.

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