Importance of miR-UL-148D Expression Pattern in Cytomegalovirus Infected Transplant Patients

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ABSTRACT

Background: MicroRNAs (miRNAs) are endogenous, 18-22 nucleotide non-coding RNA molecules. Human cytomegalovirus (HCMV) is a ubiquitous and particular herpes virus that encodes miRNAs, which increases gradually in the presence of infection. One of the important viral miRNAs is HCMV-miRUL-148D, which plays a role in establishing and maintaining viral latency.

Objective: The current study aimed to evaluate the expression levels of HCMV-miRUL-148D in active and inactive HCMV infected transplant patient groups compared to healthy individuals.

Methods: Total RNA was extracted from blood samples of 60 solid organ transplant patients and 30 healthy controls. In-house SYBR Green Real-Time PCR evaluated the expression levels of studied miRNA and gene.

Results: The expression level of the *UL-148D* gene was significantly higher in the active HCMV infected patients ($p=0.001$) compared to other groups. While the miRUL-148D expression level significantly increased in the inactive HCMV-infected patients ($p<0.001$) compared to other groups.

Conclusion: Increased miRUL-148D expression level in the inactive HCMV-infected transplant patients indicates the potential role of this miRUL-148D as a biomarker of the HCMV latent stage.

KEYWORDS: Human Cytomegalovirus; miRNA; Transplantation; miRUL-148D

INTRODUCTION

Despite considerable advances in preventive antiviral strategies, human cytomegalovirus (HCMV) infection is still a common post-transplantation problem [1]. Viral infections and diseases pose significant challenges after using antiviral prophylaxis [2, 3] or therapy [4] in organ transplanted patients [5]. Several factors are involved in HCMV infection, such as the transplanted organ, the serological status of donor and recipient, and the immunosuppressive drugs [6]. The prevalence of HCMV ranges from 50% to 90% worldwide [7, 8]. In developing countries like Iran, most children are exposed to HCMV infection, and experienced at least one history of positive serum HCMV infection. HCMV has a linear dsDNA genome with an extended programming capacity between the lytic and latent phases in specific cells [9].

HCMV primary infection usually starts with

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no symptoms, followed by a persistent latent infection [10]. HCMV primary infection in healthy people is not detectable due to the production of a robust immune response through neutralizing antibodies and cellular responses that culminate in the elimination of the lytic virus [11].

The virus replicating machinery stops by silencing the immediate-early (IE) genes as soon as the start of latency [12]. Major products of HCMV IE genes, IE1 and IE2 proteins, are responsible for establishing the lytic cycle by producing the early and late gene cascades [13]. Although the HCMV latent infection can be detected via immune response, molecular methods are not potent enough to detect this latent infection. Whereas, during the lytic phase, the positive result of the antigenemia test, which distinguishes the immunoglobulin production, and detection of the HCMV genome by molecular methods, can help certify the lytic infection [11, 14]. However, many studies proposed that the virus is not thoroughly inactivated during latency. Instead, some HCMV proteins, including UL138, vIL-10, US28, ORF94, and LUNA (UL81-UL82 locus antisense transcript) [15], might play various roles in viral latency.

More than 200 miRNAs are detected in viruses, and most of them are related to herpes viruses. HCMV is famous for its abundant miRNAs (26 mature miRNAs from 16 precursors) spread throughout the viral genome [16].

MiRNAs are small non-coding RNA molecules (18-22nt in length) [17] that have different roles in various biological processes, such as cell proliferation, development, differentiation, apoptosis, hematopoiesis [17], and also in regulation of viral replication [15, 18], immune modulation [19], and immune evasion [20]. The expression pattern of HCMV miRNAs is different during latent and lytic infections, but their presence in pathological stages has not been described [21].

The HCMV miRNAs have roles in transplant recipients during viral replication and might be used as biological detectors to discriminate different stages of the viral life cycle [22]. These miRNAs are significant regulators of host gene expression and help determine viral pathogenesis as post-transplant biomarkers [5]. Although the clinical relevance of viral miRNAs during HCMV reactivation has not been clearly defined, some recent data confirm the critical role of viral miRNAs in post-transplant outcomes [5]. One of the most important HCMV miRNAs is miRUL-112 [23], which downregulates natural killer (NK) group 2, member D (NKG2D), and helps the NK cells evasion process [21, 24].

The other important miRNA encoded by HCMV is miRUL-148D. This miRNA contributes to immune evasion via targeting the chemokine (C-C Motif) ligand 5 (CCL5) gene and the 3'UTR of RANTES (Regulated on Activation, Normal T Expressed and Secreted) during viral infection [7]. Furthermore, HCMV-miRUL-148D downregulates the secretion of IL-6 as a response to activin A in latently infected monocyte, which explains its involvement in the replication, infection, and ability to escape from the immune system [12].

miRNAs are essential in HCMV latency, and miRUL148-D contributes to the latent phase of HCMV infection by targeting the host genes. Host cellular immediate-early response gene 5 (IER5) presented as a target for miRUL-148D. Studies have shown that miRUL-148D can effectively inhibit IER5 production during latent viral infection, which controls transcription by preserving the CDC25B and CDK1 activities [12].

miRUL-148D expresses by the UL-150 gene and can be detected in clinical strains while not found in the attenuated AD169 strain (Laboratory strain). HCMV UL-150 gene belongs to a gene family that codes a small new gene called *UL-148D* [25]. The *UL-148D* gene encodes a viral glycoprotein, has an essential effect on the viral tropism programming and promotes glycoprotein O (gO) expression, which is necessary for HCMV infection. Disruption of the *UL-148D* gene in strain TB40/E causes a remarkable viral tropism elevation for epithelial cells [26].

Table 1: The PCR conditions for the HCMV *UL-148D* gene expression.

Gene	Primer	Sequence	PCR Product Length	Thermal cycling Conditions	PCR Mix
UL-148D	UL-148D: F	5'-CAGAAAAGAAGCTTAGAAAGA-3'	151	95°C/30 sec, 35 cycles of 95°C/15 sec, 60°C/20 sec, and 72°C/30 sec	Premix (5µL; 2X), Dye (0.2 µL; 50X). Forward primers (0.4 µL; 5 pM), Reverse primers (0.4 µL; 5 pM).
	UL-148D: R	5'-GTCGGAGAGTCTAGACTGGG-3'			
GAPDH	GAPDH: F	5'-GCACACTCGCATGCAGTCACG-3'	119	95°C/30 sec, 35 cycles of 95°C/15 sec, 58°C/20 sec, and 72°C/30 sec	
	GAPDH: R	5'-GGCACTCTGCACGTCACGT-3'			

Therefore, the current study aimed to evaluate the expression levels of the *UL-148D* gene and miRUL-148D in active HCMV-infected transplant patients vs. inactive ones.

MATERIALS AND METHODS

Patient Population

The present study was conducted on 60 solid organs of adult transplant recipients, including 30 livers and 30 kidneys, who were admitted to the Transplant Unit of Namazi hospital affiliated with SUMS between 2014 and 2016. Pediatric patients and adult transplanted recipients with a history of viral hepatitis and other herpes viral infections were ruled out from the study. All patients were examined for HCMV viremia. Those transplanted patients with detectable HCMV viral loads were considered members of the active HCMV infected patient group (n=30). Patients with negative Real-time PCR results were included in the inactive HCMV infected patient group (n=30). The control group consisted of 30 healthy non-transplanted individuals.

The immunosuppressive regimen utilized for all patients was composed of cyclosporine, prednisolone, and mycophenolate mofetil. EDTA-treated blood samples (3 mL) were collected from each patient.

Real-time PCR Analysis

Quantitation of HCMV DNA

The Real-time PCR protocol was performed using the Q-HCMV Real-time complete kit based on the manufacturer's instructions (GeneProof, Czech). HCMV UL-123 and the human β-globin genes as the internal control were simultaneously amplified. Briefly, HCMV-DNA was isolated from 200µl plasma of EDTA-treated blood samples using the DNA blood mini kit (STRATEC Molecular, Berlin, Germany). Extracted DNA sample (5µl) and reaction mix (20µl) were added. Negative controls also were used for determining the accuracy of tests. The PCR conditions were as follows: decontamination at 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec. each, and at 60°C for 1 min.

HCMV-UL-148D Gene Expression

Total RNA was isolated from 250 µl of buffy coats using a TRIZOL kit (RNAsol, biobasic, Canada). Then, the total extracted RNAs were converted to cDNA for the HCMV gene analysis using Prime Script RT Reagent Kit (Takara Bio, Otsa and Shiga, Japan) by considering the manufacturer's instructions. The reaction conditions were: 37°C for 15 min and 85°C for 5 sec. The synthesized cDNAs were stored at -20°C for further studies.

Table 2: The PCR conditions for the HCMV miR-UL-148D analysis.

Gene	Primer	Sequence	PCR Product Length	Thermal cycling Conditions	PCR Mix
miR-UL-148D	miR-UL-148D: F	5'-TCGTCCTCCCCTTCTTCACCG-3'	74	95°C/10min, 40 cycles of 95°C/15 sec, 63°C/35 sec	Premix (5µL; 2X), Dye (0.2 µL; 50X). Forward primers (0.4 µL; 5 pM), Reverse primers (0.4 µL; 5 pM).
miR-U6	miR-U6: F	5'-GACGGTGAGATCCAGGCTTGAGAGC-3'	75	95°C/10min, 40 cycles of 95°C/15 sec, 63°C/35 sec	
Revers	Universal Revers	5'-CAGAGAGTAGATGAGCGTGCAGT-3'			

The Real-time PCR assay was performed using an SYBR Prime Script Kit (Takara Bio, Otsa and Shiga, Japan) according to the manufacturer's instruction. The PCR mix ingredients and other relevant information is shown in Table 1. The *GAPDH* gene normalized all the samples as an internal control.

HCMV miRUL-148D Expression

HCMV miRNA extraction was performed according to the Trizol kit instructions (RNA-sol, biobasic, Canada). The cDNA synthesis for HCMV miRNAs was conducted in two steps using an in-house cDNA synthesis protocol. First: Reagent buffer (Takara Bio, Otsa and Shiga, Japan), universal stem-loop primer, and ddH2O were mixed and put at 65°C for 5min and then 25°C for 10 min. Second: RT enzyme (Takara Bio, Otsa and Shiga, Japan), total RNA (100 ng), and ddH2O were added. Both reaction mixes were added to each other, and the new mixture was put at 37°C for 15 min and 85°C for 5 sec. Finally, the synthesized cDNAs were stored at -20°C for further studies.

The cDNA library was used in the qRT-PCR analysis for mature miRNAs by utilizing the miScript SYBR Green PCR Kit (Takara Bio, Otsa and Shiga, Japan) with a forward specific mature miRNA primer and the universal reverse stem-loop primer. MiR-U6 was used as an internal control gene. The Real-time PCR mix and conditions are presented in Table 2.

Ethical Consideration

The study protocol was designed according to ethical guidelines of the 1975 Helsinki declaration and was approved by the local ethics committee of Shiraz University of Medical Sciences. Written informed consents were also taken from all study participants.

Statistical Analysis

The expression levels of the HCMV gene and miRNA were evaluated by intra- and inter-group analyses in active and inactive HCMV infected recipients and controls using the $2^{-\Delta\Delta CT}$ (Livak) method. SPSS 24.0 statistical software package (IBM Corporation, USA) was used for saving and analyzing the data. All data are presented as the mean ± SD. Parametric and non-parametric tests were used to study the differences between groups that were applicable. The p-value ≤0.05 was considered statistically significant. The area under the ROC curve (AUC) was identified with the highest sensitivity and specificity. The binary logistic regression model was used to determine the effect of confounding variables on grouping.

RESULTS

Patients Demographic Data

The active HCMV infected patient group was composed of 30 patients (mean age=30±14 years, range=25-75), out of which 53.3% (n=16) were male. Another 30 patients considered the inactive HCMV infected patient group (mean age=50.9 years, range=26-70), out of which

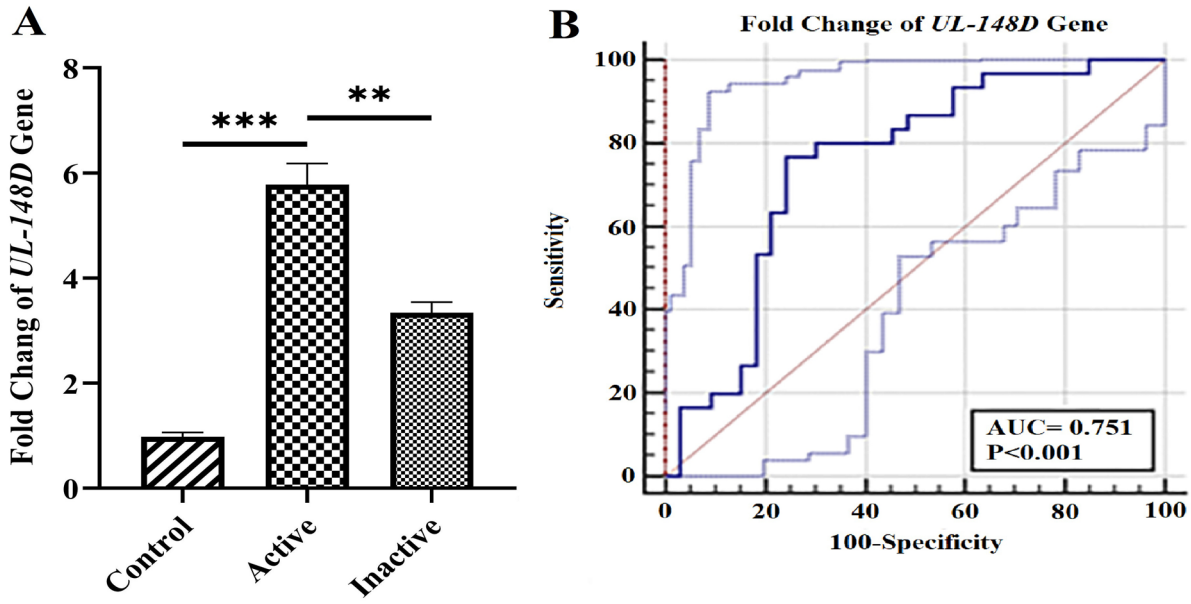

Figure 1: The expression level of the *UL-148D* gene in active and inactive HCMV infected patients. (A) shows the comparison of *UL-148D* gene expression in the active and inactive HCMV infected patient groups and controls, (B) specificity and sensitivity of *UL-148D* gene estimated by ROC curve (**p<0.05-0.001 and ***p<0.001).

56.6% (n=17) were male.

Comparison of *UL-148D* Gene Expression Level between Studied Groups

The *UL-148D* gene expression level was significantly higher in the active (p=0.001) than in the inactive HCMV infected patient group (1.80 times). *UL-148D* gene had a significantly higher expression level (5.79 times) in the active HCMV infected patient group compared with the controls (p<0.001). The expression level of the *UL-148D* gene was increased (3.21 times) in the inactive HCMV infected patient group compared with the controls (p=0.61) (Fig 1A). The ROC curve analysis has shown that the sensitivity and specificity of *UL-148D* gene expression were significant in both active and inactive HCMV infected patient groups (p<0.001) (Fig 1B). As shown in Fig 1B, the sensitivity and specificity of *UL-148D* gene expression are 80% and 79.5%, respectively. AUC index indicated the sensitivity and specificity of the factor under study equal to 0.751.

Comparison of HCMV miRUL-148D Expression Level between Studied Groups

The HCMV miRUL-148D expression level was increased in the inactive HCMV infected patient group (p=0.099) compared to the active HCMV infected ones (1.81 times). Moreover, miR-UL148D has a significantly higher expression level (5.2 times) in the inactive HCMV infected patient group compared with the control group (p<0.001). The expression level of this miRNA was increased 2.87 times in the active HCMV infected patient group versus the controls (p=0.15) (Fig 2A). The ROC curve analysis showed that the sensitivity and specificity of miR-UL-148D expression were not significant in both active and inactive HCMV infected patient groups (p=0.084) (Fig 2B), and the sensitivity and specificity of miR-UL-148D expression are 50% and 63.6%, respectively. Finally, the AUC index of this analysis is equal to 0.625.

Comparison of *UL-148D* Gene and miRUL-148D Expression Levels in the Studied Groups

The miRUL-148D expression levels were significantly lower than the *UL-148D* gene in the active HCMV infected patient group (p=0.001). While in the inactive HCMV infected patient group, these two studied molecules showed no statistically significant difference (p=0.98) (Fig 3A).

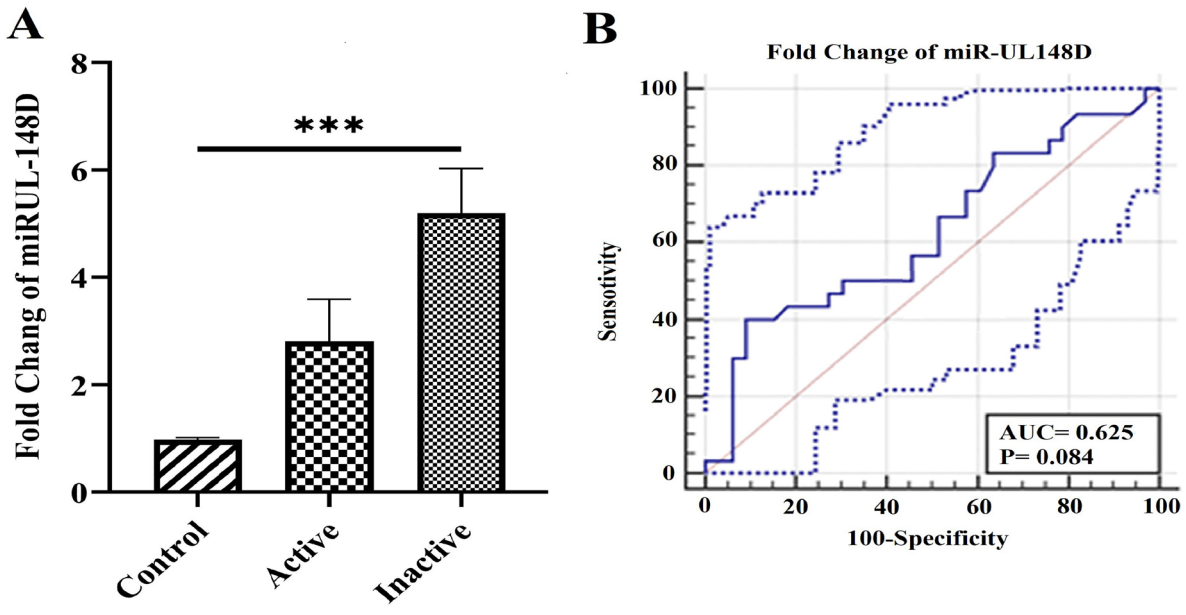

Figure 2: The expression level of HCMV miRUL-148D in active and inactive HCMV infected patients. (A) shows a comparison of HCMV miR-UL-148D expression in the active and inactive HCMV infected patient groups and controls. (B) specificity and sensitivity of HCMV miR-UL-148D estimated by ROC curve (***p<0.001).

Comparative regression analysis of *UL-148D* gene and miR-UL-148D expression levels in the active and inactive HCMV infected patient groups showed no linkage between *UL-148D* gene and miR-UL-148D expressions (Fig 3B, 3C).

DISCUSSION

Human cytomegalovirus uses effective strategies by encoding many immune-modulatory genes that can regulate host immune response processes [27]. Notably, amongst these strategies, the miRNA-based mechanism has been experimentally validated. HCMV miRNAs were implicated in many essential processes of cells and tissues [23]. Some crucial miRNAs that help HCMV replicate and escape from the immune system are HCMV miRUL-112, -UL-22A, -US-25-1, and -UL-148D. In this study, we compared the expression levels of HCMV miR-UL-148D in active and inactive HCMV-infected transplant patients for the first time. Moreover, this study evaluated the regulatory effect of HCMV miR-UL-148D on the *UL-148D* gene in solid organ transplant patients.

HCMV miRUL-148D plays a vital role in the pathogenicity of the HCMV strains [24, 26]; however, the absence of this miRNA in attenuated AD169 strain proposes that its accumulation happens during the latent phase of HCMV infection [12, 28]. The target genes for HCMV miRNAs have not been investigated widely, but it has been detected that chemokine RANTES and IEX-1 are the targets of miRUL-148D during viral infection [29].

RANTES is a pro-inflammatory chemokine involved in recruiting T cells, basophils, and eosinophils [24] to the infection area. HCMV virus with mutated miR-UL-148D cannot cause a decrease in RANTES level [20].

Pan et al. [12] showed that in infected monocytes, reduction in IL-6 is related to HCMV miRUL-148D through targeting activin A receptor (ACVR1B) [12, 15]. Lau et al. [30] also showed that the expression of the mentioned viral miRNA is increased during latent infection through targeting CCL5 [7, 30, 31]. The *UL-148D* gene has been identified as an essential HCMV glycoprotein, expressed more in the viral lytic stage, and plays an important role in virus replication.

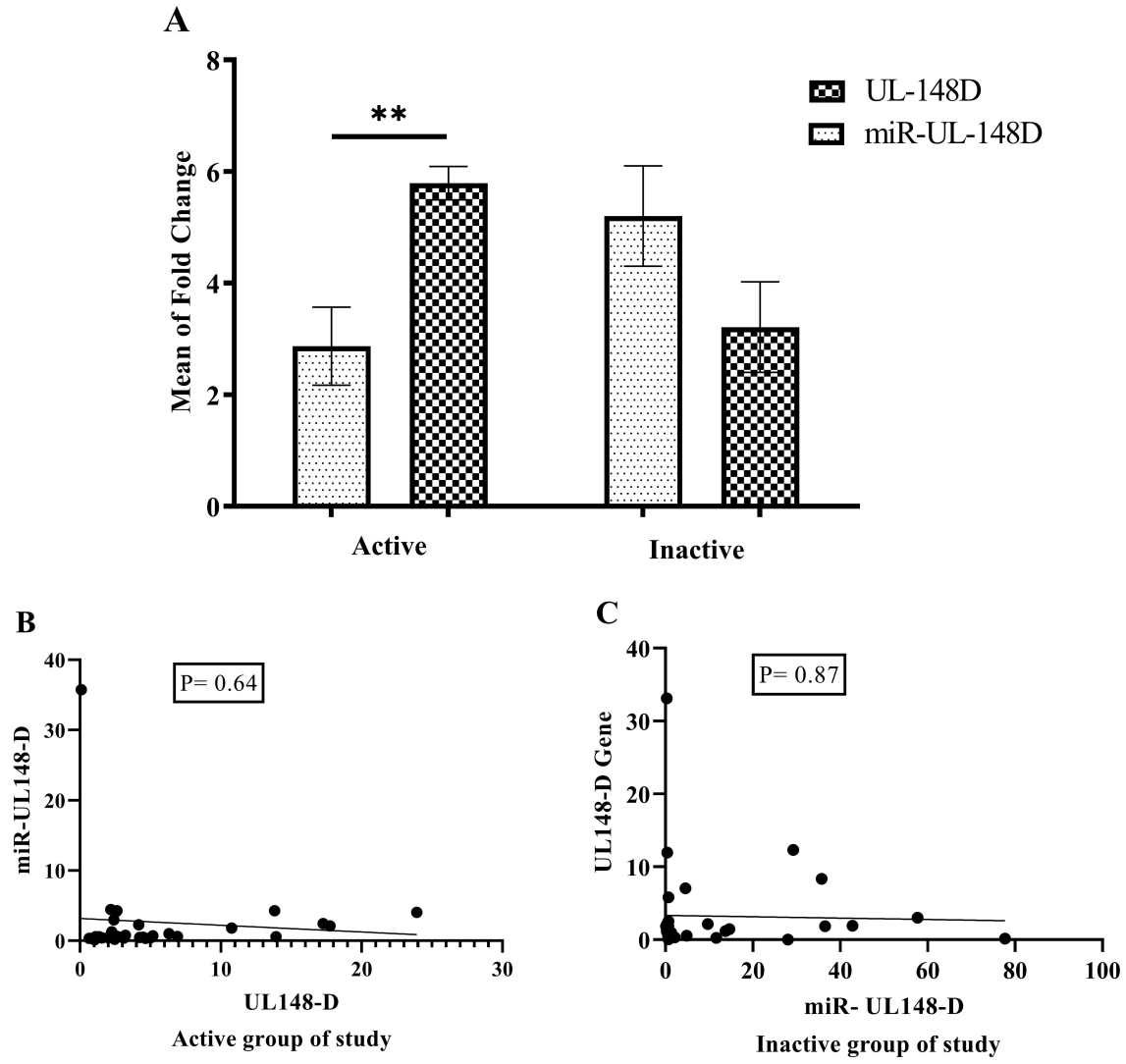

Figure 3: Comparison of UL-148D gene and miR-UL-148D expression levels in the active and inactive HCMV infected patients. (A) shows a comparison of UL-148D gene expression with the miR-UL-148D level in the active and inactive HCMV infected patient group (B) Comparative regression curve of UL-148D gene expression with the miR-UL-148D level in the active HCMV infected patient group. (C) Comparative regression curve of UL-148D gene expression with miR-UL-148D level in the inactive HCMV infected patient group (**p<0.05-0.001)

According to previous studies, the *UL-148D* gene production prepares necessary capsid glycoproteins and plays a significant role in the viral lytic stage [26].

Another study [12] proposed that miRUL-148D inhibits viral IE1 expression by maintaining the expression level of CDC25B [32]. Functional assays illustrated that this miRNA probably plays a role in simplifying HCMV latency by controlling the IER5-CDC25B-CDK1 pathway [12].

The current study showed that miRUL-148D increases in the latent stage of the viral life cycle. Therefore, based on the results of this study and the others, it can be claimed that this miRNA is one of the molecular biomarker candidates for detecting the viral latent stage. However, more research on other HCMV miRNAs is needed.

Previously, HCMV miRUL-22A was identified as a biomarker in transplanted patients and indicated that viral miRNAs might have multifaceted effects on gene expression and are associated with explicit virology and clinical consequences, which propose their potential future biomarkers [5]. Also, they play a significant role in the viral lytic stage [26].

In the current study, the expression levels of the HCMV miRUL-148D and *UL-148D* gene were compared between inactive and active HCMV infected groups. The results showed that this miRNA was expressed more than the studied gene in the latent stage. In addition, it was demonstrated that miRUL-148D expression is reduced compared to *UL-148D* gene in the active HCMV infected groups, but this reduction was not significant.

As is shown in Fig 3, there was no significant correlation between the expression level of HCMV miRUL-148D and its possible related gene, *UL-148D*, in none of the studied HCMV infected groups. These findings confirm that the expression of this miRNA and its related gene is markedly different. Previously, it was shown that the regulation of miRNA expression is quite different from that of genes [33], which is consistent with the current study results. The pattern of the *UL-148D* gene and miR-UL-148D expressions differed at each stage of the virus life cycle. Present study results showed that this miRNA had more expression in the inactive HCMV infected group.

Finally, viral miRNAs facilitate viral infection by changing the host immune evasion mechanisms. They can represent a targeted plan for future personalized therapy and diagnostic approaches and help researchers find more efficient and less toxic therapeutic strategies. This study suffered some limitations, including a small sample size, unavailability of patients for follow-up, and lack of evaluation of the protein expression in studied samples.

In conclusion, confirmation of the increased miR-UL-148D expression level in the inactive HCMV infected transplant patients compared with active HCMV infected ones presents the importance of this viral miRNA as a molecular biomarker of HCMV latent infection. This subject needs more evaluation in future studies with longer patient follow-up post-transplantation.

CONFLICTS OF INTEREST: None declared.

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