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Evaluation of up-regulated miR-UL22A-3p gene expression in miscarriages women infected with Human cytomegalovirus as biomarker for pregnancy complications

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ABSTRACT

Human cytomegalovirus-miR-UL22A-3p (hcmv-miR-UL22A-3p) is a small, non-coding RNA molecule involved in the regulation of gene expression, playing a key role in various biological processes and diseases, including viral infections such as those caused by herpesviruses. This study aimed to evaluate the expression levels of hcmv-miR-UL22A-3p in women infected with Human cytomegalovirus (HCMV) and to assess its potential role in pregnancy outcomes, particularly miscarriage. A case-control study was conducted involving 50 women who experienced HCMV-related miscarriages and had a high viral load (>10×10^3 copies/mL), alongside a control group of 50 healthy pregnant women with no history of miscarriage. Total RNA was extracted from buffy coat samples, and gene expression of hcmv-miR-UL22A-3p was quantified using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). Results demonstrated a significant upregulation of hcmv-miR-UL22A-3p in the miscarriage group, with a 2.92-fold change compared to the control group (1±0.0). Receiver Operating Characteristic (ROC) curve analysis revealed high diagnostic performance, with 92% sensitivity, 98% specificity, a confidence interval of 0.88–1.0, an area under the curve (AUC) of 0.91±0.3, and a cutoff value of 1.1 pg/mL (P = 0.000**). These findings suggest that elevated expression of hcmv-miR-UL22A-3p may be associated with HCMV-induced pregnancy loss and could serve as a potential biomarker for predicting miscarriage risk in HCMV-infected women.
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Introduction

MiRNAs in eukaryotic organisms are involved in numerous biological processes, including development, cellular differentiation, proliferation, and apoptosis. They have also been implicated in various diseases, such as cancer, cardiovascular disorders, neurodegenerative diseases, and viral infections. In the case of viral infections, miRNAs play a dual role by regulating both host and viral gene expression (Jiao et al., 2021).

Herpesviruses, a family of DNA viruses, have been shown to encode their own miRNAs. These viral miRNAs can target both viral and host mRNAs, influencing viral replication, immune evasion, and the establishment of viral latency. By manipulating host gene expression, viral miRNAs can create an environment favorable for viral survival and persistence within the host (Zhang, Yu, and Liu, 2020; Letafati et al., 2022).

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Viral miRNAs can target host cell mRNAs, leading to the repression of specific genes involved in antiviral defense mechanisms or immune responses. By downregulating host genes that are critical for mounting an effective immune response, these miRNAs help the virus establish and maintain infection within the host. This allows the virus to evade immune detection and clearance, promoting its survival and replication (Dass et al., 2023).

Additionally, viral miRNAs can target viral mRNAs, affecting viral gene expression and replication. By regulating the expression of viral genes, these miRNAs can modulate the production of viral proteins involved in various stages of the viral life cycle. This enables the virus to control its own replication, promote the establishment of latency, or enhance the production of infectious viral particles (Abu-Izneid et al., 2021; Diggins & Hancock, 2023).

Understanding the functions and mechanisms of miRNAs has opened new avenues for research and potential therapeutic interventions. The ability of miRNAs to regulate gene expression with high specificity makes them attractive targets for developing diagnostic tools and therapeutic strategies for various diseases, including viral infections (Abdel Halim et al., 2023).

MicroRNAs produced by cytomegalovirus (CMV) can have functional significance throughout the short-lived lytic replication cycle by exerting precise regulatory effects (Luan et al., 2022). The virus induces the expression of numerous genes and triggers various cellular responses. To be functionally relevant within this short timeframe, CMV-encoded miRNAs are believed to primarily target specific genes induced upon infection or repress the synthesis of short-lived proteins (Afshari, Yaghobi, and Golshan, 2022).

By targeting and inhibiting the expression of specific host genes induced by CMV infection, viral miRNAs can interfere with host cellular processes and immune responses. This allows the virus to manipulate the cellular environment to create a more favorable setting for viral replication. The targeted host genes also assist CMV in counteracting host antiviral defenses and evading the immune system (Qin et al., 2023).

In addition, viral miRNAs can repress the synthesis of short-lived proteins. This mechanism enables the rapid attenuation of specific cellular effects induced by CMV infection. Thus, viral miRNAs can modulate specific cellular pathways and prevent the production of proteins that might interfere with viral replication or immune evasion (Diggins, Skalsky, and Hancock, 2021).

MiRNAs are a class of small non-coding RNAs that have the ability to control gene expression by binding to and regulating the translation or degradation of messenger RNAs (mRNAs). During CMV infection, both viral and host-encoded miRNAs can come into play (Zhang, Yu, and Liu, 2020). CMV has its own set of miRNAs that can regulate both viral and host genes. These viral miRNAs may help the virus evade the host immune response and maintain latency (Janković et al., 2022).

This study aims to investigate whether the increased expression of the miR-UL22A-3p gene, produced by Human Cytomegalovirus (HCMV), is linked to miscarriages in women. By exploring this viral microRNA as a possible early warning sign or biomarker, the research seeks to better understand how HCMV may contribute to pregnancy complications and whether monitoring this gene could help identify women at higher risk.

Materials and Methods

Sampling

Five milliliters of freshly collected venous blood were obtained from each woman with a miscarriage and from the control group (healthy women).

Two milliliters of blood were placed in an EDTA tube for miRNA extraction. From this, one milliliter was transferred to a Trizol tube (3 ml capacity) for microRNA gene expression analysis. Total RNA concentration and integrity were assessed using the NanoDrop ND-2000 UV spectrophotometer (Thermo Scientific, USA) by measuring the A260/280 nm ratio.

The remaining three milliliters of blood were placed in a gel tube and allowed to clot. After clot formation, the samples were centrifuged at 2500 rpm for 15 minutes, and the serum was collected and stored at -20°C until use.

Serum samples were analyzed for CMV IgM and IgG antibodies using the Vitek Immunodiagnostic Assay System (VIDAS) technique. The CMV viral load was measured using a DNA CMV viral load kit with quantitative Real-Time PCR (qPCR). Samples with viral loads exceeding 10×10^3 copies/ml were considered positive for active CMV infection.

Molecular Assays: RNA Isolation and cDNA Synthesis

Total RNA was extracted from buffy coats using a modified protocol with the Trizol kit (AccuZoL™, Korea). The RNA concentration and purity were evaluated using the NanoDrop ND-2000 UV spectrophotometer (Thermo Scientific, USA) based on the A260/280 nm ratio.

cDNA synthesis for hcmv-miR-UL22A-3p was performed using a universal stem-loop primer (USTLP) specific for this study, as described in Table 1.

Table 1 Primer sequences designed for amplification and detection of HCMV miRNA hcmv-miR-UL22A-3p.

Primer	Sequence		Primer sequence5' - 3'	Tm(°C)	GC%
hcmv-miR-UL22A-3p	Stem loop		GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACCTACAA	75.8	54
	F		CGCGCCTCACCAGAATGC	64.3	67
	GAPDH	F	CACTAGGCGCTCACTGTTCTC	62	57
		R	AATCCGTTGACTCCGACCTT	61.4	50
	Universal R		CCAGTGCAGGGTCCGAGGTA	68.2	65

F: Forward, R: Reverse
GAPDH:glyceraldehyde-3-phosphate dehydrogenase

The PCR reaction mixture consisted of 10 µl of 2x PerfectStar® Green qPCR Super Mix, 1 µl of forward primer (10 pmol/µl), 1 µl of universal reverse primer (10 pmol/µl), 5 µl of cDNA, and 3 µl of nuclease-free water. The optimized conditions for microRNA detection are detailed in Table 2.

Table 2 Optimized reaction conditions for the detection of hcmv-miR-UL22A-3p microRNA.

No.	Phase	T (°C)	Time	No. of cycle
1	Initial Denaturation	94°C	10min	1
2	Denaturation -2	94°C	20sec	45
3	Annealing	60°C	30sec	
4	Extension-1	72°C		
5	Extension -2	72°C	10 min	1

SYBR Green RT-PCR

The PerfectStar® Green qPCR Super Mix, a SYBR Green-based real-time PCR premix from China, was used for amplification. The expression levels of hcmv-miR-UL22A-3p and the internal control gene GAPDH were quantified using primers designed specifically for this study.

Statistical Analysis

Data analysis was conducted using GraphPad Prism version 9.0 and SPSS statistical software version 16.0. A p-value of less than 0.05 was considered statistically significant (Cary, 2012).

Results:

Serological Results for CMV IgM and IgG in Women with Miscarriage

The serological analysis of CMV IgM and IgG in women with miscarriage showed that 3 (6.0%) samples were seropositive for CMV IgM alone, 8 (16.0%) samples were seropositive for both CMV IgM and IgG, and 39 (78.0%) samples were seropositive for CMV IgG alone. These results revealed a highly significant difference ($P < 0.01$), as shown in figure 1.

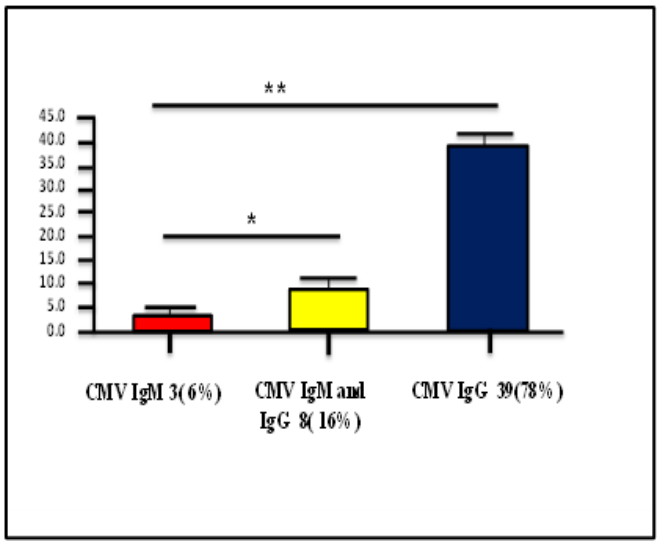

Fig 1. Results of positive CMV IgM and IgG in women with miscarriage (*$P < 0.05$; $P < 0.01$, statistically significant).

Expression of the hcmv-miR-UL22A-3p Gene in Women with Miscarriage and Control Group

The relative gene expression was determined using the RT-PCR amplification program and confirmed by melting curve analysis, following the manufacturer's simulation software, as shown in figure 2.

The fold change in hcmv-miR-UL22A-3p expression among women with miscarriage was 2.92, compared to 1.0 ± 0.0 in the control group. The mean ± SE of the threshold cycle (Ct) value for hcmv-miR-UL22A-3p expression in women with miscarriage was 32.41 ± 3.6, compared to 35.28 ± 3.6 in the control group. However, this difference in Ct values was not statistically significant (P = 0.412, not significant), as shown in Table 3.

The relative expression, calculated by the 2^-ΔΔCT method, was 2.77 ± 0.78 in women with miscarriage, compared to 0.7197 ± 0.02 in the control group, with a highly significant difference (P = 0.011*), as shown in figure 3.

Table 3 Expression levels of hcmv-miR-UL22A-3p in women with miscarriage compared to the control group.

The expression level of hcmv-miR-UL22A-3p (Mean±SE)						
Groups	U6 CT	CT	ΔCT	ΔΔCT	2^- ΔΔCT	Fold change
Control	20.8±2.7	35.28±3.6	12.286±3.1	0.676±0.01	0.7197±0.02	1±0.0
Patient	20.28±2.1	32.41±3.6	12.028±1.6	-1.272±0.2	2.77±0.78	2.92
P. Value	0.981 NS	0.412 NS	0.44 NS	0.041 *	0.011 *	----

NS: Non-significant; *Significant (*P*<0.05).

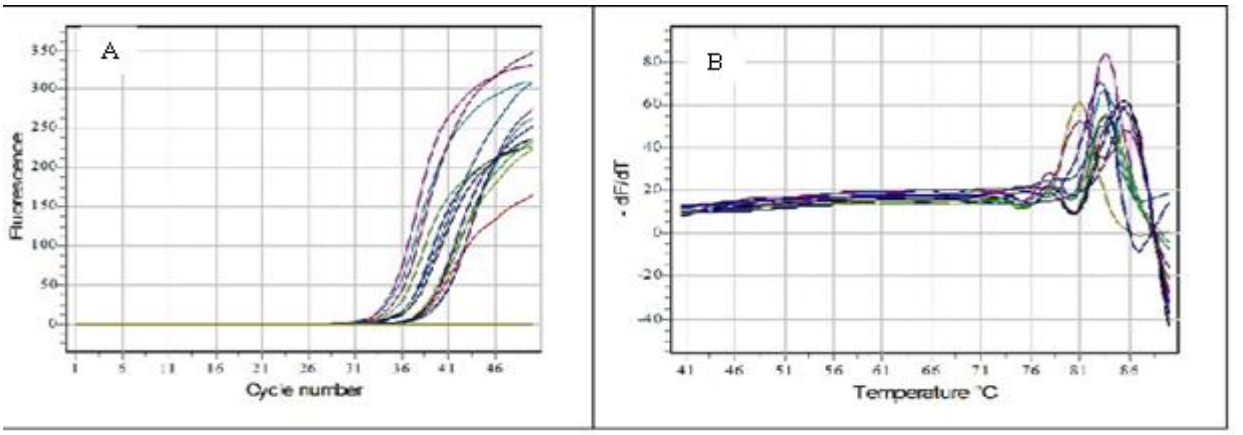

Fig 2. (A) Threshold cycle (Ct) values for miR-UL22A-3p in Real-Time PCR analysis. (B) Melting curve analysis of miR-UL22A-3p in RT-PCR.

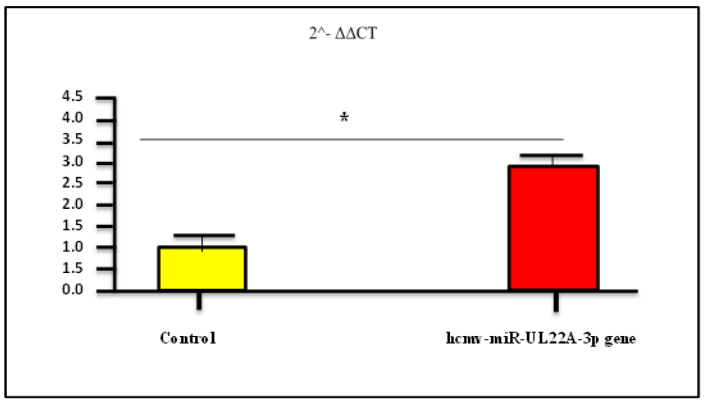

Fig 3. Relative expression (2^−ΔΔCt) of hcmv-miR-UL22A-3p compared to the control group. P < 0.05, statistically significant.

Specificity and Sensitivity of hcmv-miR-UL22A-3p

The ROC curve analysis demonstrated that hcmv-miR-UL22A-3p has potential as a significant diagnostic marker in the infected group. The sensitivity of the marker was 92%, the specificity was 98%, and the area under the curve (AUC) was 0.91 ± 0.03, with a 95% confidence interval of 0.88 to 1.0. The cutoff value was determined to be 1.1 pg/mL, with a highly significant difference (P = 0.000**), as shown in figure 4.

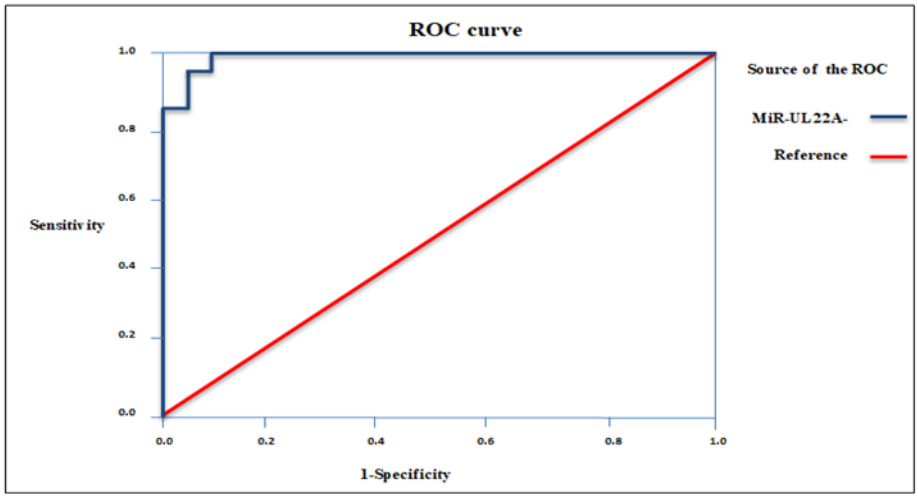

Fig. 4. ROC curve analysis using the Wilson/Brown method showing Sensitivity (%) versus 1 − Specificity (%) for hcmv-miR-UL22A-3p in women with miscarriage.

Discussion

The present study revealed novel findings indicating that the expression of HCMV miRNA in women who experienced miscarriage has an impact on the occurrence of miscarriage in those infected with HCMV. These observations support the involvement of CMV miRNAs in viral pathogenesis and suggest their potential use as indicators of virological progression. This study demonstrated the expression of hcmv-miR-UL22A-3p in a group of women with miscarriage infected with CMV compared to the control group. The fold change of 2.92 shows that the expression level of hcmv-miR-UL22A-3p is higher in women with miscarriage and active CMV infection than in the control group, indicating that this gene is upregulated in the patient group.

This finding suggests that viral miRNAs may have diverse effects on gene expression and are linked to specific virological and clinical outcomes. Consequently, these findings imply that viral miRNAs could serve as promising biomarkers in the future. Moreover, they play a crucial role during the viral lytic phase (Ji et al., 2006). The upregulation of hcmv-miR-UL22A-3p is associated with CMV infection and may contribute to pregnancy loss. Specifically, miR-UL22A-3p is mostly related to the induction of apoptosis (Skaletskaya et al., 2001).

Viral recurrence is induced by miR-UL22A via downregulation of SMAD3, an important factor in maintaining latency in infected hematopoietic progenitor cells (HPCs) (Hancock et al., 2020). Another study confirmed that miR-UL22A specifically targets C-MYC and suppresses heat-shock proteins, thereby promoting the recurrence of CMV viremia in patients undergoing solid organ transplantation. This novel immunological modulation mechanism enhances our understanding of CMV pathogenesis and could serve as a biomarker for clinical diagnosis after transplantation, pending further validation (Lisboa et al., 2015).

Although few studies have examined the role of CMV miR-UL22A-3p, it appears that these miRNAs are regulated to promote viral recurrence (Lau et al., 2016). A study by Gao et al. (2021) found that miR-UL22A-3p, with a fold change of 1.23, may serve as an indicator of adverse pregnancy outcomes (APO) in pregnant women infected with CMV. This result aligns with our findings, which also showed an increase in gene expression.

A study by Golshan et al. (2021) reported a reduction in miR-UL22A-3p levels in individuals with latent CMV infection compared to controls. They also identified the involvement of miRNAs in both the lytic and latent phases of CMV infection. Our study further revealed a significantly elevated expression of hcmv-miR-UL22A-3p in active CMV-infected kidney transplant recipients (KTRs) compared to those with latent infection. This supports the idea that miR-UL22A-3p plays a critical biological role in CMV development and confirms that increased gene expression correlates with higher viral

loads. These findings are consistent with our study outcomes.

A Receiver Operating Characteristic (ROC) curve is a plot of sensitivity versus 1-specificity of a diagnostic test and serves as a valuable tool for assessing diagnostic accuracy. The Area Under the Curve (AUC) summarizes overall diagnostic performance, with values ranging from 0 (completely inaccurate) to 1 (perfect accuracy). An AUC of 0.5 indicates no discrimination, 0.7–0.8 is considered good, 0.8–0.9 excellent, and above 0.9 exceptional (Scheff et al., 2011).

Our study demonstrated that hcmv-miR-UL22A-3p had a sensitivity of 92%, specificity of 98%, an AUC of 0.91 ± 0.03 with a 95% confidence interval of 0.88 to 1.0, and a cutoff value of 1.1 pg/mL. These results were highly significant (P = 0.000), confirming the important role of this miRNA in diagnosing CMV infection.

Conclusion

The increased expression of hcmv-miR-UL22A-3p in women infected with HCMV highlights its importance in miscarriage. These miRNAs may contribute to the pathophysiology of HCMV infection and its harmful effects causing pregnancy complications. Notably, significant alterations in viral miRNAs during active CMV infection in miscarriage cases appear to be important targets for future investigation.

Study Design and Setting

This case-control study was conducted in Babylon between February 2023 and August 2023, within the Obstetrics and Gynecology Departments of Babylon Maternity and Children's Teaching Hospital and Al-Hashimiya General Hospital.

Inclusion and Exclusion Criteria

Women aged 18 to 41 years within the reproductive age group and experiencing miscarriage from the first to fifth day were included. Women with chronic diseases, miscarriage with low viral load ($<10\times10^3$ copies/mL), infection by other agents, or immunocompromised status were excluded.

Ethical Consideration

Verbal consent was obtained from each woman before sampling. The study was approved by the Publication Ethics Committee of the Babylon Health Directorate, Ministry of Health-Iraq, under reference No. 01 dated 17-01-2023.

Conflict of interest

The authors of this paper do not have any competing interests.

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