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Research Article

Association of hcmv-miR-UL36-5P Gene Expression with Miscarriages in Women with Human Cytomegalovirus Infection in Babylon, Iraq

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Abstract

Background: HCMV is a prevalent virus that affects a significant section of the human population, causes severe disease, and affects the fetus in pregnant women. hcmv-miRNAs are important regulatory molecules in miscarriages in women CMV-infected. ***Objective***: To evaluate the association of hcmv-miR-UL36-5P gene expression with miscarriages in women with HCMV. ***Methods***: A case-control study was designed to include 140 women who had miscarriages at random, and 50 of them who had miscarriages with a high CMV viral load were categorized as miscarriage groups. Additionally, 50 healthy pregnant women who had not previously experienced miscarriages were included as a control group. After diagnosis of HCMV IgM and IgG via the VIDAS assay and CMV viral load detected by qPCR, RNA was extracted from blood samples to measure the hcmv-miR-UL36-5P gene expression by qPCR technique. ***Results***: The results revealed that out of 140 women with miscarriages, 50(35.7%) were seropositive for CMV, while 90(64.3%) were seronegative for CMV, and show that only 3(6.0%) samples were seropositive to CMV IgM, 8(16.0%) seropositive for both CMV IgM and IgG, and 39(78.0%) were seropositive for CMV IgG. The expression of hcmv-miR-UL36-5P in miscarriage women was 3.11-fold higher than the control group. ***Conclusions***: Up-regulated expression of the hcmv-miR-UL36-5P gene in women with miscarriage confirms its role in miscarriage. These miRNAs may contribute to the pathogenesis of HCMV infection and its involvement in pregnancy complications.

Keywords: Gene expression, HCMV, hcmv-miR-UL36-5P, Miscarriage.

الارتباط بين التعبير الجيني hcmv-miR-UL36-5P و الإجهاض لدى النساء المصابات بعدوى فيروس المضخم للخلايا البشري في بابل، العراق

الخلاصة

الخلفية: فيروس المضخم للخلايا البشرية هو فيروس منتشر يؤثر على جزء كبير من البشر، ويسبب مرضًا يؤثر على الجنين في النساء الحوامل. تعتبر miRNAs الخاصة بـ هذا الفيروس جزيئات تنظيمية مهمة في النساء المصابات بالفيروس مع حصول الإجهاض. **الهدف:** أجريت هذه الدراسة لقياس التعبير الجيني (hcmv-miR-UL36-5P) في حالات الإجهاض لدى النساء بسبب فيروس المضخم للخلايا البشرية. **الطريقة:** تم تصميم دراسة حالة وشاهد لتشمل 140 امرأة تعرضن للإجهاض بشكل عشوائي، حيث تم أخذ 50 امرأه من إجمالي عدد النساء اللاتي تعرضن للإجهاض لديهن حمولة فيروسية عالية لـ CMV (مجموعة الإجهاض). بالإضافة إلى ذلك، تم أخذ مجموعة ضابطة مكونة من 50 امرأة حامل سليمة لم تتعرض للإجهاض من قبل. بعد تشخيص HCMV IgM و IgG بواسطة جهاز VIDAS والحمولة الفيروسية لـ CMV التي تم اكتشافها بواسطة qPCR، تم استخراج RNA من عينات الدم لقياس التعبير الجيني hcmv-miR-UL36-5P بواسطة تقنية qPCR. **النتائج:** كشفت الدراسة أن من بين 140 امرأة مصابة بالإجهاض، كانت 50 (35.7%) مصابة بفيروس المضخم للخلايا، بينما كانت 90 (64.3%) غير مصابة بفيروس المضخم للخلايا، وأظهرت الدراسة أن 3 (6.0%) فقط من العينات كانت تحتوي على IgM لفيروس المضخم للخلايا، و8 (16.0%) عينات كانت تحتوي على IgM و IgG لفيروس المضخم للخلايا، و39 (78.0%) عينات تحتوي على IgG لفيروس المضخم للخلايا. كان التعبير عن hcmv-miR-UL36-5P في النساء المصابات بالإجهاض 3.11 ضعفًا مقارنة بالضابطة (1±0.0). **الاستنتاجات:** ان زيادة التعبير الجيني hcmv-miR-UL36-5P لدى النساء اللاتي يعانين من الإجهاض بسبب عدوى HCMV الدور المهم لهذا الجين في الإجهاض؛ وقد تساهم هذه miRNAs في التسبب في عدوى HCMV وعواقبها السلبية، والتي تتسبب في مضاعفات الحمل.

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INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the β-herpesvirinae subfamily, which was first identified in the 1950s [1]. MiRNAs are a group of small noncoding RNAs, ranging from 19 to 22 nucleotides in length, that play a regulatory function in all types of multicellular organisms by controlling gene expression in various biological processes [2,3]. CMV is recognized for its ability to develop efficient immune evasion tactics [4]. CMV-encoded miRNAs possess efficient strategies for evading the immune system by altering immunological responses. These strategies are involved in regulating interactions

between viral miRNAs and host genes across different species [5,6]. Herpesviruses are a kind of enveloped dsDNA viruses that are found everywhere. They are classified into three subfamilies (α, β, and γ) based on their similarity in genetic sequence. Human cytomegalovirus (HCMV) is classified under the β-herpesvirus subfamily. HCMV suppresses its own gene expression to prevent viral multiplication and release, resulting in latent infection. Meanwhile, the virus is poised to reengage in activity given suitable stimulus. The capacity of HCMV to establish a dormant infection, experience intermittent reactivation, and elude the host's immune response affects its success in infecting the host. Reports indicate that over 60% of persons in industrialized nations possess IgG antibodies against HCMV, but this percentage is almost 100% in developing countries [7]. MicroRNAs are small, non-coding RNA molecules that play a crucial role in gene regulation. Recently, circulating cytomegalovirus (CMV) encoded microRNAs (miRNAs) have been recognized as valuable biomarkers for diagnosing and distinguishing different illnesses. Furthermore, their impact on the formation and progression of diseases, both in terms of normal physiological processes and abnormal pathological conditions, has been recently validated [8]. This study aims to evaluate the association of hcmv-miR-UL36-5P gene expression with miscarriages in HCMV-infected pregnant women.

METHODS

Study design and setting

A case-control study was conducted in the Obstetrics and Gynecology Departments of Babylon Maternity and Children Teaching Hospital and Al-Hashimiya General Hospital between February 2023 and August 2023. We randomly selected one hundred and forty women who had miscarriages, choosing 50 of them as a miscarriage group due to their high CMV viral load. We also recruited a control group of 50 healthy pregnant women who had no previous miscarriages.

Inclusion and exclusion criteria

The inclusion criteria for the patient cohort comprise women of reproductive age, specifically those aged between 18 and 41 years. During the miscarriage phase, from the initial day of the miscarriage to the fifth day thereafter, 50 women experiencing miscarriage and 50 healthy women (control group) were selected. The exclusion criteria encompass women with chronic conditions, those who have experienced miscarriage with a viral load of less than 10×10^3 copies/mL, women who have miscarried due to other infectious agents, and immunocompromised women.

Sample collection and outcome evaluation

Five milliliters of freshly drawn venous blood were obtained from each woman experiencing a miscarriage and from the control group, which consisted of healthy pregnant women without a history of miscarriage and without a CMV viral load. Two milliliters were preserved in an EDTA tube for miRNA extraction, while one milliliter was transferred from the blood EDTA tube to a three-milliliter Trizol tube for microRNA gene expression analysis. The NanoDrop ND-2000 UV spectrophotometer (Thermo Scientific, USA) was employed to assess the quantity and integrity of RNA by the 260/280 nm ratio. The remaining three milliliters of blood were retained in the gel tube until coagulation occurred. The sample was subjected to centrifugation at 2500 rpm for 15 minutes, after which the serum was extracted and stored at -20 °C until analysis for CMV IgM, IgG, and CMV viral load.

Estimation of IgM, IgG and CMV viral load

CMV IgM and IgG antibodies are identified using VIDAS (Vitek Immunodiagnostic Test technology), a BioMerieux-developed automated immunoassay technology. The VIDAS system is intended to detect a wide range of infectious disorders, including viruses, bacteria, parasites, and health-related indicators. VIDAS uses enzyme-linked fluorescence assay (ELFA) technology. CMV IgM Positive: If the CMV IgM result is 0.90 AU/mL or more. Negative: CMV IgM results of less than 0.70 AU/mL are deemed negative. The results: CMV IgG Positive: If the CMV IgG test indicates (≥ 6 AU/mL), Negative: If the CMV IgG result is <4 AU/mL. CMV viral load determined by kit (RealBest DNA CMV viral load/Best-Russian) and qPCR SaCycler-96 Real Time PCR SECASE/Italy was greater than 10×103 copy/mL, indicating an active viral infection.

RNA isolation and cDNA synthesis

All samples had their total RNA extracted from bufy coats using the Trizol kit (AccuZoLTM, Korea) and the FavorPrep Total RNA Mini Kit (Favorgen, Korea). CDNA synthesis was carried out using EasyScript®cDNA Synthesis SuperMix (Tran-China) in a two-step technique. A universal stem loop primer (USTLP) is employed in this approach to synthesize cDNA for hcmv-miR-UL36-5P, as described in Table 1. Ingredients for PCR reaction (2x PerfectStar® Green qPCR super) To identify microRNAs, mix 10µL of forward primer (10 picomols/µL) and universal reverse primer (10 picomols/µL) with 5µL of cDNA and 3µL of nuclease-free water. See Table 2 for optimal conditions.

SYBR green RT-PCR

RT-PCR mix SYBR Green Premix (PerfectStar® Green qPCR Super Mix, China) was used. The mRNA expression level of hcmv-miR-UL36-5P and housekeeping gene (GAPDH) was measured using designed primers from Microgen-Korea.

Table 1: The primer sequence hcmv-miR-UL36-5P

Primer	Sequence	Primer sequence5'- 3'	Tm (°C)	GC (%)
hcmv-miR-UL36-5P	Stem Loop	GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACTCTTTC	75.3	54
	F	CGCGCGTCGTTGAAGACA	62.5	61
U6	F	CACTAGGCGCTCACTGTTCTC	62	57
	R	AATCCGTTGACTCCGACCTT	61.4	50

F: Forward, R: Reverse

Table 2: The optimum condition of detection hcmv-miR-UL36-5P

No.	Phase	Tm (°C)	Time (sec)	No. of cycles
1	Initial Denaturation	94	600	1.0
2	Denaturation-2	94	20	
3	Annealing	60	30	45
4	Extension-1	72		
5	Extension-2	72	600	1.0

Ethical consideration

Verbal consent was taken from each miscarriage woman before sampling. This study was approved by the Publication Ethics Committee of the Babylon Health Directorate, Ministry of Health-Iraqi, under reference No. 01 at 17-01-2023.

Statistical analysis

The statistical study was performed using GraphPad Prism 9.0 and SPSS statistical software, specifically version 16.0. A *p*-value less than 0.05 is considered significant.

RESULTS

The serological diagnosis for CMV seropositive in miscarried women reveals that only 3 (6.0%) samples were seropositive to CMV IgM, 8 (16.0%) specimens were seropositive for both CMV IgM and IgG, and 39 (78.0%) specimens were seropositive for CMV IgG. The results were highly significant, as seen in Figure 1.

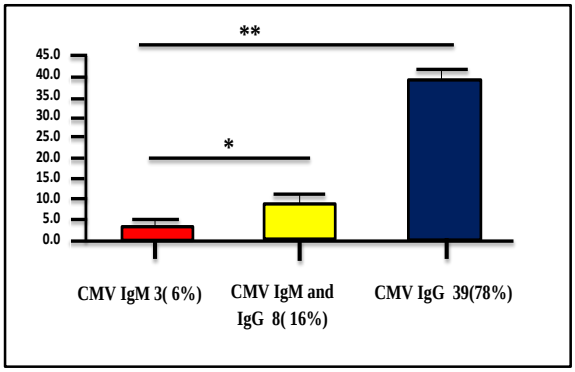

Figure 1: Seropositive CMV IgM and IgG of miscarriages women. * $p<0.05$; ** $p<0.01$.

The current study found that women undergoing miscarriages in rural locations had the greatest percentage of positive CMV IgM (9/18%), with p = 0.0001, indicating high relevance. In contrast, women experiencing miscarriages in urban regions had the lowest percentage of positive CMV IgM (4%), with p = 0.0001, indicating significant results. The *p*-values for seropositive CMV IgM in rural and urban groups were 0.0004 and 0.0001, respectively, indicating high significance, as shown in Table 3.

Table 3: Distribution miscarriages women with positive CMV IgM according to location of residence

IgM Habitation	Positive n(%)	Negative n(%)	Total n(%)	***p*-value**
Rural	9(18)	39(78)	48(96)	0.0001
Urban	2(4)	0(0.0)	2(4)	0.0001
Total	11(22)	39(78)	50(100)	---
p-value	0.0004	0.0001	---	---

The results showed that the highest number and percentage of positive CMV IgG was 33 (60%) in misarrange women who lived in rural areas, with a highly significant difference (p = 0.0001), and the lowest number and percentage of positive CMV IgG was 12 (24%) in misarrange women who lived in urban areas, with a highly significant difference (p = 0.0001). Table 4 shows that the *p*-values between rural and urban seropositive CMV IgG for positive and negative were 0.0007 and 0.0001, respectively, indicating a highly significant difference.

Table 4: Distribution miscarriages women with positive CMV IgG according to location of residence

IgG Habitation	Positive n(%)	Negative n(%)	Total n(%)	*p*-value
Rural	33(66)	5(10)	38(76)	0.0001
Urban	12(24)	0(0.0)	12(24)	0.0001
Total	45(90)	5(10)	50(100)	---
p-value	0.0007	0.0001		

Melting curve analysis was used to assess relative gene expression in the RT-PCR amplification program, as illustrated in Figure 2. In women who miscarried, the expression of hcmv-miR-UL36-5P was 3.11-fold higher than in the control group (1±0.0). The study found that women who miscarried had higher mean hcmv-miR-UL36-5P gene

expressions (33.51±5.6) than the control group (35.78±4.6) (p = 0.73).

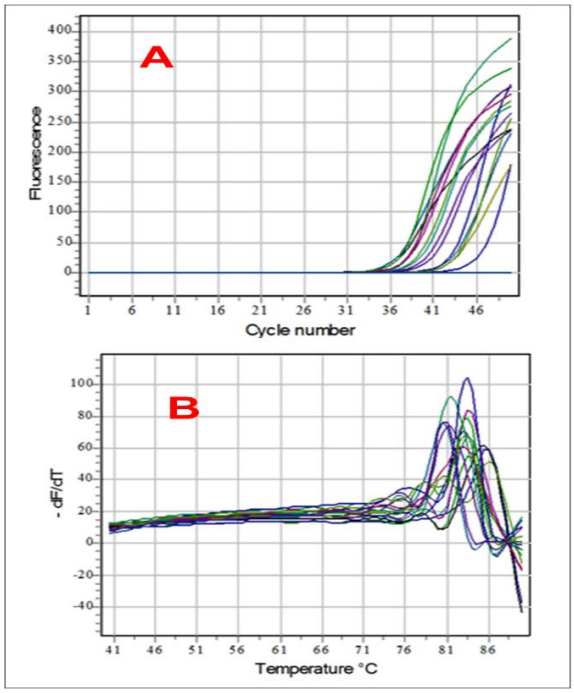

Figure 2: A) Threshold Cycle (Ct) values in Real-Time PCR Analysis of miR-UL36-5P; B) Melting Curve of hcmv-miR-UL36-5P in RT-PCR.

Table 5 shows that miscarriage women had a substantially higher 2^- ΔΔCT (3.011±0.78) than the control group (1.23±0), with a p-value of 0.033. The ROC curve analysis showed that hcmv-microRNA expression was a significant diagnostic marker in the infected group (miscarriage women), with a sensitivity of 99%, specificity of 98%, and confidence interval of 1.00±0.0. The area under the curve was 1.00±0.0, and the cutoff value was 2.0 pg/mL, with a highly significant difference (p = 0.000), as shown in Figure 3.

DISCUSSION

In the current study, the prevalence of human cytomegalovirus (HCMV) infection was positive in 50 (35.7%) miscarriages of women, and the result was significant. The results of these studies were consistent with those observed by Khudhair and Al-Azzawi [9], who observed a CMV infection prevalence rate of 32.78% among the study population; however, other studies conducted in Iraq indicated higher rates of CMV infection among women, reaching 60.63%, 56%, and 85.5%, respectively [9-11].

Table 5: The expression level of hcmv-miR-UL36-5P Compared between the miscarriages women group and the control group

Groups	U6 CT	CT	ΔCT	ΔΔCT	2^- ΔΔCT	Fold change
Control	20.8±2.7	35.78±4.6	14.68±2.2	-0.02±0	1.23±0	1±0.0
Patient	20.28±2.1	33.51±5.6	13.526±3.2	-0.184±0.01	3.011±0.78	3.11
p-value	0.99	0.73	0.44	0.021	0.033	----

Values are expressed as mean±SE. The relative gene expression was calculated using fold change (Experimental/Control).

CMV infection rates may be significantly influenced by the characteristics of the study population, such as age, location, socioeconomic status, immunological condition, and preexisting medical conditions. The disparities in CMV infection rates among women who have had miscarriages may be attributed to these variations. This discrepancy between present and prior outcomes suggests that augmenting the infection rate is contingent upon enhancing the transmission of CMV.

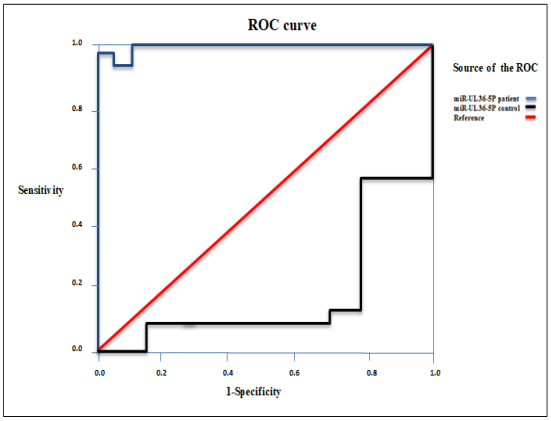

Figure 3: ROC curve analysis (Wilson/Brown method) Sensitivity% and 1-Specificity %, for (hcmv-Mir-UL36-5P) p<0.05 in misarrange women and control group. ROC curve of hcmv-Mir-UL36-5P as biomarker (AUC = 1.00±0.0).

The present study revealed that the seroprevalence of CMV was markedly elevated among women experiencing miscarriage in rural areas compared to their urban counterparts, with a positive CMV IgM rate of 9 (18%) in rural residents, contrasted with 2 (4%) in urban residents. In addition, the rate of positive CMV IgG in the miscarriage women who were residents of rural areas was 33 (66%), while this rate was 12 (24%) in women who were residents of urban areas. These findings agreed with many studies, such as Al-Rawazq *et al*. [12], who showed that the infection rate among rural residents was 74%, which was significantly higher than the infection rate among urban residents (24%). The cases of 1, 2, and 4-5 miscarriages scored the highest percentage in pregnant women who were living in rural areas than women who were living in urban areas [13]. However, a study conducted by Hama and Abdurahman [14] indicated that the occurrence of cytomegalovirus IgG and IgM among women who have had miscarriages varies according to their place of residence. Specifically, there is a significant increase of 91% in the number of miscarriaged women who test positive for high levels of CMV in their serum in metropolitan areas. Furthermore, a separate study revealed a 96% rise in the occurrence of miscarriage among women with high serum

positives for CMV in urban regions, compared to a mere 4% in rural areas [15]. According to our findings, it can be said that the severity of virus infections varies across regions due to various factors, including healthy habits, cultural differences in dietary habits, levels of education, primary healthcare programs, and early detection of infections. Practices related to cleanliness and personal care, as well as hygiene practices and sanitary conditions, may vary between rural areas and urban areas [16]. The availability of clean water, adequate sanitation facilities, and instruction on hygiene habits might differ between rural and urban locations. Adopting better hygiene measures, such as frequent handwashing and appropriate disposal of body fluids, may effectively decrease the spread of CMV. Insufficient access to resources or a lack of information about preventative measures in villages may lead to an increased prevalence of CMV infection. CMV infection rates may be influenced by socioeconomic variables, such as poverty and poor healthcare access. Rural regions, especially in developing nations, may exhibit elevated poverty rates and encounter restricted availability of healthcare infrastructure. These variables may influence general well-being and raise the probability of infections by CMV. In the current study, the expression of hcmv-miR-UL36-5P in miscarriaged women compared with the control group was 3.11-fold change, while in the control group it was 1±0.0. According to another study, hcmv-miR-UL36-5P has been shown to bind to SLC25A6 (ANT3), which may suppress the apoptotic process [17]. Although this miRNA was discovered to boost viral replication in in vitro models, research indicated that an increase in the level of hcmv-miR-UL36-5P is associated with a prolonged persistence of viremia during the early phases of antiviral treatment in the solid organ transplant (SOT) patient. Aso suggested that other opposing factors, such as therapeutic immune suppression, may be responsible for the impact indicated [18]. The expression pattern of microRNAs encoded by human cytomegalovirus in circulation during the transition from virus latency to reactivation was investigated in a study conducted by Zhou et al.; the findings indicated a 2.26-fold change elevation in gene expression among individuals afflicted with CMV. In terms of enhanced gene expression [19]. This result is comparable to the findings of our study. hcmv-miR-UL36-5P has been discovered to be significantly expressed in HEK293 cells, which has been linked to an increase in HCMV DNA synthesis. This suggests that miR-UL36-5P is a viral miRNA that aids in HCMV replication [19]. According to a study by Golshan *et al*., the majority of the CMV miRNAs exhibited a substantial increase in active HCMV-infected KTRs as compared to latent ones and controls. It was shown that hcmv-miR-UL36-5P had the highest expression level in comparison to all other tested miRNAs in CMV-infected individuals [20]. In Our study also showed that miR-UL36-5P is significantly increased in the active phase of CMV infection compared with the control group, with *p*-values of 0.033 and 0.000, respectively. Additional research verified the link between changes in circulating CMV-encoded miRNA contents and pathological outcomes in several disorders, including hypertension, cardiovascular disease, cancer, diabetes, and mental and neurological diseases. One theory is that dysregulated viral miRNAs may change the host transcriptome [21]. For the first time, the relationship between human illnesses and circulating CMV miRNAs was verified in essential hypertension [22]. Reactivation of CMV infection may occur in individuals with compromised or underdeveloped immune systems, such as those undergoing transplants, and can result in significant and perhaps fatal consequences [23]. miRNAs may enhance the regulation of viral replication in solid organ transplant patients who are seropositive for cytomegalovirus (CMV) after transplantation. Han *et al*. [24] found that hcmv-miRUL36-5P (with a fold change of 0.95) has adverse pregnancy outcomes in pregnant women who have contracted CMV. This result contradicts the results of my study. Our data also showed that hcmv-miR-UL36-5P are significantly increased in the active phase of CMV infection in miscarriaged women. This increased fold change likely plays a role in CMV activity and its relationship to miscarriages in women.

Conclusion

The elevated gene expression of hcmv-miR-UL36-5P in women experiencing miscarriage due to HCMV infection underscores the gene's significant role in miscarriage; these miRNAs may play a part in the pathogenesis of HCMV infection and its detrimental effects, which are associated with pregnancy complications.

Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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