



# Assessment of HCMV-encoded microRNAs in plasma as potential biomarkers in pregnant women with adverse pregnancy outcomes

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**Background:** Human cytomegalovirus (HCMV) is the most frequent cause of congenital infections and can lead to adverse pregnancy outcomes (APOs). HCMV encodes multiple microRNAs (miRNAs) that have been reported to be partially related to host immune responses, cell cycle regulation, viral replication, and viral latency, and can be detected in human plasma. However, the relevance for HCMV-encoded miRNAs in maternal plasma as an indicator for APOs has never been evaluated.

**Methods:** Expression profiles of 22 HCMV-encoded miRNAs were first measured in plasma samples from 20 pregnant women with APOs and 28 normal controls using quantitative reverse-transcription polymerase chain reaction. Next, markedly changed miRNAs were validated in another independent validation set consisting of 20 pregnant women with APOs and 27 control subjects. Markedly changed miRNAs were further assessed in the placenta tissues. HCMV DNA in peripheral blood leukocytes (PBLs) and anti-HCMV immunoglobulin M (IgM) and anti-HCMV immunoglobulin G (IgG) in plasma were also examined in both training and validation sets. Diagnostic value and risk factors were compared between APO cohorts and normal controls.

**Results:** Analysis of the training and validation data sets revealed that plasma concentrations of hcmv-miR-UL148D, hcmv-miR-US25-1-5p and hcmv-miR-US5-1 were significantly increased in pregnant women with APOs compared with normal controls. Hcmv-miR-US25-1-5p presented the largest area under the receiver-operating characteristic (ROC) curve (AUC) (0.735; 95% CI, 0.635–0.836), with a sensitivity of 68% and specificity of 71%. Furthermore, plasma levels of hcmv-miR-US25-1-5p and hcmv-miR-US5-1 correlated positively with APOs ( $P=0.029$  and  $0.035$ , respectively). Hcmv-miR-US25-1-5p in the placenta tissues were dramatically increased in APOs, and correlated with plasma hcmv-miR-US25-1-5p. Nevertheless, neither the concentration of HCMV DNA in PBLs nor the positivity rates of anti-HCMV IgM and anti-HCMV IgG in plasma showed a statistically significant correlation with APOs.

**Conclusions:** We identified a unique signature of HCMV-encoded miRNAs in pregnant women with APOs that may be useful as a potential noninvasive biomarker for predicting and monitoring APOs during HCMV infection.

**Keywords:** Human cytomegalovirus (HCMV); microRNA (miRNA); fetal structural anomaly (FSA); spontaneous abortion (SA); plasma

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## Introduction

Fetal structural anomaly (FSA) and spontaneous abortion (SA) are two important types of adverse pregnancy outcomes (APOs). In the clinically recognized pregnancies, the incidence of FSA is approximately 3% (1,2), and the cumulative risk of SA is approximately 11–20% (3,4). Although various types of genetic variations can cause FSA and SA, human cytomegalovirus (HCMV) is one of the most important nonhereditary causes of APO (5-8). Nearly 30–40% of primary HCMV infected pregnant women have congenitally infected fetuses due to mother-child transmission (9,10). In the United States, 4% to 5% of live births have congenital HCMV infection (11), and approximately 10–15% have birth defects (12). To date, there is no evidence that pregnant women who are immune have a lower congenital HCMV infection risk (13). Furthermore, although HCMV-DNA positive pregnant women show a 3-fold greater chance of congenital infection, there is no positive correlation between HCMV DNA positivity and APO (14). Therefore, the identification of noninvasive predictors of APO for pregnant women with HCMV infection is an important gap in our knowledge.

MicroRNAs (miRNAs) are a type of noncoding RNA nucleotide ~22 nt in length that play a critical roles in various biological processes (15). Previous studies have revealed that a variety of miRNAs derived from the host itself and other species are highly stable in plasma and serum, and may serve as noninvasive biomarkers for various diseases (16-21). In addition, small noncoding RNAs can be transmitted through the mammalian placenta and directly regulate fetal gene expression (22).

HCMV encodes at least 26 mature miRNAs during its infectious life cycle (23). HCMV miRNAs can target both host and viral genes to regulate various aspects of cellular and viral biology, such as host immune responses (24-26), cell cycle regulation (27), viral replication (28), and viral

latency (29-32). HCMV-encoded miRNAs have been confirmed to exist in plasma, and they are associated with hypertension, chronic hepatitis B and oral lichen planus (33-35). However, the plasma signature of HCMV-encoded miRNAs in pregnant women with APO and its clinical relevance have not been studied.

We postulated the existence of a unique plasma profile of HCMV-encoded miRNAs in pregnant women with APO, and used quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assays to test this hypothesis. Furthermore, we evaluated the diagnostic potential of the significantly altered HCMV miRNAs in the plasma as a noninvasive predictor of APO for pregnant women with HCMV infection. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-7354>).

## Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Qiqihar Medical University [[2017]19] and individual consent for this retrospective analysis was waived.

### *Patient characteristics and clinical features*

A total of 95 pregnant women were enrolled from the First, Second and Third Affiliated Hospitals of Qiqihar Medical University and Jianhua Hospital (Qiqihar, China), and obtained relevant permits. Normal or APO were determined based on clinical examinations and laboratory assessments. As shown in *Table 1* and *Table S1*, 40 pregnant women with APO and 55 with normal pregnancy participated in this study. All blood samples were collected in EDTA tubes, and centrifuged at room temperature at 3,000 ×g for 10 min. The

**Table 1** Demographic and clinical features of pregnant women with APO and controls in the present study

Variables	Training set			Validation set			HCMV titers set			Placenta tissues set		
	APO	Controls	P value	APO	Controls	P value	APO	Controls	P value	APO	Controls	P value
Number	20	28		20	27		40	55		20	20	
Age, years <sup>a</sup>	27±5.04	29±6.24	0.28 <sup>b</sup>	28±3.87	29±5.76	0.47 <sup>b</sup>	27±4.49	29±5.95	0.20 <sup>b</sup>	28±3.60	30±6.30	0.27 <sup>b</sup>
Type of APO, n (%)												
FSA	13 (65.0)			12 (60.0)			25 (62.5)			10 (50.0)		
SA	7 (35.0)			8 (40.0)			15 (37.5)			10 (50.0)		
Anti-HCMV IgM, n (%)												
Positive	1 (5.0)	0 (0.0)	0.42 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	1 (1.8)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>
Negative	19 (95.0)	28 (100.0)		20 (100.0)	27 (100.0)		40 (100.0)	54 (98.2)		20 (100.0)	20 (100.0)	
Anti-HCMV IgG, n (%)												
Positive	19 (95.0)	27 (96.0)	0.63 <sup>c</sup>	20 (100.0)	27 (100.0)	>0.99 <sup>c</sup>	38 (95.0)	54 (98.2)	0.57 <sup>c</sup>	1 (5.0)	1 (5.0)	>0.99 <sup>c</sup>
Negative	1 (5.0)	1 (4.0)		0 (0.0)	0 (0.0)		2 (5.0)	1 (1.8)		19 (95.0)	19 (95.0)	
HBV, n (%)												
Positive	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>
Negative	20 (100.0)	28 (100.0)		20 (100.0)	27 (100.0)		40 (100.0)	55 (100.0)		20 (100.0)	20 (100.0)	
HIV, n (%)												
Positive	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>
Negative	20 (100.0)	28 (100.0)		20 (100.0)	27 (100.0)		40 (100.0)	55 (100.0)		20 (100.0)	20 (100.0)	
Syphilis, n (%)												
Positive	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>	0 (0.0)	0 (0.0)	>0.99 <sup>c</sup>
Negative	20 (100.0)	28 (100.0)		20 (100.0)	27 (100.0)		40 (100.0)	55 (100.0)		20 (100.0)	20 (100.0)	

<sup>a</sup>, age data are presented as the mean ± SD; <sup>b</sup>, Student *t*-test; <sup>c</sup>, two-sided  $\chi^2$  test. APO, adverse pregnancy outcome; HCMV, human cytomegalovirus; FSA, fetal structural anomaly; SA, spontaneous abortion; IgM, immunoglobulin M; IgG, immunoglobulin G.

supernatant (plasma) and blood cells were stored separately at  $-80^{\circ}\text{C}$  until analysis.

### **RNA isolation and RT-qPCR**

Total RNA was extracted from plasma using phenol/chloroform purification protocol. Briefly, 100  $\mu\text{L}$  of plasma was diluted with 300  $\mu\text{L}$  of ribonuclease-free water and 200  $\mu\text{L}$  of acid phenol (Sigma, P4682), and then 20  $\mu\text{L}$  of synthetic MIR2911 was added. After the mixture was vortex-mixed vigorously, 200  $\mu\text{L}$  of chloroform was added. The sample was incubated at room temperature for 15 minutes and then centrifuged at 16,000  $\times g$  for 20 minutes. The aqueous supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and mixed with 2 volumes of isopropyl alcohol and 1/10-volume of 3 M sodium acetate (pH 5.3). Next, the mixture was stored at  $-20^{\circ}\text{C}$  for 2 h and then centrifuged at 16,000  $\times g$  for 20 minutes. Subsequently, the RNA pellet was collected, washed with 75% ethanol and dried for 10 minutes at room temperature. Finally, the pellet was dissolved in 30  $\mu\text{L}$  of ribonuclease-free water and stored at  $-80^{\circ}\text{C}$  until further analysis. Total RNA was extracted from placenta tissues using TRIzol reagent (Invitrogen, CA, USA).

We performed a TaqMan probe-based RT-qPCR assay (Table S2) to investigate the differential expression of HCMV-encoded miRNAs between normal and APO groups as described previously (33,34). The RT-qPCR assays were performed in triplicate using an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA). Expression levels of miRNAs were calculated using the CT values. We assessed the detection limits of the RT-qPCR assay by performing calibration curves developed with synthetic miR-16 oligonucleotides (Figure S1). All reactions were performed in triplicate.

### **HCMV titers**

To investigate the copy numbers of HCMV in peripheral blood leukocytes (PBLs) and placenta tissues, RT-qPCR was performed on samples from 40 pregnant women with APO and 55 with normal pregnancy. DNA was extracted from PBLs and placenta tissues using Blood Genomic DNA Midi Kit (CW BIO, CW0541) and TIANamp Genomic DNA Midi Kit (TIANGEN, DP304-03) according to the manufacturer's protocols. We amplified HCMV DNA by TaqMan real-time PCR with the following HCMV specific primers: HCMV DNA forward: 5'-CACGGTCCCGGTTTAGCA-3', HCMV DNA reverse:

5'-CGTAACGTGGACCTGACGTTT-3'. A 10-fold diluted recombinant plasmid containing the HCMV target sequence was used as a template for standard curve preparation (Figure S2). The procedure consisted of 45 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 seconds, annealing at  $60^{\circ}\text{C}$  for 30 seconds and extension at  $72^{\circ}\text{C}$  for 30 seconds. Plasmid DNA containing the HCMV target sequence was used in separate reactions serving as a positive control. Results were expressed as copies per 1 mL blood and 10 mg placenta tissues.

### **Enzyme-linked immunosorbent assay (ELISA)**

Anti-HCMV immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies were detected with a commercially available ELISA kit (Dia.Pro Diagnostic Bioprobes s.r.l, CMVG.CE/S and CMVM.CE/S) according to the manufacturer's instructions. For the IgG-ELISA, a value of  $<0.5$  IU/mL was considered a negative result, and a value of  $>0.5$  IU/mL was considered a positive result, indicating prior exposure to HCMV. For the IgM-ELISA, the test results were calculated using the optical density (OD) value at 450 nm, and the cut-off value for positivity was  $\text{OD} >1.2$ .

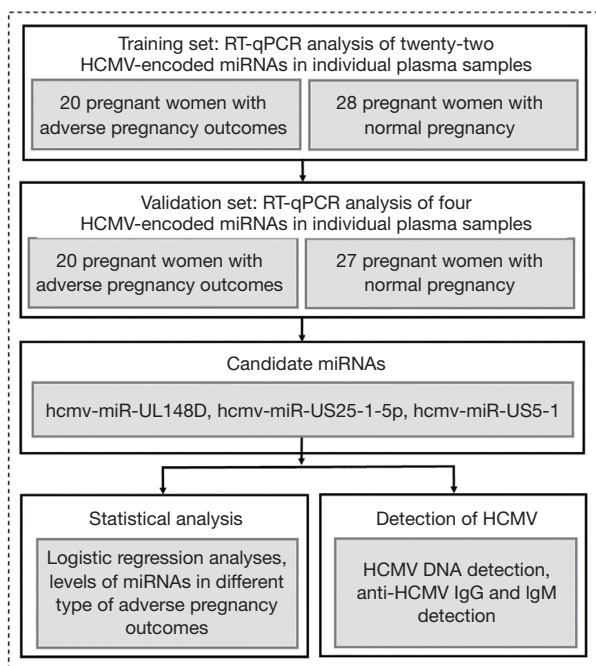
### **Statistical analysis**

Clinical characteristics between the two study cohorts were statistically analyzed using a Student's *t*-test and  $\chi^2$  test (Table 1). Data from the RT-qPCR assays were also statistically analyzed using a Student's *t*-test. The results are presented as the mean  $\pm$  SEM for miRNAs and the mean  $\pm$  SD for other variables. Univariate logistic regression analyses were performed to analyze associations between plasma HCMV-encoded miRNAs and APO. In addition, linear regression models were performed to analyze associations between plasma HCMV-encoded miRNAs and variables. A P value of  $<0.05$  was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, USA).

## **Results**

### **Expression profiles of HCMV-encoded miRNAs in plasma from pregnant women**

The experimental design of this study is shown in Figure 1. We enrolled 95 pregnant women to participate in our study, and the samples were randomly divided into a training set and a validation set. In the training set, we detected 22



**Figure 1** Overview of the experimental design. RT-qPCR, quantitative reverse-transcription polymerase chain reaction; HCMV, human cytomegalovirus; miRNA, microRNA; IgG, immunoglobulin G; IgM, immunoglobulin M.

HCMV-encoded miRNAs in plasma from 20 pregnant women with APO and 28 normal controls by TaqMan probe-based RT-qPCR. Our data showed that the plasma of all pregnant women contained a specific level of the examined HCMV-encoded miRNAs. As shown in *Table 2*, 4 of 22 HCMV-encoded miRNAs, including hcmv-miR-US25-1-5p, hcmv-miR-UL148D, hcmv-miR-US33-3p, and hcmv-miR-US5-1, were expressed at higher levels in plasma from pregnant women with APO than in plasma from normal controls ( $P < 0.05$ ). These results indicate that the altered levels of plasma HCMV-encoded miRNAs may be specific to pregnant women with APO.

#### **Further validation of HCMV-encoded miRNAs in the plasma from pregnant women by RT-qPCR**

Subsequently, we confirmed the 4 altered HCMV miRNAs in an additional independent sample set (validation set) consisting of 20 pregnant women with APO and 27 normal subjects (*Figure 2*). As shown in *Figure 2A,B,C* and *Table S3*, the pregnant women with APO had higher plasma levels of hcmv-miR-US25-1-5p, hcmv-miR-US5-1 and hcmv-miR-

UL148D than the normal controls in the validation set. However, the plasma levels of hcmv-miR-US33-3p showed no significant difference between pregnant women with APO and normal controls (*Figure 2D* and *Table S3*). The observed differences in the 3 identified miRNAs between 40 pregnant women with APO and 55 control individuals are illustrated in *Figure 3A,B,C* and *Table S4*.

#### **Plasma expression levels of 3 identified HCMV-encoded miRNAs in different types of APO**

FSA and SA are common categories of APO. These APOs are devastating for couples, and most FSAs and SAs are unexplained. We analyzed the altered expression of the above 3 HCMV miRNAs in the pregnant women with different types of APO [FSA ( $n=25$ ) and SA ( $n=15$ )] enrolled in this study. The results showed that hcmv-miR-UL148D and hcmv-miR-US25-1-5p were only elevated in pregnant women with FSA but that hcmv-miR-US5-1 was significantly upregulated in pregnant women with both FSA and SA (*Figure 3D,E,F* and *Table S5*). Additionally, the plasma level of hcmv-miR-US25-1-5p was much higher in pregnant women with FSA than in pregnant women with SA (*Figure 3E* and *Table S5*).

#### **Diagnostic value of 3 altered HCMV-encoded miRNAs as a predictor of APO for pregnant women with HCMV infection**

To assess the diagnostic accuracy of the 3 plasma HCMV miRNAs for APO, we performed receiver-operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) of the 3 altered miRNAs (hcmv-miR-UL148D, hcmv-miR-US25-1-5p, and hcmv-miR-US5-1) was 0.689, 0.735 and 0.688, respectively (*Table S6*). Among them, hcmv-miR-US25-1-5p presented the largest AUC (0.735; 95% CI, 0.635–0.836), with a sensitivity of 68% and specificity of 71%. Although we also investigated the diagnostic value of the combinations of the 3 selected miRNAs, the AUC of all the combinations was lower than that of hcmv-miR-US25-1-5p alone (*Table S6*). Our data indicate that plasma hcmv-miR-US25-1-5p is a potential predictor of APO in pregnant women with HCMV infection.

#### **Increased plasma levels of HCMV-encoded miRNAs in pregnant women correlate with APO**

To further evaluate the ability of the three increased HCMV miRNAs in plasma from pregnant women to



**Table 2** Expression profile of HCMV-encoded miRNAs in pregnant women with APO and controls in the training set

HCMV encoded miRNAs	APO (n=20)	Controls (n=28)	Fold change	P value <sup>a</sup>
hcmv-miR-UL148D	6.12±0.89	3.54±0.31	1.73	0.003
hcmv-miR-US25-1-5p	0.17±0.02	0.10±0.01	1.75	0.003
hcmv-miR-US33-3p	0.11±0.02	0.06±0.01	1.76	0.018
hcmv-miR-US5-1	3.70±0.55	2.32±0.27	1.59	0.019
hcmv-miR-UL112-3p	5.82±1.01	4.03±0.43	1.44	0.078
hcmv-miR-US4-3p	1.30±0.31	0.80±0.13	1.63	0.105
hcmv-miR-US29-5p	20.58±2.42	16.77±1.63	1.23	0.181
hcmv-miR-UL36-3p	0.44±0.12	0.28±0.06	1.59	0.183
hcmv-miR-US29-3p	0.21±0.06	0.13±0.03	1.54	0.197
hcmv-miR-US25-1-3p	0.83±0.31	0.47±0.12	1.74	0.24
hcmv-miR-UL22A-5p	1.03±0.22	0.76±0.13	1.36	0.259
hcmv-miR-US25-2-5p	3.73±1.14	2.53±0.54	1.47	0.304
hcmv-miR-US33-5p	17.33±6.67	11.97±2.35	1.45	0.397
hcmv-miR-UL112-5p	1.17±0.50	0.78±0.16	1.5	0.401
hcmv-miR-US22-3p	0.97±0.22	0.80±0.11	1.22	0.439
hcmv-miR-US5-2-3p	0.10±0.02	0.08±0.01	1.24	0.446
hcmv-miR-US22-5p	39.84±8.33	33.44±6.52	1.19	0.543
hcmv-miR-UL22A-3p	0.19±0.07	0.16±0.04	1.21	0.651
hcmv-miR-US4-5p	0.46±0.11	0.40±0.09	1.14	0.692
hcmv-miR-UL36-5p	16.21±1.54	17.00±1.44	0.95	0.713
hcmv-miR-US5-2-5p	5.92±1.71	5.23±1.03	1.13	0.72
hcmv-miR-US25-2-3p	8.21±1.90	7.99±0.88	1.03	0.908

Data are presented as the mean ± SEM. <sup>a</sup>, Student *t*-test. HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome.

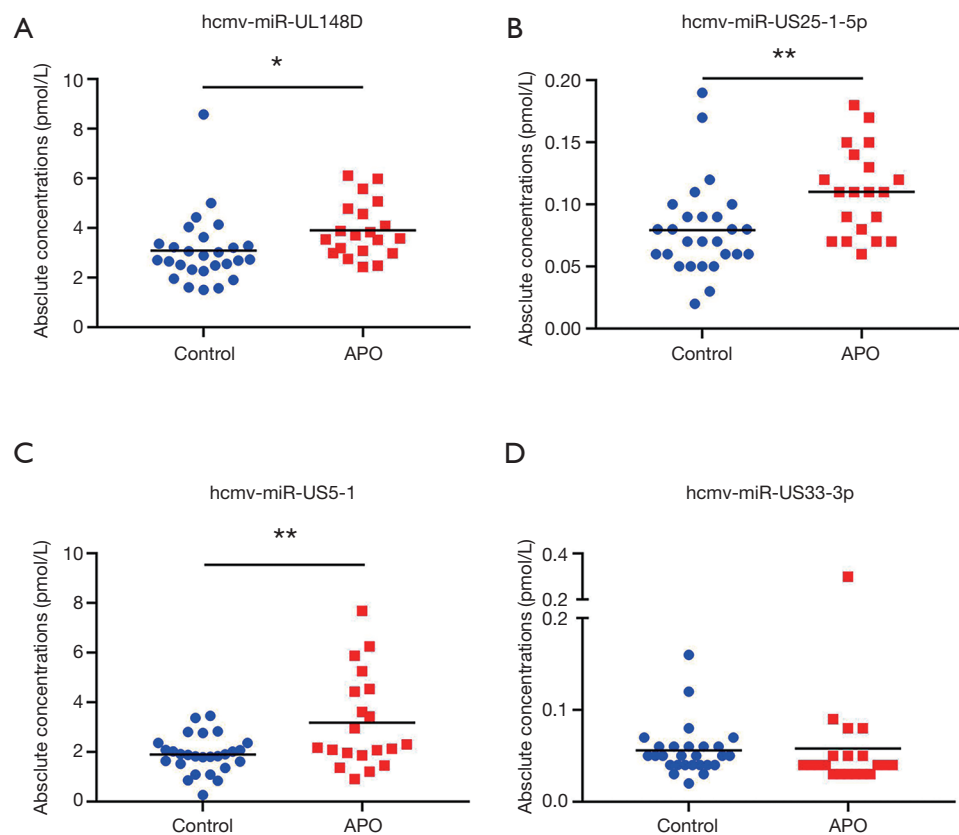
predict risk, we used APO as the dependent variable and the risk score as the covariate to conduct a univariate logistic regression analysis. Hcmv-miR-US25-1-5p and hcmv-miR-US5-1 correlated independently with APO (Table S7). The odds ratios (ORs) of these 2 HCMV miRNAs for pregnant women with APO were as follows: hcmv-miR-US25-1-5p (OR =26500.657, 95% CI, 2.848–246,567,602.202, P=0.029) and hcmv-miR-US5-1 (OR =1.406, 95% CI, 1.024–1.931, P=0.035). The results suggest that elevated plasma levels of hcmv-miR-US25-1-5p and hcmv-miR-US5-1 are potential risk factors for APO.

The levels of hcmv-miR-UL148D, hcmv-miR-US25-1-5p, and hcmv-miR-US5-1 in the placenta tissues of 20 pregnant women with APO and 20 with normal pregnancy

were further assessed. Our results showed that hcmv-miR-US25-1-5p in the placenta tissues were dramatically increased in APO compared with normal controls (Figure 4A), but hcmv-miR-UL148D and hcmv-miR-US5-1 could not be detected in the placenta tissues. Pearson correlation scatter plot analysis showed significant correlation between the levels of hcmv-miR-US25-1-5p in plasma and placenta tissues (P=0.0009) (Figure 4B). These data indicated that hcmv-miR-US25-1-5p could be transfer through placenta to the fetal side.

#### Detection of HCMV in pregnant women

Because expression of the 3 HCMV miRNAs was



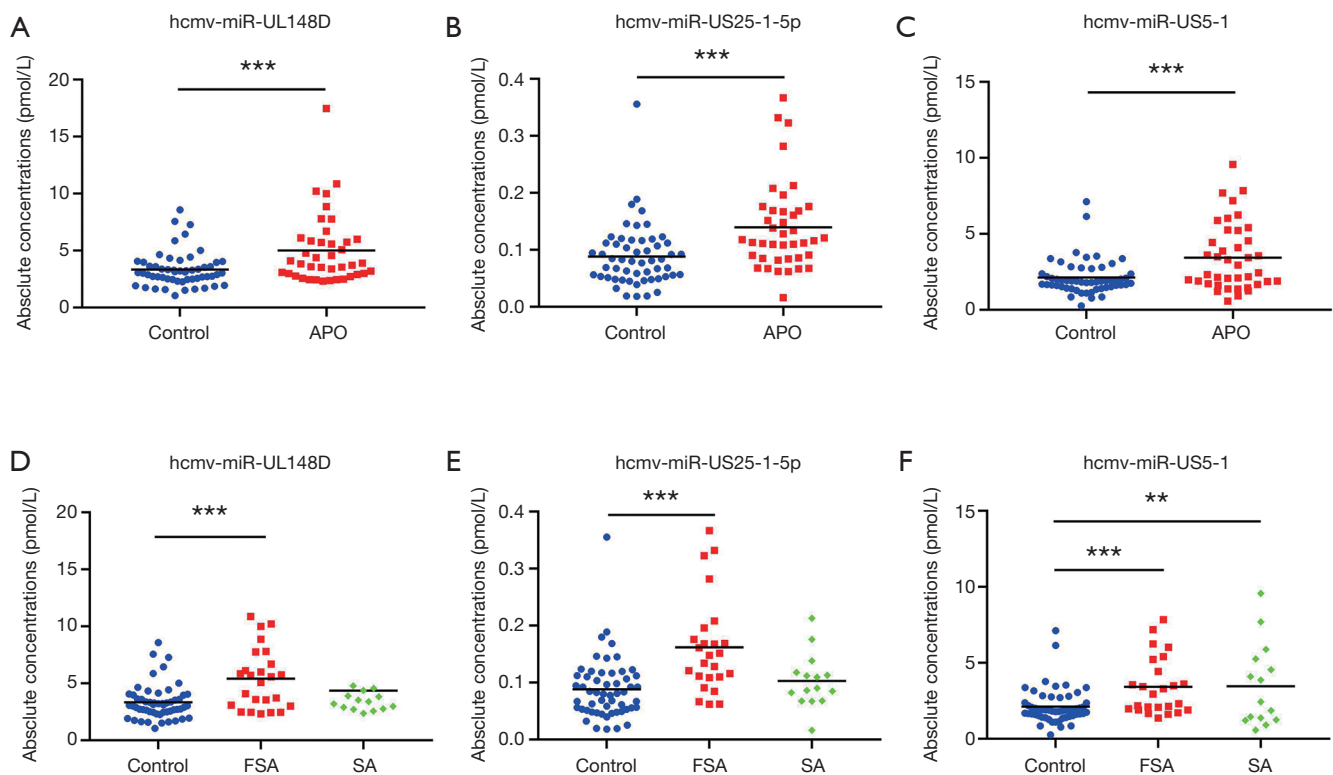
**Figure 2** Elevated plasma levels of HCMV-encoded miRNAs in pregnant women with APOs in the validation set. (A,B,C,D) Concentrations of 4 HCMV-encoded miRNAs were measured in 20 pregnant women with APOs and 27 normal controls using RT-qPCR. Cq values were converted to absolute values based on the standard curve. Each P value was derived from a two-sided Student's *t*-test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome; RT-qPCR, quantitative reverse-transcription polymerase chain reaction.

significantly increased in the plasma from pregnant women with APO, we investigated the discrepancy in HCMV load between pregnant women with APO and normal controls. A previous study reported that HCMV DNA in PBLs is more sensitive for diagnosing HCMV infection than that in plasma (36). Therefore, we used RT-qPCR to detect HCMV DNA in PBLs from both the training and validation sets. Nevertheless, we found no significant differences in HCMV DNA levels between pregnant women with APO and normal controls, with HCMV virus titers of (33.46–2,307)  $\pm$ 678.9 copies/mL in APO and (9.08–6,370)  $\pm$ 1,088.0 copies/mL in normal controls ( $P=0.60$ , Figure S3A). However, HCMV DNA in placenta tissues was negative in participants (20 pregnant women with APO and 20 with normal pregnancy). We further examined concentrations of anti-HCMV IgM and anti-HCMV IgG in plasma from all

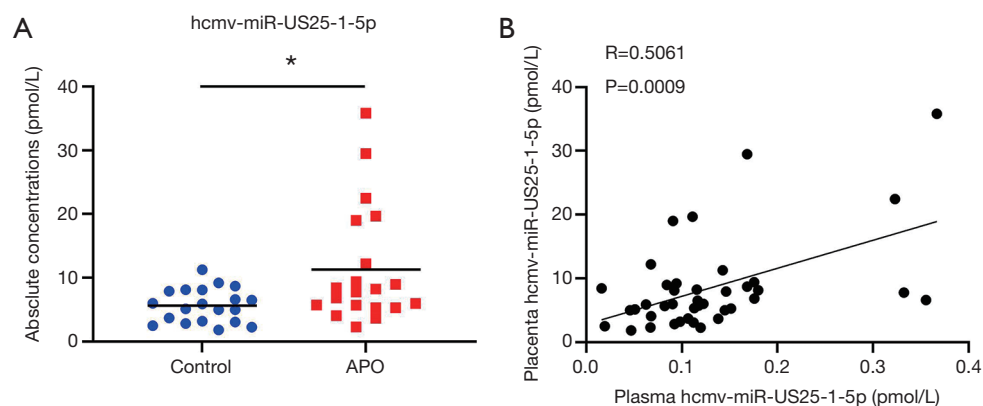
40 pregnant women with APO and 55 control individuals by ELISA. Our results indicated no significant difference in the positivity rates of anti-HCMV IgM and anti-HCMV IgG or in the plasma concentrations of anti-HCMV IgG between the two study cohorts (0% vs. 1.8%,  $P > 0.99$ ; 95.0% vs. 98.2%,  $P=0.57$ , respectively) (Table 1 and Figure S3B). In addition, linear regression was used to examine associations between clinical characteristics and plasma hcmv-miR-US25-1-5p. Nevertheless, plasma hcmv-miR-US25-1-5p was not correlated with age of pregnant women, gestational age, HCMV DNA titers in PBLs and anti-HCMV IgG concentrations (Table S8).

## Discussion

Although various types of genetic variations can cause FSA



**Figure 3** Plasma levels of 3 HCMV-encoded miRNAs upregulated in pregnant women with different types of APOs. (A,B,C) In the training and validation sets, the concentrations of 3 HCMV-encoded miRNAs were measured in 40 pregnant women with APOs and 55 normal controls using RT-qPCR. (D,E,F) The concentrations of 3 upregulated HCMV-encoded miRNAs were measured by RT-qPCR in 25 women with fetal structural anomalies, 15 women with SAs and 55 normal controls. Each P value was derived from ANOVA. Cq values were converted to absolute values based on the standard curve. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome; RT-qPCR, quantitative reverse-transcription polymerase chain reaction; SA, spontaneous abortion.



**Figure 4** Associations between placenta hcmv-miR-US25-1-5p concentration and plasma hcmv-miR-US25-1-5p concentration. (A) Concentrations of hcmv-miR-US25-1-5p in placenta were measured in 20 pregnant women with APOs and 20 normal controls using RT-qPCR. P value was derived from a two-sided Student's *t*-test. \*,  $P < 0.05$ . (B) Pearson's correlation scatter plot of hcmv-miR-US25-1-5p levels in placenta tissues and plasma. Cq values were converted to absolute values based on the standard curve. APO, adverse pregnancy outcome; RT-qPCR, quantitative reverse-transcription polymerase chain reaction.



and SA, congenital HCMV infection is one of the most important nongenetic causes of APO. However, there are no reliable qualitative or quantitative methods for the early prediction of APO due to HCMV infection in pregnant women. This study examined for the first time the relationship between HCMV-encoded miRNAs in maternal plasma and APO. Furthermore, our results indicated that plasma hcmv-miR-US25-1-5p may serve as a noninvasive biomarker of APO for pregnant women with HCMV infection.

Our study identified 3 of the 22 HCMV-encoded miRNAs, hcmv-miR-US25-1-5p, hcmv-miR-US5-1, and hcmv-miR-UL148D, to be markedly increased in pregnant women with APO. More importantly, we observed a significant difference in the concentrations of detected miRNAs among pregnant women with different types of APO. Compared with normal controls, the 3 identified HCMV miRNAs in plasma were all significantly increased in pregnant women with FSA, whereas only hcmv-miR-US5-1 was significantly upregulated in pregnant women with SA. Most notably, pregnant women with FSA had higher hcmv-miR-US25-1-5p levels than did pregnant women with SA. These findings suggest that increased expression of these three HCMV miRNAs may have a greater impact on FSA. Among the 3 miRNAs, hcmv-miR-US25-1-5p and hcmv-miR-US5-1 were significantly related to APO, suggesting that these miRNAs are potential risk factors for APO. Hcmv-miR-US25-1-5p presented the largest AUC (0.735; 95% CI, 0.635–0.836), indicating that plasma hcmv-miR-US25-1-5p may serve as a predictor of APO for pregnant women with HCMV infection. Additionally, hcmv-miR-US25-1-5p in the placenta tissues were dramatically increased in APO and correlated with plasma hcmv-miR-US25-1-5p. These results suggest that hcmv-miR-US25-1-5p can be transferred to the fetal side through the placenta, which may affect the development of the fetus.

In the present study, we assessed HCMV DNA in PBLs from our studied pregnant women and found no significant difference between pregnant women with APO and normal controls. HCMV DNA in maternal blood and/or serological investigation has become a key indicator for the diagnosis of primary infection (36). However, the correlation between HCMV DNA and congenital infection remains controversial (14,36-38). Additionally, HCMV DNA does not correlate with APO (14), consistent with our finding. Moreover, HCMV DNA in placenta tissues of pregnant women was negative in this study, while hcmv-

miR-US25-1-5p was upregulated in placenta tissues of pregnant women with APO, indicating hcmv-miR-US25-1-5p is easier to pass through the placenta than HCMV. Our results showed that more than 90% of the pregnant women in both study cohorts were anti-HCMV IgG positive, with no significant difference in positivity rates for anti-HCMV IgG or IgM. There is also no strong evidence that antiviral antibody responses provide protection against maternal infection, intrauterine transmission, or APO (39). Currently, predictors of APO in pregnant women with HCMV infection are not well defined, and solving this problem has important clinical value. Our results show that HCMV-encoded miRNAs are able to distinguish pregnant women with APO from normal controls.

In general, prognosis can only be determined until at late pregnancy if the fetus has no ultrasound features or severe ultrasound abnormalities (40,41). Sometimes invasive operations are required to obtain fetal blood for some prognostic parameter measurements (42-44). Therefore, the use of plasma HCMV miRNAs as a noninvasive predictor of APO for pregnant women with HCMV infection has great clinical significance.

Our study identified three altered HCMV-encoded miRNAs in pregnant women. Based on the fact that exogenous/endogenous small noncoding RNAs in the maternal system can be transferred through the mammalian placenta and influence fetal development and health by directly regulating fetal gene expression (22), determining whether the three HCMV-encoded miRNAs can influence fetal development warrants future study.

The validated downstream target genes for hcmv-miR-UL148D include *IER5*, *ACVR1B*, *RANTES*, and *IEX-1* (23,29,45,46), and those for hcmv-miR-US25-1-5p include *CD147*, *cyclin E2*, *BRCC3*, *YWHAE*, *UBB*, *NPM1*, and *HSP90AA* (27,47-49); those for hcmv-miR-US5-1 include *GMNN*, *IKK $\alpha$* , *IKK $\beta$* , and *US7* (50-53). These validated target genes can be divided into four categories: viral latency (*IER5*, *ACVR1B*, and *RANTES*), viral replication (*YWHAE*, *UBB*, *NPM1*, *HSP90AA*, *GMNN*, and *US7*), immune evasion (*ACVR1B*, *RANTES*, *IKK $\alpha$* , and *IKK $\beta$* ), and cell processes (*IEX-1*, *CD147*, *cyclin E2*, *BRCC3*). Downregulation of these genes by HCMV-encoded miRNAs may interfere with host immune responses and the cell cycle, leading to abnormalities in embryonic development. The above results indicate that the HCMV-encoded miRNAs identified in our study may contribute to the pathogenesis of HCMV infection-related diseases as well as APO, but further research is needed.

Overall, our study shows that HCMV-encoded miRNAs in plasma are safe, stable, and specific biomarker candidates for APO monitoring and prognosis. Moreover, the required testing costs are relatively low. As the factors leading to APO in pregnant women are complex, it is necessary to further exclude the known confounding factors that affect the results of the maternal cohort study. This study only compared the two major types of APO (FSA and SA) with normal pregnant women, and more detailed classification and larger sample sizes for prospective studies are needed. Although we clarified the relationship between plasma HCMV-encoded miRNAs and APO in pregnant women, the function of these identified HCMV miRNAs on intrauterine transmission and the pathogenic mechanism have not yet been elucidated. Therefore, further careful design and implementation of prospective studies are required.

In summary, we first report a distinctive plasma signature of HCMV-encoded miRNAs in pregnant women with APO and that plasma levels of hcmv-miR-US25-1-5p and hcmv-miR-US5-1 in pregnant women are associated with APO. Furthermore, hcmv-miR-US25-1 may constitute a noninvasive biomarker for predicting APO in pregnant women during HCMV infection. These findings indicate that HCMV-encoded miRNAs may provide pathophysiological clues for the development of APO and warrant further study.

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## Footnote

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**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Qiqihar Medical University {[2017]19} and individual consent for this retrospective analysis was waived.

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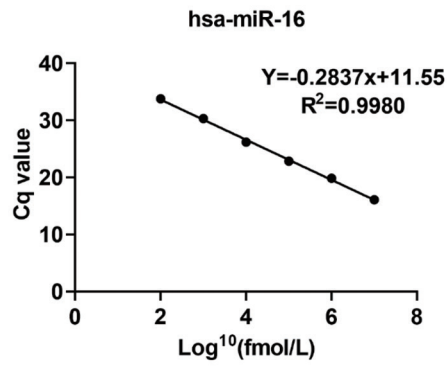
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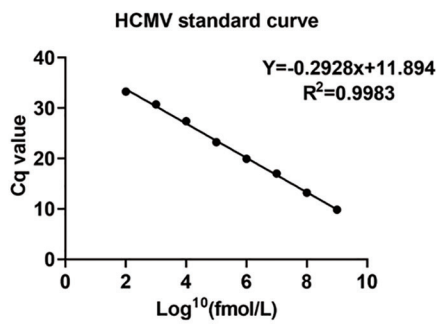
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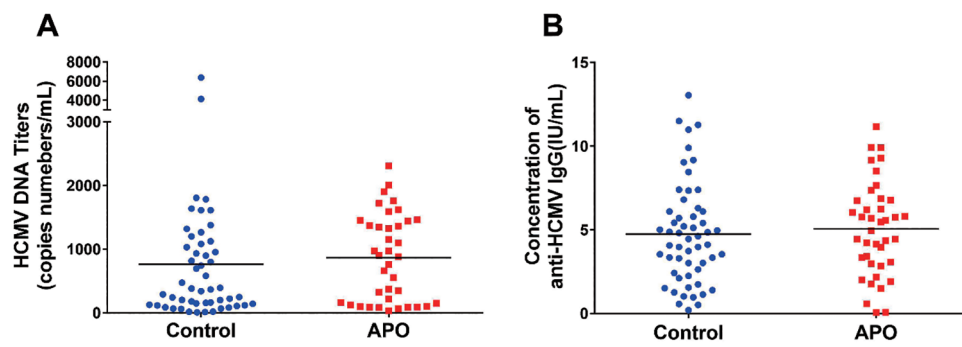
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**Figure S1** Standard curves of miR-16 using synthetic miRNAs. miRNA, microRNA.



**Figure S2** Standard curve of recombinant plasmid that contained the HCMV target sequence. HCMV, human cytomegalovirus.



**Figure S3** Detection of HCMV and its relationship with HCMV-encoded miRNAs. (A) The HCMV DNA titers were calculated in PBLs from pregnant women with APOs and compared with those from normal controls. (B) Comparison of the concentrations of anti-HCMV IgG in the APO group (n=40) versus the control group (n=55). HCMV, human cytomegalovirus; miRNA, microRNA; PBL, peripheral blood leukocyte; APO, adverse pregnancy outcome; IgG, immunoglobulin G.



**Table S1** Gestational age of pregnant women with adverse pregnancy and normal controls in the present study

Training set			Validation set			Placenta tissues set		
Case number	Gestational age	Type of pregnancy	Case number	Gestational age	Type of pregnancy	Case number	Gestational age	Type of pregnancy
N1	20	Normal	N29	29	Normal	N1	20	Normal
N2	36	Normal	N30	18	Normal	N2	36	Normal
N3	6	Normal	N31	37	Normal	N3	6	Normal
N4	40	Normal	N32	13	Normal	N4	40	Normal
N5	19	Normal	N33	39	Normal	N5	19	Normal
N6	38	Normal	N34	39	Normal	N7	9	Normal
N7	9	Normal	N35	9	Normal	N11	28	Normal
N8	39	Normal	N36	39	Normal	N12	18	Normal
N9	38	Normal	N37	38	Normal	N23	5	Normal
N10	37	Normal	N38	40	Normal	N24	11	Normal
N11	28	Normal	N39	16	Normal	N25	8	Normal
N12	18	Normal	N40	38	Normal	N26	13	Normal
N13	37	Normal	N41	38	Normal	N27	7	Normal
N14	40	Normal	N42	40	Normal	N28	9	Normal
N15	38	Normal	N43	19	Normal	N29	29	Normal
N16	38	Normal	N44	33	Normal	N30	18	Normal
N17	37	Normal	N45	40	Normal	N26	13	Normal
N18	39	Normal	N46	17	Normal	N45	40	Normal
N19	39	Normal	N47	16	Normal	N48	40	Normal
N20	39	Normal	N48	40	Normal	N49	27	Normal
N21	39	Normal	N49	27	Normal	C4	27	FSA
N22	38	Normal	N50	36	Normal	C8	25	FSA
N23	5	Normal	N51	13	Normal	C11	17	FSA
N24	11	Normal	N52	16	Normal	C13	23	FSA
N25	8	Normal	N53	17	Normal	C21	38	FSA
N26	13	Normal	N54	16	Normal	C22	15	FSA
N27	7	Normal	N55	5	Normal	C23	20	FSA
N28	9	Normal	C21	38	FSA	C24	39	FSA
C1	30	FSA	C22	15	FSA	C26	23	FSA
C2	24	FSA	C23	20	FSA	C28	27	FSA
C3	33	FSA	C24	39	FSA	C15	10	SA
C4	27	FSA	C25	20	FSA	C17	9	SA
C5	19	FSA	C26	23	FSA	C18	27	SA
C6	22	FSA	C27	14	FSA	C19	8	SA
C7	24	FSA	C28	27	FSA	C33	6	SA
C8	25	FSA	C29	22	FSA	C34	12	SA
C9	26	FSA	C30	29	FSA	C35	10	SA
C10	25	FSA	C31	20	FSA	C36	9	SA
C11	17	FSA	C32	26	FSA	C39	12	SA
C12	17	FSA	C33	6	SA	C40	12	SA
C13	23	FSA	C34	12	SA			
C14	18	SA	C35	10	SA			
C15	10	SA	C36	9	SA			
C16	11	SA	C37	9	SA			
C17	9	SA	C38	26	SA			
C18	27	SA	C39	12	SA			
C19	8	SA	C40	12	SA			
C20	7	SA						

FSA, fetal structural anomaly; SA, spontaneous abortion.

**Table S2** TaqMan advanced miRNA assays (Applied Biosystems)

Assay name	Mature miRNA sequence	Assay ID
hcmv-miR-UL22A-5p	UAACUAGCCUUCGGUGAGA	007677
hcmv-miR-UL22A-3p	UCACCAGAAUGCUAGUUUGUAG	006040
hcmv-miR-UL36-5p	UCGUUGAAGACACCGUGAAAGA	197212-mat
hcmv-miR-UL36-3p	UUUCCAGGUGUUUUAACGUGC	006481
hcmv-miR-UL112-5p	CCUCCGGAUCACAUGGUUACUCA	469687
hcmv-miR-UL112-3p	AAGUGACGGUGAGAUCAGGCU	006621
hcmv-miR-UL148D	UCGUCCUCCCCUUCUACCG	197215-mat
hcmv-miR-US33-5p	GAUUGUGCCCGACCGUGGGCG	197227-mat
hcmv-miR-US33-3p	UCACGGUCCGAGCACAUCCAA	467895-mat
hcmv-miR-US5-1	UGACAAGCCUGACGAGAGCGU	004641-mat
hcmv-miR-US25-1-5p	AACCGCUCAGUGGCUCGGACC	197211-mat
hcmv-miR-US25-1-3p	UCCGAACGCUAGGUCGGUUCU	467895-mat
hcmv-miR-US25-2-5p	AGCGGUCUGUUCAGGUGGAUGA	197201-mat
hcmv-miR-US25-2-3p	AUCCACUUGGAGAGCUCCCGCGGU	468261-mat
hcmv-miR-US4-5p	UGGACGUGCAGGGGAUGUCUG	469977-mat
hcmv-miR-US4-3p	UGACAGCCCGCUACACCUCU	469699-mat
hcmv-miR-US5-2-5p	CUUUCGCCACACCUAUCCUGAAAG	469274-mat
hcmv-miR-US5-2-3p	UAUGAUAGGUGUGACGAUGUCU	469255-mat
hcmv-miR-US29-5p	UGGAUGUGCUCGGACCGUGACG	CS1RUMR
hcmv-miR-US29-3p	CCCACGGUCCGGGCACAAUCA	468621-mat
hcmv-miR-US22-5p	UGUUUCAGCGUGUGUCCGCGGG	468736-mat
hcmv-miR-US22-3p	UCGCCGGCCGCGCUGUACCAGG	468548-mat
peu-MIR2911	GGCCGGGGGACGGGCUGGGA	242025-mat
hsa-miR-16	UAGCAGCACGUAAAUUUGGCG	00391

miRNA, microRNA.

**Table S3** The concentration of HCMV-encoded miRNAs in pregnant women with APOs and normal controls in the validation set

HCMV encoded miRNAs	APOs (n=20)	Controls (n=27)	Fold change	P value <sup>a</sup>
hcmv-miR-UL148D	3.91±0.25	3.08±0.27	1.27	0.035
hcmv-miR-US25-1-5p	0.11±0.01	0.08±0.01	1.38	0.006
hcmv-miR-US5-1	3.18±0.43	1.90±0.14	1.67	0.003
hcmv-miR-US33-3p	0.06±0.01	0.06±0.01	1.05	0.875

Data are presented as the mean ± SEM. <sup>a</sup>, Student *t*-test. HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome.**Table S4** The concentration of HCMV-encoded miRNAs in pregnant women with APOs and normal controls

HCMV encoded miRNAs	APOs (n=40)	Controls (n=55)	Fold change	P value <sup>a</sup>
hcmv-miR-UL148D	5.01±0.49	3.32±0.20	1.51	<0.001
hcmv-miR-US25-1-5p	0.11±0.01	0.08±0.01	1.58	<0.001
hcmv-miR-US5-1	3.44±0.35	2.11±0.16	1.63	<0.001
hcmv-miR-US33-3p	0.09±0.01	0.06±0.01	1.43	0.041

Data are presented as the mean ± SEM. <sup>a</sup>, Student *t*-test. HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome.**Table S5** The concentration of HCMV-encoded miRNAs in two types of pregnant women with APOs and normal controls

HCMV encoded miRNAs	FSA (n=25)	SA (n=15)	Controls (n=55)	Fold change			P value <sup>a</sup>		
				FSA vs. controls	SA vs. controls	FSA vs. SA	FSA vs. controls	SA vs. controls	FSA vs. SA
hcmv-miR-UL148d	5.41±0.53	4.35±0.96	3.32±0.20	1.63	1.31	1.24	<0.001	0.105	0.298
hcmv-miR-US25-1-5p	0.16±0.02	0.10±0.01	0.09±0.01	1.83	1.16	1.57	<0.001	0.356	0.018
hcmv-miR-US5-1	3.42±0.38	3.46±0.70	2.11±0.16	1.62	1.64	0.99	<0.001	0.005	0.954

Data are presented as the mean ± SEM. <sup>a</sup>, Student *t*-test. HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome; FSA, fetal structural anomaly; SA, spontaneous abortion.

**Table S6** The respective AUCs of three candidate miRNAs in ROC curve analyses for control and APO cohorts

Test result variable(s)	APOs vs. controls				
	Area	Std. error	Asymptotic sig.	Asymptotic 95% CI	
				Lower bound	Upper bound
hcmv-miR-UL148D	0.689	0.055	0.002	0.581	0.797
hcmv-miR-US25-1-5p	0.735	0.051	<0.0001	0.635	0.836
hcmv-miR-US5-1	0.688	0.058	0.002	0.574	0.801
The panel <sup>a</sup>	0.685	0.058	0.002	0.573	0.798

<sup>a</sup>, combination of three miRNAs. AUC, area under the ROC curve; miRNA, microRNA; ROC, receiver-operating characteristic; APO, adverse pregnancy outcome.

**Table S7** Univariate logistic regression analyses of plasma HCMV miRNAs for pregnant women with APOs

Variables	B	Std. error	OR	95% CI for OR		P value
				Lower	Upper	
hcmv-miR-US25-1-5p	10.185	4.662	26,500.657	2.848	246,567,602.202	0.029
hcmv-miR-US5-1	0.341	0.162	1.406	1.024	1.931	0.035

HCMV, human cytomegalovirus; miRNA, microRNA; APO, adverse pregnancy outcome; OR, odds ratio.

**Table S8** Clinical characteristics associated with the change of plasma hcmv-miR-25-1-5p in linear regression model

Variables	Unstandardized coefficient	P value
Age	4.973E-04	0.711
Gestational age	-3.438E-04	0.585
HCMV DNA titers in PBLs	1.423E-05	0.072
Anti-HCMV IgG concentrations	-2.168E-04	0.931

HCMV, human cytomegalovirus; PBLs, peripheral blood leukocyte; IgG, immunoglobulin G.