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Downregulations of circulating miR-31 and miR-21 are associated with preeclampsia

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ABSTRACT

MicroRNAs (miRNAs/miRs) are highly stable in circulating, which suppress target gene expression by base-pairing to the 3′-untranslated region. We compared the expressions of 3 circulating miRs (miR-31, miR-21, and miR-16), which are related to the control of cell apoptosis, invasion, angiogenesis and immune tolerance in non-pregnancy (n = 10), 20–34 gestational weeks normal pregnancy (20–34 GW NP, n = 20), early onset preeclampsia (EOPE, n = 20), 34–41 gestational weeks normal pregnancy (34–41 GW NP, n = 20) and late onset preeclampsia (LOPE, n = 20). Using quantitative RT-PCR, we found the levels of miR-31, miR-21 and miR-16 changed throughout different stages of pregnancy with the non-pregnancy as the calibrator. The plasma miR-31 levels were significantly down-regulated in EOPE rather than in LOPE when compared to gestational age matched normal pregnancy ($P < 0.001$). MiR-21 levels were significantly lower in LOPE compared to healthy controls ($P < 0.001$), while no significant difference was found between EOPE and 20–34 gestational weeks normal pregnancy ($P = 0.376$). The miR-16 expressions were at similar levels between preeclampsia (PE) and normal pregnancy. Receiver operating characteristic (ROC) curve analyses indicated the miR-31 differentiated EOPE patients from healthy controls with an area under the curve (AUC) of 0.875 with 95.0% sensitivity and 70.0% specificity. ROC curves also discriminated the LOPE patients from healthy pregnancy with an AUC of 0.793, 65.1% sensitivity and 90.3% specificity for plasma miR-21 levels. This study is the first to demonstrate the difference, and circulating miR-31 may serve as a diagnostic biomarker for early onset preeclampsia meanwhile miR-21 might be a diagnostic biomarker for late onset preeclampsia.

1. Introduction

Preeclampsia (PE), a multisystem disorder that occurs in 3–5% of pregnancies, complicates with maternal and fetal morbidity [1]. It develops after 20 gestational weeks and is characterized by hypertension combined with proteinuria, which may result from defective placentation eliciting inadequate blood perfusion and ischemia [2]. Preeclampsia, especially early onset PE, is a major cause of maternal and fetal morbidity, preterm birth and intrauterine growth restriction. Most studies have implicated that inadequate invasion of cytotrophoblasts into the uterine artery, leading to reduced utero-placental perfusion, placental ischemia and altered maternal immune response may play important roles in the development of preeclampsia [3]. Despite extensive research, there still lack adequate accurate diagnostic biomarkers for PE. In the search for diagnostic markers, those that may be obtained from the circulation are preferred alternatives to those derived from organ tissue samples.

MicroRNAs (miRNAs/miRs) are endogenous small noncoding RNA molecules, usually 22–24 nucleotides long, which regulate target gene expression by pairing with the 3′-untranslated region (UTR), resulting in translation suppression of target mRNA [4]. MiRNAs play important roles in diverse cell processes such as differentiation, proliferation, apoptosis, metabolic homeostasis, tumorigenesis and immune response. In addition, these miRNAs are highly stable in circulation, allowing their use as biomarkers in a host of disease states such as cancer [5,6], cardiovascular disease [7,8] and so on. Some studies have evaluated many miRs related to placenta and circulating in preeclampsia [9–12]. Although increasing numbers of studies about circulating miRs are becoming available in the literature, few studies have evaluated miR-31, miR-21 and miR-16 in plasma as markers for preeclampsia. These

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miRNAs regulate diverse cellular and developmental processes by targeting genes involved in proliferation, apoptosis, invasion, angiogenesis and immune tolerance [13–15].

In this current study, we compared the circulating levels of the three microRNAs in plasma from non-pregnancy, 20–34 gestational weeks normal pregnancy (20–34 GW NP), early onset preeclampsia (EOPE), 34–41 gestational weeks normal pregnancy (34–41 GW NP) and late onset preeclampsia (LOPE), in order to identify noninvasive miRNA biomarkers for preeclampsia.

2. Materials and methods

2.1. Subjects

Ninety women were recruited in this study between January 2017 and March 2018, and completed informed consent forms. The study design was approved by the Qilu Hospital Ethical Committee on human research.

The study was comprised the following five groups: 1. non-pregnancy (n = 10) plasma collected from premenopausal normal women at 3-5th day after menstruation as control; 2. 20–34 gestational weeks normal pregnancy (n = 20) plasma collected from normal pregnant women who underwent routine outpatient antenatal examinations and did not develop preeclampsia; 3. early onset preeclampsia (n = 20) plasma collected from pregnant women manifesting the disease at 20–34 gestational weeks; 4. 34–41 gestational weeks normal pregnancy (n = 20) plasma collected from normal pregnant women who underwent routine outpatient antenatal examinations and did not develop preeclampsia; 5. late onset preeclampsia (n = 20) plasma collected from pregnant women manifesting the disease at 34–41 gestational weeks. All pregnant were singleton, non-smoker and not in labour. Preeclampsia was diagnosed by the elevation of blood pressure above 140/90 mmHg measured at four-hour intervals and proteinuria above 0.3 g/day after 20 gestational weeks [16]. The five groups blood samples were collected prior to any surgery and chemotherapy. Additionally, the plasma samples were collected again from preeclampstic patients again at the time of delivery. Women with pregnancy complications such as pregnancy-induced diabetes or any other medical problem that would predispose them to an alteration in their immune response such as nephritis, cancer, systemic lupus erythematosus (SLE) were excluded from the study.

2.2. Samples processing and RNAs extraction

5 ml venous blood was obtained from women who had given written informed consent, collected in EDTA vacutainer tubers. The tubers were centrifuged at 4 °C and 1500*g* for 10 min. The plasma was Frozen immediately at −80 °C without an RNA perservative until used to measured. Samples were processed and frozen within thirty minutes of collection from each woman.

MicroRNAs were extracted from plasma (200ul) using the miRcute Serum/Plasma miRNA Isolation Kit (TIANGEN, China) following the manufacturer's protocol. The OD260/280 of the microRNAs samples ranged between 1.8 and 2.0. MicroRNAs, 50 ng, were used for reverse transcription in a final volume of 20uL, using Thermo Scientific RevertAid First Strand cDAN Synthesis Kit together with miRNA specific stem-loop RT (synthesized by GenePharma), including 4ul of 5x reaction buffer, 0.5ul RNase inhibitor, 0.75ul dNTP mix (10 mM), 0.2ul reverse transcriptase, and 1.2ul RT primer in a thermal cycler (25 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min). The cDNA samples were stored at −20 °C before analysis.

2.3. MicroRNAs quantification

Real-time quantitative polymerase chain reaction (qRT-PCR) was performed by using TB Green™ Premix Ex Taq™ Kit (Takara, Japan) in the Step One Plus Real-Time PCR System. The reaction conditions were as follows: 95 °C for 30 s, 40 cycles of denaturing at 95 °C for 5 s, annealing and extending 60 °C for 30 s. U6 was used as an internal control. The relative expression values were calculated using the comparative $2^{-\Delta\Delta Ct}$ method with the non-pregnancy group as the calibrator [17].

2.4. Statistical analysis

Data is expressed as mean ± standard deviation (SD). SPSS22.0 (SPSS, Chicago, Illinois) was used for statistical analysis. Comparisons among groups involved analysis of variance (ANOVA) followed by Student-Newman-Keuls method to determine the differences between groups. $P < 0.05$ was considered statistically significant. Analysis was made by using sensitivity and specificity, and the best cut-off values were determined by the receiver operating characteristic (ROC) curve. All data were processed through GraphPad Prism 7 software.

3. Results

3.1. Characteristics of subjects

The clinical characteristics of the study groups are summarized in Table 1. All women were similar in maternal age and body mass index (BMI) ($P > 0.05$). There were no significant differences between non-pregnancy, 20–34 GW NP and 34–41 GW NP groups in systolic blood pressure and diastolic blood pressure ($P > 0.05$). The blood pressure of preeclampsia women was significantly higher than that in gestational week matched normal pregnancy ($P < 0.001$). In the EOPE group, 24 h urine protein was significantly greater than that in LOPE ($P = 0.001$), however no significant difference was observed in the blood pressure between EOPE and LOPE groups ($P = 0.142$).

3.2. Clinical outcomes of normal pregnancy and preeclampa

The clinical outcomes of 20–34 GW NP, 34–41 GW NP, early onset PE and late onset PE groups were shown in Table 2. The gestational week at delivery, mode of delivery, newborn sex, birth and placental weight showed no significant differences between 20–34 GW and 34–41

Table 1
Clinical Characteristics of Study Groups.

Variable	Non-pregnancy (n = 10)	20–34GW NP (n = 20)	EOPE (n = 20)	34–41GW NP (n = 20)	LOPE (n = 20)
Maternal age (years)	28.80 ± 5.29	29.6 ± 4.88	29.10 ± 6.03	30.05 ± 4.91	29.15 ± 5.13
BMI(kg/m^2)	21.59 ± 1.05	22.17 ± 1.24	22.29 ± 1.35	22.47 ± 1.31	22.22 ± 1.67
GW at blood collection	ND	27.78 ± 3.95	27.81 ± 3.87	37.16 ± 1.94	36.85 ± 1.88
Nulliparity n (%)	2 (20%)	11(55%)	9(45%)	10(50%)	8(40%)
24 h urine protein (g)	ND	ND	4.86 ± 2.19	ND	2.73 ± 1.33
Systolic blood pressure (mmHg)	108.00 ± 8.33	105.15 ± 9.53	160.05 ± 8.61	105.30 ± 9.52	155.85 ± 8.34
Diastolic blood pressure (mmHg)	70.50 ± 6.29	71.20 ± 5.36	102.90 ± 6.38	73.10 ± 8.51	103.35 ± 6.54

Abbreviations: 20–34GW NP, 20–34 gestational week normal pregnancy; EOPE, early onset preeclampsia; 34–41GW NP, 34–41 gestational week normal pregnancy; LOPE, late onset preeclampsia; BMI, body mass index; ND, not determined. Date are presented as mean ± standard deviation or numbers (n%).

Table 2
Clinical Outcomes of Normal Pregnancy and Preeclampsia.

	Normal Pregnancy			Preeclampsia		
	20–34GW NP (n = 20)	34–41GW NP (n = 20)	P Value	Early onset PE (n = 20)	Late onset PE (n = 20)	P Value
GW at delivery (weeks)	38.87 ± 0.92	39.27 ± 1.13	0.23	29.83 ± 3.96	37.21 ± 1.55	< 0.001
Mode of delivery						
Vaginal delivery	17	18	0.63	6	9	0.33
Caesarean section	3	2		14	11	
Newborn sex						
Female	11	10	0.75	8	13	0.11
Male	9	10		12	7	
Birth weight (gram)	3325 ± 395.87	3470 ± 384.71	0.25	966.5 ± 456.79	2597.5 ± 347.7	< 0.001
Placental weight (gram)	538.71 ± 67.85	578.06 ± 64.26	0.07	153.39 ± 71.97	404.45 ± 53.38	< 0.001

Abbreviations: 20–34GW NP, 20–34 gestational week normal pregnancy; 34–41GW NP, 34–41 gestational week normal pregnancy; PE, preeclampsia; Date are presented as mean ± standard deviation or numbers.

GW normal pregnancy. In the early onset PE group, gestational week at delivery, birth and placental weight were significantly lower than that in late onset PE, while there were no significant differences in mode of delivery and newborn sex.

3.3. Expressions of plasma miR-31, miR-21 and miR-16

Quantitative RT-PCR analysis was applied to analyze plasma miR-31, miR-21 and miR-16 expression levels in five groups: non-pregnancy (n = 10), 20–34 GW NP (n = 20), early onset PE (n = 20), 34–41 GW NP (n = 20) and late onset PE (n = 20). Melting curve analysis revealed a single peak in each sample.

The miRNA expressions in non-pregnancy, gestational weeks matched normal pregnancy, early and late onset PE were markedly different. With the non-pregnancy group as the calibrator, plasma miR-31 levels were significantly reduced in early onset PE group compared with 20–34 GW NP group (0.97 ± 0.76 vs 4.20 ± 3.65, P < 0.001), however there was found no evident difference between 34–41 GW NP group and late onset PE group (2.39 ± 2.17 vs 3.46 ± 1.90, P = 0.132) (Fig. 1A). Meanwhile, the miR-31 expressions in normal pregnancy revealed up-regulated at 20–34 GW NP (4.20-fold change, P < 0.001) and 34–41 GW NP (2.39-fold change, P = 0.116), when compared to non-pregnancy.

Maternal plasma miR-21 was significantly under-expressed in late onset PE (1.67 ± 1.12) when compared to 34–41 GW NP (3.77 ± 2.33) (P < 0.001). The relative expression in EOPE group was also lower than gestational weeks matched normal pregnancy group, however there was no significant difference (0.54 ± 0.60 vs 0.94 ± 1.36, P = 0.376) (Fig. 1B). We found significantly higher expression of miR-21 in 34–41 GW NP group compared to non-pregnancy (P < 0.001) and 20–34 GW NP groups (P < 0.001), whereas there was on change in miR-21 between non-pregnancy and 20–34 GW normal pregnancy groups (P = 0.883).

MiR-16 did not significantly differ between 20–34 GW NP group and EOPE group (P = 0.376), at the meantime, there was also no significant difference between 34–41 GW NP group and LOPE group (P = 0.069) (Fig. 1C). With the growth of gestational age, miR-16 expression was increased gradually form non-pregnancy, 20–34 GW normal pregnancy and 34–41 GW normal pregnant women.

In addition, measurements of miR-31, miR-21 and miR-16 showed that there were remarkably lower in women with EOPE compared with LOPE (Fig. 1). When the preeclampsia patients were admitted to hospittal, given anti-hypertensive and spasmolytic therapy, plasma were sampled again at the time of delivery to evaluate miR-31, miR-21 and miR-16 changes. However, there were no significant changes in the three miRNAs expressions between sampled before treatment and sampled at delivery (Table 3).

3.4. Diagnosis value of plasma miR-31 and miR-21

To find out whether plasma miR-31 and miR-21 levels could be used as potential diagnostic markers for preeclampsia, ROC curves and areas under the ROC curves (AUCs) were performed. The results showed that the expression levels of miR-31 differentiated EOPE patients from 20–34 GW healthy controls, with an AUC of 0.875 (95% confidence interval [CI]: 0.762–0.988, P < 0.001) (Fig. 2A). The cut-off value was 0.947, which was associated with sensitivity of 95.0% and specificity of 70.0%. ROC curves also discriminated the LOPE patients from the gestational weeks matched healthy controls, with an AUC of 0.793 (95% CI: 0.651–0.934, P < 0.01) (Fig. 2B), 65.1% sensitivity, and 90.3% specificity for plasma miR-21 levels and the cut-off value was 2.309.

4. Discussion

Preeclampsia, especially early onset PE, is a major cause of maternal and perinatal morbidity. Early and late onset preeclampsia, defined as preeclampsia developed before and after 34 weeks of gestation, respectively [18]. Although early onset PE represents the minority of

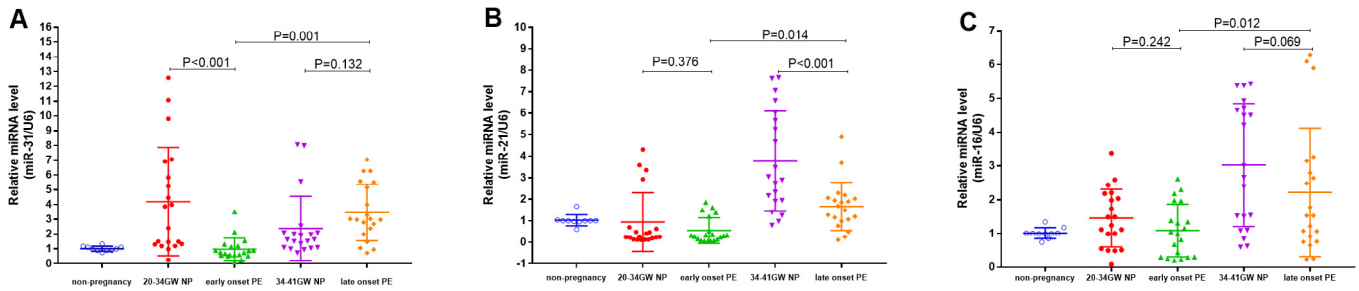

Fig. 1. Scatter plots of the miRNAs relative expressions in women plasma. The expression ratios were calculated using the non-regnancy group as the calibrator and U6 was used as an internal control. (A) Comparison of miR-31 levels. (B) Comparison of miR-21 levels. (C) Comparison of miR-16 levels. GW, gestational week; NP, normal pregnancy; PE, preeclampsia.

Table 3
Sequential miRNAs Expressions in Preeclampsia Patients.

miRNA	Early onset preeclampsia			Late onset preeclampsia		
	Sampled before treatment	Sampled at delivery	P Value	Sampled before treatment	Sampled at delivery	P Value
miR-31	0.97 ± 0.76	1.03 ± 0.78	0.80	3.46 ± 1.91	3.15 ± 1.67	0.59
miR-21	0.54 ± 0.60	0.61 ± 0.42	0.69	1.67 ± 1.12	1.52 ± 0.72	0.62
miR-16	1.09 ± 0.78	1.21 ± 0.66	0.60	2.21 ± 1.90	2.17 ± 1.58	0.93

cases, it is more closely associated with significant maternal and neonatal morbidity and mortality. Around ten-fold increased risk of perinatal death and maternal death in women with early onset preeclampsia was observed, comparing with normal pregnancy [19,20]. In addition, some studies showed biological variations and different spectrums of pathophysiology between early and late onset preeclampsia [21–23]. Thus searching diagnostic markers for preeclampsia especially EOPE may serve as a target population for improving outcomes of preeclampsia.

To date, while numerous proteins with promising predictive value for preeclampsia have been recently reported, the widespread utility of these biomarkers still requires thorough clinical validation [24–26]. The circulating microRNAs biomarkers have obvious advantage, because changes of which in gene expression might be detectable before changes in proteins. However, little is known about the value of circulating miR-31, miR-21 and miR-16 as potential biomarkers for EOPE and LOPE, when compared to gestational weeks matched normal pregnancy. With the aim of identifying the three miRNAs circulating in blood plasma as potential biomarkers of PE, we use qRT-PCR to analyze their plasma expression levels in five groups: non-pregnancy, 20–34 GW NP, early onset PE, 34–41 GW NP and late onset PE. We focused mainly in these microRNAs being reported to play roles in pathogenesis of hypertension, obesity, vascular inflammation, insulin resistance and diabetes, angiogenesis and immune tolerance [13–15]. Interestingly, we found that the miRNAs expression pattern in non-pregnancy, different normal gestational ages, EOPE and LOPE were markedly different.

In our current study, for the first time, we have identified that plasma miR-31 levels were significantly down-regulated in early onset PE rather than in late onset PE when compared to gestational age matched normal pregnancy. Although this is the first report suggesting a potential role of miR-31 in predicting EOPE, this miRNA has been already implicated in embryonic implantation and development [27]. Interestingly, we found that the miR-31 expressions in the plasma in normal pregnancy were over-expressed when compared to non-pregnancy. Kresowik at al. [27] also found that in early pregnancy compared to estrous cycle, miR-31 expression was significantly elevated in both the endometrium and serum. The observed increased miR-31 expression during normal pregnancy suggests that it may participate in the creation of an immune-tolerant maternal environment by suppressing Forkhead Box P3 (FOXP3) and C-X-C Motif Chemokine Ligand 12 (CXCL12) [27]. In addition, miR-31 can directly suppress FIH1 (factor inhibiting hypoxia-inducible factor 1), resulting in the up-regulation of VEGF and promotion of angiogenesis [28]. Reduced miR-31 expression perhaps disturb the immune-tolerant and angiogenesis environment and could contribute to pregnancy complications such as maternal abortion and preeclampsia, however the exact mechanism remains to be elucidate in future studies.

In contrast, circulating miR-21 levels were significantly lower in PE groups at 34–41 GW compared to later gestation weeks in normal pregnancy while there was no significant difference between EOPE and gestational weeks matched healthy controls. Moreover, a significant increase in the expression of miR-21 was revealed in blood plasma at 34–41 GW normal pregnancy compared to non-pregnancy, 20–34 GW normal pregnancy, EOPE and LOPE. Jairajpuri et al. [29] revealed up-regulation of circulating miR-21 in women with PE according to the severity of the disease while Choi et al. [30] found miR-21 significantly downregulated in PE. The miR-16 expression in plasma with pregnancy without PE revealed almost three times up-regulated at 34–41 GW normal pregnancy than non-pregnancy and 20–34 GW NP. In contrast, no difference was found between PE and normal pregnancy which was in agreement with the study by Hromadnikova et al. [31]. Hu et al. [32] reported significantly increase levels of miR-16 in preeclampsia placentas, however Hromadnikova at al. [33] indicated no statistical significance in miR-16 gene expression levels in placental tissues affected with preeclampsia. These discrepancies in microRNA expression may be attributed apart from no uniform way of data normalization to variability in some factors such as race. In addition, relative contributions of maternal or fetal factors or poor placentation to the whole manifestation of preeclampsia are likely to cause different physiological changes and responses. Last but not least, differences in preeclampsia sub-classifications including the timing of onset (early vs late) and the severity (mild vs severe) also further complicate the issue, and each classify might have different microRNAs expressions. Future researches need to

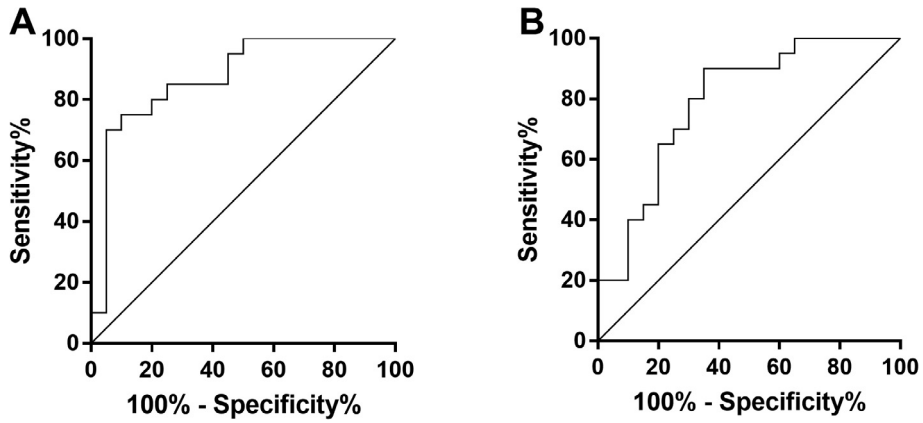

Fig. 2. ROC curves analysis of the miRNAs as potential diagnostic markers for PE. (A) ROC of miR-31 for early onset PE (AUC = 0.875, P < 0.001). (B) ROC of miR-21 for late onset PE (AUC = 0.793, P < 0.01). ROC, receiver operating characteristic; AUC, areas under the ROC. PE, preeclampsia.

be validated in big scale studies involving sufficient number of subjects.

Changes in miR-31, miR-21 and miR-16 expression in plasma samples from non-pregnant women, different periods of normal pregnancy, EOPE and LOPE were revealed in this study for the first time. Their expression levels changed throughout different stages of pregnancy with the non-pregnancy as the calibrator. Plasma levels of miR-31, miR-21 and miR-16 all showed that there were remarkably lower in women with EOPE compared with LOPE, while there were no significant changes in the three miRNAs expressions between sampled before treatment and sampled at delivery. Moreover, we demonstrated the high diagnostic value of miR-31 for early onset PE and miR-21 for late onset PE, respectively. In light of the above results, we have concluded that the expression of miRNAs in the blood plasma samples from women with preeclampsia compared to normal pregnancy should be corrected by gestational age, because their expression levels change throughout different stages of pregnancy. However, the functional significance of miR-31 for EOPE and miR-21 for LOPE pathogenesis remains to be confirmed and clarified.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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