Differential microRNA expression profile in the plasma of preeclampsia and normal pregnancies

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Abstract. Preeclampsia is a common disease in pregnant women that can only be diagnosed from 20 weeks after fertilization. Developing early diagnosis markers is urgent and would be helpful in selecting appropriate treatment strategies. The present study aimed to identify the differential expression profiles of microRNAs in the plasma between patients with preeclampsia and normal pregnancies using microarray methods. Using quantitative polymerase chain reaction (qPCR), the differentially expressed microRNAs (miRNAs or miRs) identified from the microarray analysis were validated. A total of 3 miRNAs, including hsa-miR-1304-5p, hsa-miR-320a and hsa-miR-5002-5p, were upregulated in the plasma of patients with preeclampsia pregnancies. Examination of the functions of these miRNAs demonstrated that they were involved in cell proliferation, indicating that preeclampsia affected this pathway. In addition, 26 downregulated miRNAs were identified by microarray methods. The functions of these miRNAs included immune regulation, vascular development, cancer pathology and pathology of other disease (tuberculosis, oligozoospermia, psoriasis and Alzheimer's disease). Using qPCR, the most differentially expressed miRNAs were confirmed to be hsa-miR-1304-5p, hsa-miR-320a and hsa-miR-5002-5p, which were upregulated, as well as hsa-miR-188-3p, hsa-miR-211-5p, hiv1-miR-TAR-3p, hsa-miR-4432 and hsa-miR-4498 that were significantly downregulated in the plasma of preeclampsia patients. The present findings may be useful in the development of early diagnosis markers and treatment targets for preeclampsia.

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Introduction

Preeclampsia is a common obstetric complication that represents a major factor leading to maternal perinatal mortality and a long-term impact on maternal and child health (1). Preeclampsia in pregnant women results in high risk of cerebrovascular accident, placental abruption and postpartum hemorrhage, while higher risks of fetal distress, stillbirth and neonatal asphyxia have been observed in the fetus (2). In addition, the risk of cerebral infarction and diabetes increases by 2-8 times within 20 years in preeclampsia patients (3). To date, the effect of conventional treatments on preeclampsia is not satisfactory. One of the reasons is that the intervention is always performed during the late pregnancy stages. However, once the placental pathology is formed, it is difficult for healing to occur (4,5). Thus, it is important to develop early diagnosis markers and perform early intervention in preeclampsia patients.

MicroRNA (miRNA or miR) is a small endogenous non-coding RNA molecule, which is one of the most important regulators of gene expression of protein-coding genes, serving an important role in post-transcriptional regulation (6,7). High expression levels of multiple miRNAs in the placenta have been demonstrated to regulate cell proliferation, apoptosis, migration and invasion (8). Furthermore, several miRNAs were specifically expressed in the placenta and released into the maternal blood via exocytosis (9). Comparative analysis in cases of normal pregnancy and preeclampsia patients identified abnormal miRNA expression in preeclampsia placenta. For instance, miRNA-210 is upregulated in preeclampsia placentas, and inhibits the expression levels of homeobox A9 and ephrin A3, leading to reduced cell migration and invasion (10). miR-17-92 and its paralogous miRNA-106a-363 were downregulated in the cell differentiation of trophoblasts, and their overexpression inhibited the cell differentiation (11). Previous studies have also reported the differential expression of miRNAs in the placenta at different stages of pregnancy (12,13). At the early stages of pregnancy, miRNAs that participate in tumor-derived, angiogenic, anti-apoptotic processes are highly expressed in the placenta; by contrast, miRNAs that mediate cell differentiation, tumor progression and immune regulation are highly expressed in the placenta at later stages of pregnancy (14). However, to the best of our knowledge, no previous studies have investigated the

expression profiles of miRNAs in the plasma of patients with preeclampsia pregnancies. This information may assist in the development of novel diagnosis markers.

Microarray analysis as a method to identify differential expression is a cheap and high efficient approach. In a previous study, using microarray, differentially expressed miRNAs were identified in the placenta of patients with preeclampsia and normal pregnancies (15). The present study aimed to investigate the differential expression of miRNAs in the plasma of preeclampsia and normal pregnancies. The findings of the study may be helpful for developing novel methods for the diagnosis of preeclampsia pregnancies at an early stage.

Materials and methods

Patients and sampling. Plasma was collected from women (26.83±1.17 years old) from April to May 2014 with preeclampsia pregnancies (n=3) and normal pregnancies (n=3) at the Second Xiangya Hospital of Central South University (Changsha, China). The plasma levels of placental protein 13 (PP13), placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1), pregnancy-associated plasma protein A (PAPP-A) and human chorionic gonadotropin (hCG) were determined using an AutoDELFIA immunoassay analyzer (Xi'an Yima Opto-electrical Technology Com., Ltd, Shaanxi, China), according to a previously published protocol (16). Patients with preeclampsia presented typical characteristics, according to the diagnostic criteria of preeclampsia (17). All the plasma samples were collected at the 11 weeks after fertilization without any prior clinical treatment to investigate early diagnostic index, while diagnosis was only confirmed at 20 weeks of pregnancy. Informed consents were obtained from the preeclampsia and normal pregnancy individuals. The present study was approved by the Research Ethics Committee of Second Xiangya Hospital of Central South University.

RNA isolation and analysis. The total RNAs were extracted from the plasma using RNA TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Agarose gel electrophoresis (1.2%) was used to detected RNA integrity. The quality and quantity of the total RNAs were then analyzed using Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) of >7.0 were used in subsequent analyses.

Microarray analysis. Total RNA samples from the preeclampsia pregnancies (n=3) were mixed to obtain the preeclampsia pregnancy group, while samples from normal pregnancies (n=3). The RNA groups (1 μg each) were examined by Human miRNA Expression Microarray Release 14.0 (Agilent Technologies, Inc.). The experiment and quality control were performed by CapitalBio Corp. (Beijing, China) following the manufacturer's instructions. Subsequently, the signals of the hybridization were obtained and analyzed using an Agilent G2565CA Microarray Scanner system (Agilent Technologies, Inc.). Differential expression of miRNAs between the preeclampsia and normal pregnancies was determined when P-values were <0.05. Using the Agilent GeneSpring software (version 7.3; Agilent Technologies, Inc.), the fold-changes of miRNAs were calculated.

Quantitative polymerase chain reaction (qPCR). Using qPCR, the miRNA expression profiles of the microarray analysis results were validated. cDNA was synthesized from 1 μg total RNA and AMV Reverse Transcriptase kit (Takara, Dalian, China). Next, qPCR analysis was performed on an ABI PRISM 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycling conditions of the reaction were as follows: 95˚C for 5 min, followed by 40 cycles of 10 sec at 95˚C and extension for 30 sec at 60˚C. qPCR was performed using the miScript SYBR-Green PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers (hsa-miR-1304-5p, cat. no. MS00031395; hsa-miR-320a, cat. no. MS00014707; hsa-miR-188-3p, cat. no. MS00008897; hsa-miR-211-5p, cat. no. MS00003808; hiv1-miR-TAR-3p, cat. no. MS00015393; hsa-miR-4432, cat. no. MS00041363; hsa-miR-4498, cat. no. MS00045003; hsa-miR-5002-5p, cat. no. MS00038857) were purchased from Qiagen. The expression levels of miRNAs were calculated according to the $2^{-\Delta\Delta Cq}$ method (18) and normalized to the U6 expression.

Function analysis of miRNAs. The function of the differentially expressed miRNAs was defined using the Targetscan software (www.targetscan.org) and the miRDB database (http://mirdb.org/miRDB/).

Statistical analysis. Significance differences between the two groups were analyzed using a student's t-test. The data are represented as the mean ± standard error. A value of $P<0.05$ was confirmed to indicate a statistically significant difference. All the statistical analyses were conducted using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Clinical characteristics of preeclampsia and normal pregnancies. The levels of various factors in the plasma of women with preeclampsia pregnancies are shown in Table I. Certain of these characteristics were significantly different in preeclampsia compared with the values in normal pregnancies. More specifically, PlGF was significantly lower in the preeclampsia patients compared with the control group, while hCG and PP13 were significantly increased in the preeclampsia group. However, sFlt-1 and PAPP-A levels were not markedly different in the plasma between the control and preeclampsia groups.

Quality control of RNAs. The quality of the total RNA in each plasma sample was determined using an Agilent 2100 bioanalyzer, and the RIN values of samples were found to be >7.0; 9.3 for patients with preeclampsia and 8.7 for control patients (Fig. 1A). The 18S and 28S ribosomal RNA (rRNA) peaks were clearly high, and the 5S rRNA peak was hardly observed. Agarose gel electrophoresis also demonstrated clear 28S and 18S peaks, while the 5S peak was hardly observed (Fig. 1B). Thus, the quality and quantity of these total RNAs were in accordance with the requirements for performing microarray analysis.

Table I. Characteristics of women with preeclampsia and normal pregnancies included in the present study.

Parameter	Control group (n=3)	Preeclampsia patients (n=3)
sFlt-1 (pg/ml)	1,427.84±465.44	1,579.45±478.96
PlGF (pg/ml)	49.52±5.81	38.21±6.78[a]
PAPP-A (mU/l)	5,549.33±431.47	4,732.74±786.48
hCG (mIU/ml)	23,736.33±67.43	61,250.67±4,127.69[a]
PP13 (pg/ml)	433.12±37.77	580.08±43.72[a]

[a]P<0.05 vs. control group. sFlt-1, soluble fms-like tyrosine kinase-1; PlGF, placental growth factor; PAPP-A, pregnancy-associated plasma protein A; hCG, human chorionic gonadotropin; PP13, placental protein 13.

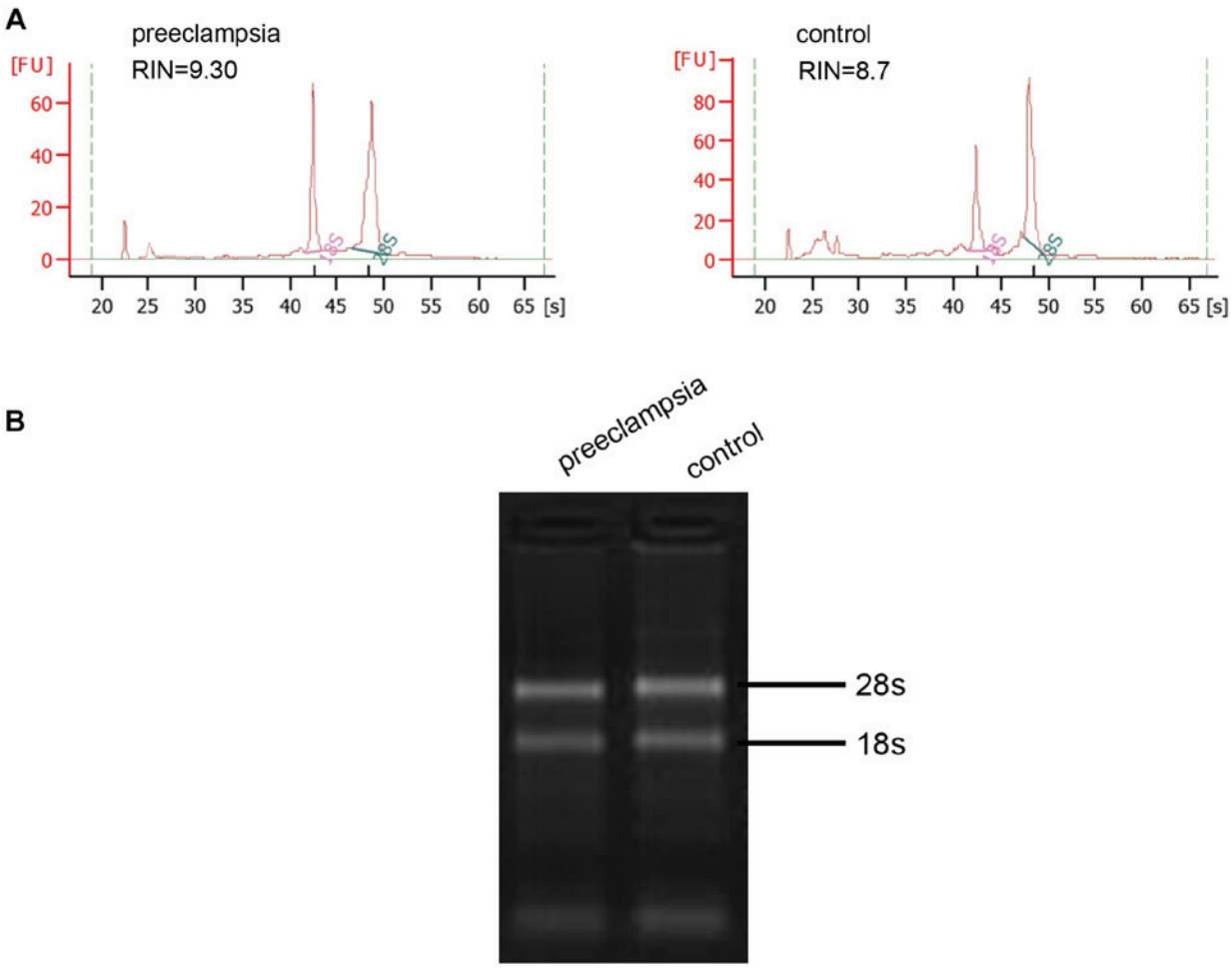

Figure 1. Quality control of the mixed total RNAs of three individuals in each group. (A) RNA quality analysis by Agilent 2100 Bioanalyzer, and (B) agarose gel analysis of total RNA obtained from the plasma of women with preeclampsia and normal pregnancies.

Differential expression of miRNAs in the plasma of preeclampsia and normal pregnancies. Microarray analysis demonstrated that 3 miRNAs were upregulated and 26 miRNAs were downregulated in the plasma of women with preeclampsia pregnancies, when compared with their expression in normal pregnancies (Fig. 2; Table II). The upregulated miRNAs were hsa-miR-1304-5p (2.10-fold), hsa-miR-320a (2.26-fold) and hsa-miR-5002-5p (1.63-fold). The fold-change of downregulated miRNAs was between 0.26 and 0.66, and the 5 most downregulated miRNAs were hsa-miR-188-3p, hsa-miR-211-5p, hiv1-miR-TAR-3p, hsa-miR-4432 and hsa-miR-4498. These results identified by microarray were then confirmed by qPCR analysis. The results of qPCR were similar to the microarray findings, indicating that hsa-miR-1304-5p, hsa-miR-320a and hsa-miR-5002-5p had significantly higher expression levels in the plasma of preeclampsia pregnancy patients (Fig. 3A). Furthermore, hsa-miR-188-3p, hsa-miR-211-5p, hiv1-miR-TAR-3p,

Table II. Differential expression of miRNAs between preeclampsia and normal pregnancies.

A, Upregulated in preeclampsia

miRNA	Fold-change	P-value
hsa-miR-1304-5p	2.10	0.05
hsa-miR-320a	2.25	0.05
hsa-miR-5002-5p	1.63	0.04

B, Downregulated in preeclampsia

miRNA	Fold-change	P-value
hsa-miR-188-3p	0.26	<0.01
hsa-miR-211-5p	0.32	0.05
hiv1-miR-TAR-3p	0.33	<0.01
hsa-miR-4498	0.40	0.03
hsa-miR-4432	0.40	0.05
hsa-miR-3184-5p	0.42	<0.01
hsa-miR-92a-2-5p	0.45	0.05
hsa-miR-424-3p	0.45	<0.01
hsa-miR-5582-3p	0.47	0.05
hsa-miR-1273c	0.47	0.03
hsa-miR-3171	0.5	0.04
hsa-miR-203a-3p	0.51	0.04
ebv-miR-BART1-5p	0.53	<0.01
hsa-miR-5009-3p	0.53	0.04
hsa-miR-892b	0.54	0.04
hsa-miR-5000-5p	0.57	0.02
hsa-miR-107	0.58	0.03
hsa-miR-3649	0.6	<0.01
hsa-miR-4482-3p	0.6	0.03
hsa-miR-506-5p	0.62	<0.01
hsa-miR-2392	0.62	0.04
hsa-miR-642b-3p	0.64	0.04
hsa-miR-4758-5p	0.64	0.05
hsa-miR-369-3p	0.65	<0.01
hsa-miR-4329	0.65	0.02
hsa-miR-3064-5p	0.66	0.02

The fold-change was calculated by Agilent GeneSpring software. miR or miRNA, microRNA.

hsa-miR-4432 and hsa-miR-4498 demonstrated significantly lower expression in preeclampsia compared with normal pregnancies (P<0.05; Fig. 3B).

Functional analysis of differentially expressed miRNA. The upregulated miRNAs in preeclampsia pregnancies, including hsa-miR-1304-5p, hsa-miR-320a and hsa-miR-5002-5p, were involved in the cell proliferation function. Furthermore, the 26 downregulated miRNAs in preeclampsia pregnancies were assigned into several functional categories, including immune regulation, vascular development, cancer pathology and pathology of other disease (tuberculosis, oligozoospermia, psoriasis and Alzheimer's disease). The functions of these miRNAs are shown in Table III.

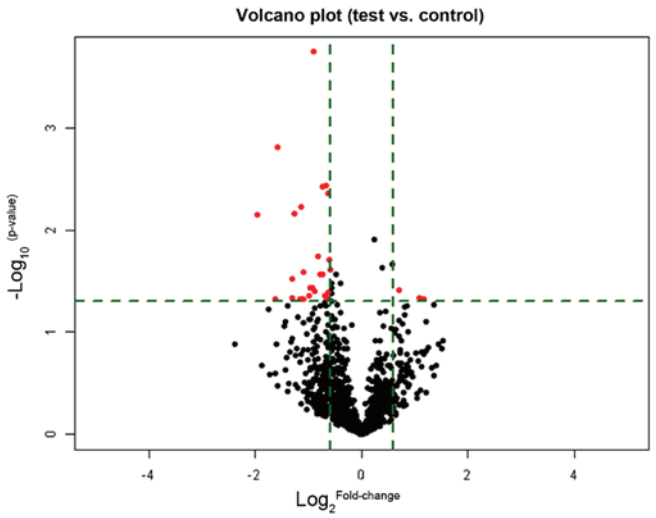

Figure 2. Differences in miRNA expression in the plasma between the preeclampsia and normal pregnancies are shown by a volcano plot. Red dots represent the significantly differentially expressed miRNAs.

Discussion

Preeclampsia is a common disease in human pregnancy, and its characteristics include elevation of blood pressure after 20 weeks of pregnancy and high expression of protein markers in urine (19,20). The symptoms in pregnant women contain headaches, abdominal pain and visual impairment (21,22). In addition, the miscarriage and stillbirth risks are high in preeclampsia patients (23). Due to the late diagnosis of preeclampsia (20 weeks), the efficacy of treatment is poor. Developing novel markers for earlier diagnosis is urgent in order to establish new treatment strategies. In the present study, the miRNAs that were differentially expressed in the plasma of preeclampsia patients were identified by microarray chip analysis. The candidate differentially expressed miRNAs are potential markers for early diagnosis of preeclampsia. In addition, these miRNAs may be new therapeutic target for the treatment of preeclampsia in the future.

Among all the miRNAs identified from the microarray analysis, only 3 miRNAs were significantly upregulated in the plasma of preeclampsia patients. These miRNAs had cell proliferation functions. hsa-miR-1304-5p has previously been demonstrated to be associated with human non-small cell lung cancer cell growth (24). Furthermore, hsa-miR-320a has been shown to mediate cell proliferation of colon cancer cells via targeting β-catenin (25). Although the evidence on hsa-miR-5002-5p is currently limited, a previous study revealed that it participates in tumorigenesis and cancer cell proliferation (26). Thus, based on results of the current study, cell proliferation-associated miRNAs were upregulated significantly in preeclampsia. Preeclampsia presents with high blood pressure, while it also has characteristics of cell proliferation (27). The present results provided a

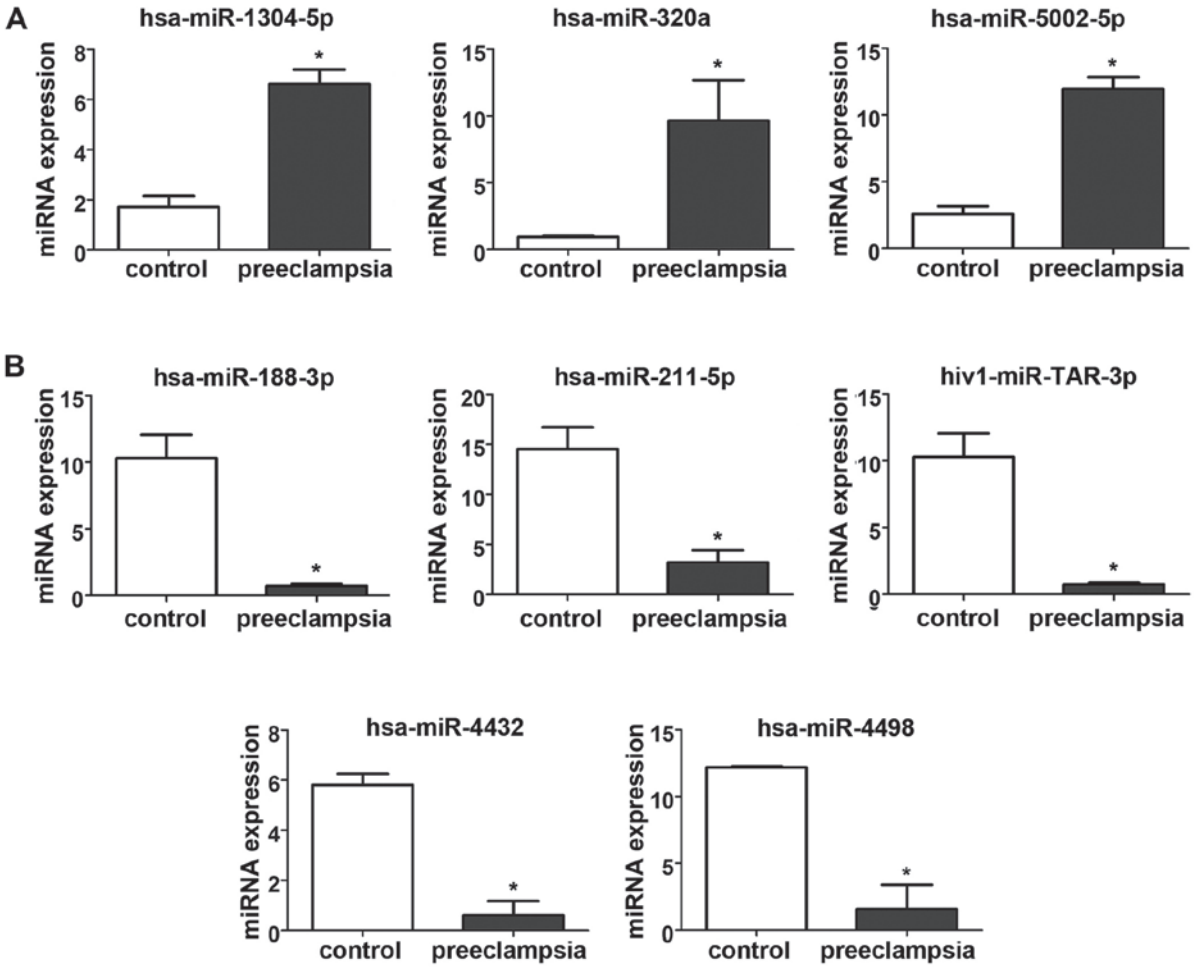

Figure 3. qPCR results demonstrating the significant differences in miRNA expression in the plasma between the preeclampsia and normal pregnancies. (A) The 3 upregulated and (B) 5 most downregulated miRNAs in preeclampsia pregnancies were analyzed by qPCR. *P<0.05 vs. normal pregnancy (control) group. qPCR, quantitative polymerase chain reaction.

molecular explanation for this phenomenon. At the early stage of preeclampsia, rapid growth of cells guarantees the requirements for embryos and placenta (28). In the present study, the highly expressed miRNAs suggested abnormal cell proliferation in preeclampsia, and these miRNAs may be novel markers for early diagnosis of preeclampsia.

Among all the downregulated miRNAs in the plasma of preeclampsia patients, the main functional category was immune function. The miRNAs with the lowest expression, including hsa-miR-188-3p, hsa-miR-211-5p, hiv1-miR-TAR-3p, hsa-miR-4432 and hsa-miR-4498, were demonstrated to participate in immune regulation. For instance, hsa-miR-188-3p participates in the growth of T cells (29), hsa-miR-211-5p is dysregulated in the plasma after hepatitis C virus infection (30), and hiv1-miR-TAR-3p expression is altered following human immunodeficiency virus-1 infection (31). Furthermore, hsa-miR-4432 is associated with the pathogenesis of chronic lymphocytic leukemia, which also represents immune dysfunction (32). Alteration of hsa-miR-4498 was also observed in major depressive disorder, which is associated with immune dysfunction (33). Based on these previous observations, the downregulation of several immune function-associated miRNAs in the plasma of preeclampsia patients indicated a depressive effect of preeclampsia on the immune system. In addition, the present study demonstrated that certain miRNAs associated with cancer pathology were downregulated in preeclampsia, and these miRNAs may suppress the pathway of cell migration and cell cycle progression. Finally, other miRNAs were identified that participated in several diseases, including tuberculosis (34), oligozoospermia (35), psoriasis (36) and Alzheimer's disease (37). These results revealed that the abnormal expression of the miRNAs may lead to the disease pathology.

In conclusion, the present study identified the differentially expressed miRNAs between preeclampsia and normal pregnancies in the plasma of patients by microarray analysis. The upregulated miRNAs in preeclampsia pregnancies participated in cell proliferation, while the downregulated miRNAs mainly participated in immune regulation, cancer pathology and the pathology of other disease. The significant difference in the expression of these miRNAs was further confirmed by qPCR. The differentially expressed miRNAs in the plasma of preeclampsia and normal pregnancies may serve as novel early diagnostic markers and treatment targets.

Acknowledgements

Not applicable.

Table III. Function of miRNAs with differential expression between preeclampsia.

A, Upregulated in preeclampsia	
miRNA	Functions
hsa-miR-1304-5p	Associated with human non-small cell lung cancer cell growth
hsa-miR-320a	Mediate cell proliferation of colon cancer cells
hsa-miR-5002-5p	Tumorigenesis and cancer cell proliferation
B, Downregulated in preeclampsia	
miRNA	Functions
hsa-miR-188-3p	Growth of T cells
hsa-miR-211-5p	Associated with Hepatitis C virus infection
hiv1-miR-TAR-3p	Associated with human immunodeficiency virus-1 infection
hsa-miR-4498	Associated with immune dysfunction
hsa-miR-4432	Pathogenesis of chronic lymphocytic leukemia
hsa-miR-3184-5p	Associated with breast cancer
hsa-miR-92a-2-5p	Associated with nasopharyngeal carcinoma metastasis
hsa-miR-424-3p	Associated with lung cancer metastasis
hsa-miR-5582-3p	Associated with differentiated thyroid carcinoma
hsa-miR-1273c	Associated with gastric cancer
hsa-miR-3171	Associated with glioblastoma
hsa-miR-203a-3p	Associated with epstein-Barr virus infection
ebv-miR-BART1-5p	Associated with nasopharyngeal carcinoma
hsa-miR-5009-3p	Associated with Colon cancer
hsa-miR-892b	Pathogenesis of glioblastoma multiforme
hsa-miR-5000-5p	Pathogenesis of Alzheimer's disease
hsa-miR-107	Associated with human non-small cell lung cancer
hsa-miR-3649	Pathogenesis of glioblastoma
hsa-miR-4482-3p	Pathogenesis of thrombocytopenia
hsa-miR-506-5p	Microvascular proliferation in glioblastoma
hsa-miR-2392	Pathogenesis of colon cancer
hsa-miR-642b-3p	Associated with breast cancer
hsa-miR-4758-5p	Associated with pancreatic cancer
hsa-miR-369-3p	Associated with psoriasis
hsa-miR-4329	Associated with tuberculosis
hsa-miR-3064-5p	Associated with ovarian cancer
hsa-miR-188-3p	Growth of T cells

miR or miRNA, microRNA.

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Availability of data and materials

All the data in the current article are available from the authors on request.

Authors' contributions

YZ and YD participated in project conception and in the study design. FZ and YZ analyzed the data and corrected the manuscript. YD wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Informed consents were obtained from the preeclampsia and normal pregnancy individuals. The present study was approved

by the Research Ethics Committee of Second Xiangya Hospital of Central South University (Changsha, China).

Consent for publication

All the patients provided written informed consent for the publication. The associated data and accompanying images were approved to publish by the patients.

Competing interests

The authors declare that they have no competing interests.

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