Research article

Identification of differentially expressed miRNAs in plasma exosomes from patients with early-onset pre-eclampsia using next generation sequencing

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ABSTRACT

Pre-eclampsia (PE), a major cause of perinatal morbidity and mortality, accounts for up to 14 % mortality of maternal and 18 % of fetal or infant mortalities. However, the pathogenesis process of PE remains unclear. The aim of this study was to identify differentially expressed microRNAs (miRNAs) in the peripheral blood exosomes of early-onset PE patients versus healthy pregnant women using high-throughput sequencing, and to find candidate miRNAs as molecular markers. *Methods*: Peripheral blood samples were collected from five preeclamptic patients and five healthy women. Exosomal miRNAs were sequenced using the Illumina HiSeq4000 sequencing platform. The target gene prediction, biological function enrichment, and signaling pathway prediction of the miRNAs with significant differences were carried out using the Starbase database software, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively. Our results showed 65 significantly differentially expressed miRNAs in the exosomes of early-onset PE patients compared to control group, with 17 up-regulated and 48 down-regulated (P < 0.05). A total of 2231 target genes were predicted for all differentially expressed miRNAs. Biological functions enriched by these target genes were mainly associated with Ras protein signal transduction, GTPase-mediated signal transduction regulation, histone modification, and β-transforming growth factor regulatory process. Key regulatory signaling pathways included TGF-β signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, tumor necrosis factor signaling pathway and EGFR tyrosine kinase inhibition signaling pathways. QPCR validation in 40 independent samples for 10 miRNAs, identified three miRNAs were confirmed in the second population. MIR7151 was a most significant differentially expressed miRNAs, and predicted its downstream regulatory gene, *KCNQ1OT1*, using Starbase software. There were significant differences in miRNA expression profiles between peripheral blood exosomes of early-onset PE patients and normal pregnant women, suggesting that these

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miRNAs may contribute to the pathophysiology of early-onset PE by regulating various biological functions and signaling pathways.

1. Introduction

Pre-eclampsia is a primary contributor to perinatal morbidity and mortality, with a mortality rate of up to 14 % in pregnant women and 18 % in fetuses or infants [1]. PE is categorized into early and late-onset PE, with the former having a more rapid progression and leading more severe consequences even prior to the manifestation of clinical symptoms [2]. Currently, there is no effective strategy to prevent or treat PE. The most viable treatment is termination of pregnancy and placental delivery. The complexity of the pathogenesis of PE and the lack of effective biochemical diagnostic system poses significant challenges to early intervention and diagnosis of PE.

Several hypotheses regarding the genetic contributors to PE have been proposed, but definitive diagnosis of PE can only be made when the mother presents symptoms, making early screening and diagnosis of PE difficult. Nowadays, advances in molecular biology technology have made prenatal screening and diagnosis of a large number of genetic diseases and gene mutations possible. Clinical observations have shown a clear familial predisposition towards PE, suggesting a genetic link that could potentially facilitate early screening and diagnosis. During the 1990s, an Icelandic study found both recessive and dominant genes associated with PE. The rarity of familial aggregation in PE allowed the implementation of genome-wide linkage analyses to identify susceptibility loci and potential candidate genes. Arngrímsson et al. performed the first genome-wide assay in 343 patients with PE and identified a susceptibility locus on chromosome 2p13 [3]. Subsequently, Fitzpatrick et al. identified additional genetic susceptibility loci for PE, which was located on chromosome 2q23,2p11-12 [4]. In 2001, Lachmeijer et al. analyzed 38 Dutch families with a history of PE and found a locus on chromosome 10q22 exhibiting a parental effect [5]. Although many studies have shown that there is a genetic predisposition to PE, evidence supporting PE as a Mendelian genetic disease is weak. PE, as a complex disorder, is thought to be arise from a combination of genetic alterations and environmental influences.

Current studies show that specific genes are involved in the pathological and physiological regulation of PE, with significant differences in expression between patients and normal subjects. These genes may be used as potential molecular markers. However, the relationship between genotypes and phenotypes of PE has not yet been fully understood. PE is a very complex disorder that is not only related to fetal and placental genes, but also to the pregnant woman's own resilience to reproduction. For example, several genes have been associated with the pathophysiology of pathophysiology of PE, including vascular endothelial growth factor (*VEGF*), transforming growth factor beta (*TGF-β*), hypoxia-inducible factor (*HIF*), tumor necrosis factor-alpha (*TNF-α*), interleukin 6 (*IL-6*), and others [6–12].

In recent years, exosomes, nanovesicles released by a variety of cells or tissues, have emerged as potential contributor to the pathology of numerous disease, including PE [13–15]. Exosomes facilitate intercellular communication and regulate functions of receptor cells, tissues, and organs by delivering biologically active components. In particular, exosomal miRNAs are closely related to the pathogenesis of PE. Recent studies have shown that the expression levels of exosomal miRNAs in preeclamptic patients are significantly altered, thereby impacting invasive capacity of maternal trophoblasts and the function and angiogenesis of endothelial cells [16]. This implies that exosomal miRNAs are ideal candidate targets for specific molecular markers.

This study aims to identify the core miRNAs involved in the regulatory pathway of early-onset PE and candidate molecular markers of early-onset PE, and investigate the mechanism and mode of action of these miRNAs. The findings of this study provide a new scientific basis for the early diagnosis and treatment of early-onset PE.

2. Materials and methods

2.1. Patients selection

This study included pregnant women who gave birth at Jiaxing Maternal and Child Health Hospital from January 2020 to June 2020. Patients were divided into two group: normal group and those with early-onset PEaccording to the routine examination results. The recruitment criteria for the normal group: a. Age between 20 and 40; b. No high risk factors in pre-pregnancy examination; c. Blood collection at 28–34 weeks; d. No foetal anomalies and complications in follow-up deliveries.

A total of 50 participants were enrolled, for the first part, miRNA sequencing, we had 10 participants, 5 in each group. For the second part, clinical sample validation, we had 40 participants, 20 in each group. All participants provided informed consent. This study was approved by the Medical Ethics Committee of Jiaxing Maternal and Child Health Hospital (Approval No. 2019 (LUN)-6). The eligibility criteria of this study included a diagnosis of early-onset PE. singleton pregnancy, gestational age of 28–34 weeks, complete pregnancy data, and age between 20 and 40 years.

2.2. Sample collection and exosome isolation

Maternal blood from each participant was drawn into EDTA anticoagulation tubes at the time of delivery. Blood was separated into plasma (upper layer) and erythrocyte (lower layer) by centrifugation at 4 °C, 4000 g for 10 min using density gradient method (Eppendorf 5810 R). The plasma and erythrocytes were stored at a temperature of - 80 °C until further experiments.

Plasma exosomes were extracted by ultracentrifugation, with the samples speed-melted at 37 °C. Subsequently, the supernatant

was filtered through 0.45 μm filter membrane and the filtrate was collected. The filtrate was transferred to a new centrifuge tube and centrifuged again at 4 °C, 100,000×*g* for 70 min with the ultra-speed rotor selected. Next, the supernatant was removed and resuspended with 10 mL of pre-cooled 1 × PBS, and then centrifuged again at 4 °C, 100,000×*g* for 70 min with the ultra-speed rotor selected. The supernatant was removed and resuspended with 250 μL of pre-cooled 1 × PBS, and 20 μL of the supernatant was electronically extracted. The collected exosomes were stored at −80 °C.

2.3. Exosomes identification

Exosome morphology was confirmed through electron microscopy. First, 10 μL of exosomes sample was added to the copper mesh to precipitate for 1 min. The floating liquid was absorbed on the filter paper. Next, 10 μL of hydrogen peroxide acetate was added to the copper mesh to precipitate for 1 min, and we absorbed the floating liquid on the filter paper. Then, we dried the exosomes at room temperature for a few minutes and carried out the imaging of electron microscopy detection at 100 kv. Finally, the results of imaging were obtained by transmission electron microscopy.

CD9, an exosome-specific protein, was detected via protein electrophoresis. After completing the electrophoresis of exosome protein, the membrane was rotated and subsequently blocked with milk at room temperature for 1 h. Post-blocking, the membrane was washed with TBST (Tris-buffered saline and Tween 20). Next, the CD9 primary antibody was added and incubated overnight at 4 °C. The membrane was then washed three times with TBST. A secondary antibody was added and incubated at room temperature for 1 h, followed by three TBST washes. Color developed and the results were photographed. In addition, nanoparticle trajectory analysis (NTA) was conducted to measure the particle size and concentration of exosome samples. This involved diluting the samples with 1 × PBS buffer, with measurement subsequently taken using ZetaViewpmx110 analyser. Data calibration was performed using 110 nm polystyrene particles.

2.4. High-throughput sequencing and analysis of exosomes miRNAs

We conducted library construction, sequencing and analysis of small RNA from 2 mL of serum samples of 5 participants in each group. Total RNA, including miRNA, was extracted by using a TRIzol kit. Quality control was performed on the total RNA from exosomes to ensure it met the prerequisites for library construction.

The unique special structure of the 3′ and 5′ ends of the Small RNA (a complete phosphate group at the 5′ end and a hydroxyl group at the 3′ end) was leveraged in the subsequent steps. With the total RNA serving as the starting sample, we synthesized cDNA by adding adapters directly at both ends of the Small RNA. After cDNA synthesis, we amplified it using PCR and isolated the target DNA fragments through Polyacrylamide Gel Electrophoresis (PAGE). Gel cutting was used to retrieve the cDNA. After the library was constructed, we conducted preliminary quantification using Qubit 2.0 and diluted the library to 1 ng/ul. We used the Agilent 2100 system to detect the insert size of the library. Once the insert size met the expectation, the effective concentration of the library was accurately quantified by Q-PCR (>2 nM) to ensure the quality of the library.

Sequencing was performed using a HiSeq4000 high-throughput sequencer. Statistical analysis was conducted to assess the expression of known miRNAs in each sample was performed. We standardized the expression using the TMP method. Screening criteria was set using the absolute value of the fold difference |FC | ≥1.0 and P value < 0.05. We used the DEseq2 software to identify and perform hierarchical clustering analysis on differentially expressed miRNAs.

In addition, we conducted miRNAs target gene prediction and functional analysis. Using Starbase software, we predicted the candidate target genes of miRNAs. The target genes of differentially expressed miRNAs were subjected to Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analyses.

2.5. Clinical sample validation of differentially expressed miRNAs

We selected the top 10 miRNAs with the smallest *P*-value selected for validation on clinical samples and investigated their association with PE phenotypes. Using our medical platform, we applied quantitative reverse transcription polymerase chain reaction (qRT-PCR) technology to verify the expression differences of miRNAs in the exosomes extracted from the peripheral blood of 20 women with early-onset PE and 20 healthy pregnant women. These samples were pre-collected, and we searched for the miRNAs that correlated with the phenotypes to elucidate the role of miRNAs in development of PE. The gestational week, maternal age, and sample collection date for the healthy participants were matched with those in the experimental group.

RNA was extracted from the samples and its concentration was determined using Quantus Fluorometer. The sample information was registered and the samples were immediately frozen at −80 °C.

2.6. RT-qPCR assay

A RT-qPCR was conducted on each extracted RNA sample following the specified reaction system composition and protocol. The reaction system for reverse transcription was configured with 5 μL dNTP Mix (10 mM Each), 2.5 μL Primer Mix, 3 ng of RNA Template, 3.4 ng of cel-miR 39, 10 μL of 5 × RT Buffer, 2.5 μL of RiboLock RNase Inhibitor (20 U/μL), and 2.5 μL of RevertAid *M*-MuLV RT (200 U/μL), reaching a total volume of 50 μL. Reverse transcription primer with specific sequences were used for hsa-miR-7151–5p RT, hsa-miR-584–5p RT, hsa-miR-6721-5p-RT, hsa-miR-520a-5p-RT, hsa-miR-520 h-RT, hsa-miR-518 b-RT, hsa-miR-148 b-3p-RT, hsa-miR-1301-3p-RT, hsa-miR-204-5p-RT, hsa-let-7c-5p RT, and cel-miR-39 R T.

RT-qPCR was performed by preparing the reaction system was 10 μL of 2 × UltraSYBR Mixture, 2 μL of Primers (F/R mix, 0.2 μM), and 8 μL of cDNA to make a 20 μL reaction system. The reaction was conducted through the following steps: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s, 72 °C for 25 s, another single cycle at 72 °C for 5 min. Lastly, the melting curve was generated from 65 °C to 95 °C.

The primers were designed as per the sequences mentioned for hsa-miR-7151–5p qRT-F, hsa-miR-584–5p qRT-F, hsa-miR-6721–5p qRT-F, hsa-miR-520a-5p qRT-F, hsa-miR-520 h qRT-F, hsa-miR-518 b qRT-F, hsa-miR-148 b-3p qRT-F, hsa-miR-1301–3p qRT-F, hsa-miR-204–5p qRT-F, hsa-let-7c-5p qRT-F, cel-miR-39 qRT-F, and miR generic qRT-R.

3. Results

3.1. Participants clinical information

This study included 10 participants for the miRNA-seq part, with 5 in each group. The clinical information was shown in Table 1. Participants: N1–N5 were for normal group, ab1-ab5 were for PE group. Both groups were distributed in the age range of 20–40 years old, and there was no significant difference in the age between the two groups, gestational age (GA): 32–33 weeks in the normal group, 30–33 weeks in the PE group, no significant difference between the two groups. BMI: 18.7–31.2 in the normal group, 20.8–26.1 in the PE group, there was no significant difference in the BMI values of the two groups. For proteinuria: there was a significant difference between the two groups, with proteinuria in the PE group basically being at the massive and abundant level, and proteinuria in the normal group being almost non-existent. SBP and DBP were significantly higher in the PE group than in the normal group ($p < 0.05$).

3.2. Characterization of plasma exosomes

Transmission electron microscopy showed the presence of cup-shaped or oval-shaped exosomes were in the serum (Fig. 1a). Western blot confirmed the expression of the exosome-specific protein CD9 (Fig. 1b). Non-adjusted image of gel for Western blot analysis was shown in S. Fig. 1. Nanoparticle trajectory analysis showed a particle diameter range of 30–200 nm, with the majority centered at 119 nm (Fig. 1c). These findings indicated that the isolated vesicles were exosomes.

3.3. Comparative expression profiling of exosomes in PE

Sequencing results showed that there were 65 significantly differentially expressed miRNAs in preeclamptic exosomes compared to controls. Among these, 17 were up-regulated and 48 were down-regulated (Fig. 2a). Furthermore, a clustering heat map of the differential miRNAs was generated to visualize the pattern of differential miRNAs expression between the PE group and the normal group (Fig. 2b). Table 2 lists the identified miRNAs, including their expression difference folds and associated *P*-values.

### 3.4. Bioinformatics analysis of differentially expressed miRNAs

The target genes of differentially expressed miRNAs were analyzed for GO enrichment across three classifications: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The top 10 enriched terms based on *P*-value included Ras protein signal transduction; axonogenesis, regulation of small GTPs, and the role of small GTPs, regulation of small GTPase mediated signal transduction; histone modification, response to transforming growth factor beta; positive regulation of cell cycle, muscle tissue development, and striated muscle tissue development (Fig. 3a).

The KEGG pathway analysis revealed the top 10 significantly enriched pathways in differentially expressed miRNAs, including: TGF-beta signaling pathway, EGFR tyrosine kinase inhibitor resistance, proteoglycans in cancer, PI3K-Akt signaling pathway, glioma,

Table 1
Participants clinical information.

Participants	Age (year)	Diagnosis	Week of pregnancy for blood sampling (GA)	Early weight (kg)	Weight at blood draw (kg)	Height (m)	BMI	Proteinuria	SBP (mmHg)	DBP (mmHg)
N1	32	Normal	32 W+2D	57.5	65.6	1.63	21.6	±	128	68
N2	32	Normal	32 W+2D	60	66.8	1.63	22.6	Negative	121	68
N3	29	Normal	32 W+6D	49	53	1.60	19.1	±	106	57
N4	32	Normal	33 W+1D	45	53.5	1.55	18.7	Negative	116	52
N5	37	Normal	33 W+2D	72	82	1.52	31.2	Negative	121	68
ab1	27	PE	31 W+4D	57	75.3	1.52	24.7	++++	148	94
ab2	21	PE	33 W+4D	60	77	1.63	22.6	+++	191	127
ab3	26	PE	31 W+1D	62	79	1.54	26.1	++	135	105
ab4	35	PE	30 W	56	87	1.64	20.8	+++	183	124
ab5	29	PE	30 W	57	62	1.54	24.0	+++	136	72

This table describes the patient clinical information. N: normal participants; ab: PE participants; GA: Gestational age; W: week; D: day; BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; Proteinuria (±): Few or occasional proteins; (+): small amount of protein; (++): moderate amount of protein; (+++): large amount of protein; (++++):abundant protein.
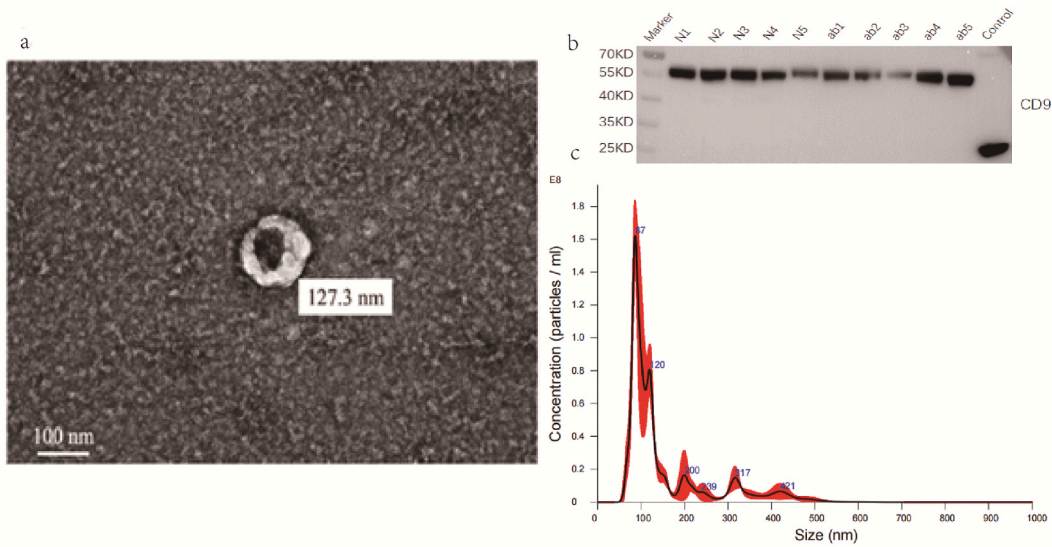
Fig. 1. Characteristics of human Plasma-derived exosomes. a: Plasma-derived exosomes as observed under electron microscope (scale bar, 100 nm); b: Western blot analysis of exosomes surface markers (CD9), control: a cell lysate used as a positive control to verify the standardisation of the Western Blot experiment, N: Normal sample; ab: PE sample; c: Representative nanoparticle tracking analysis (NTA) profile human plasma exosomes.
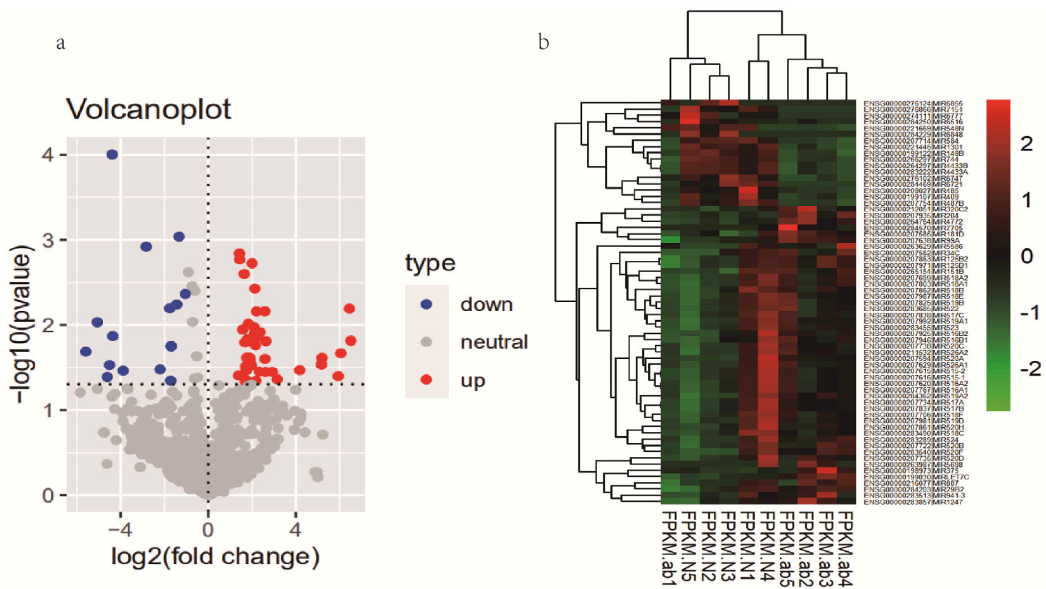
Fig. 2. Cluster analysis of differentially expressed exosomal miRNAs isolated from plasma of PE patients and normal controls. a. Volcano map showing distribution of differentially expressed microRNAs based on the p values and fold-changes. $P < 0.05$ and $|\log 2 \text{ (fold-change)}| \geq 1$ were used to show differential expression. b. Heat map showing hierarchical clustering (HCL) of normalized levels of the differentially expressed microRNAs from PE (n = 5) and normal control (n = 5). FPKM: Fragments Per Kilobase of exon model per Million mapped reads; N: Normal sample; ab: PE sample.

breast cancer, cellular senescence, MAPK signaling pathway, human papillomavirus infection, and signaling pathways regulating pluripotency of stem cells (Fig. 3b).

Starbase software was used to predict target gene of differentially expressed miRNAs, resulting in a total of 2231 target genes. Both GO analysis and KEGG analysis revealed an enrichment in biological functions such as Ras protein signaling, GTPase-mediated signal transduction regulation, histone modification and β-transforming growth factor regulatory processes. Notably, several signaling pathways were implicated, including TGF-β signaling, PI3K-Akt signaling, MAPK signaling, tumor necrosis factor signaling, and EGFR tyrosine kinase inhibition signaling (Fig. 4a and b).

Table 2
Differentially expressed miRNAs between PE patients and normal people.

| feature	id	fc	pval
gene	ENSG00000276866|MIR7151	4.942909125	9.94E-05
gene	ENSG00000207714|MIR584	2.059687179	0.000916
gene	ENSG00000284469|MIR6721	3.439378928	0.001207
gene	ENSG00000207863|MIR125B2	0.244481279	0.00145
gene	ENSG00000207971|MIR125B1	0.240244685	0.001698
gene	ENSG00000199030|MIRLET7C	0.093556357	0.001889
gene	ENSG00000199122|MIR148B	1.687393155	0.002399
gene	ENSG00000284203|MIR29B2	0.176553693	0.002517
gene	ENSG00000207594|MIR520A	0.07172262	0.003742
gene	ENSG00000266297|MIR744	1.816853521	0.004327
gene	ENSG00000221445|MIR1301	2.158244581	0.005756
gene	ENSG00000207754|MIR487B	2.433888569	0.006405
gene	ENSG00000283540|MIR520F	0	0.006453
gene	ENSG00000284362|MIR519A2	0.022967942	0.006924
gene	ENSG00000207925|MIR516B2	0.061399023	0.006953
gene	ENSG00000221669|MIR548 N	5.614159155	0.009303
gene	ENSG00000264764|MIR4772	0.128781374	0.009709
gene	ENSG00000207992|MIR519A1	0.077693787	0.010561
gene	ENSG00000207638|MIR99A	0.196608066	0.01136
gene	ENSG00000207803|MIR518A1	0.041151541	0.012177
gene	ENSG00000207699|MIR518A2	0.074390738	0.012726
gene	ENSG00000284229|MIR6848	4.915358182	0.013522
gene	ENSG00000283455|MIR523	0.060468348	0.013917
gene	ENSG00000283513|MIR941-3	0.136258708	0.014633
gene	ENSG00000284570|MIR7705	0	0.015321
gene	ENSG00000207987|MIR518E	0.110859581	0.015467
gene	ENSG00000207946|MIR516B1	0.01982157	0.015491
gene	ENSG00000207861|MIR520H	0.16704482	0.016009
gene	ENSG00000207722|MIR520B	0.067724288	0.0174
gene	ENSG00000264297|MIR4433B	2.378861869	0.017783
gene	ENSG00000283222|MIR4433A	2.379134058	0.017793
gene	ENSG00000276102|MIR6747	6.13460429	0.020628
gene	ENSG00000207562|MIR34C	0	0.021552
gene	ENSG00000207706|MIR518F	0.146058721	0.024291
gene	ENSG00000207734|MIR517A	0.122501556	0.024358
gene	ENSG00000207837|MIR517B	0.122423255	0.024362
gene	ENSG00000207862|MIR518B	0.114908515	0.024404
gene	ENSG00000283857|MIR1247	1.46E-11	0.024408
gene	ENSG00000207738|MIR520C	0.022343991	0.025099
gene	ENSG00000216077|MIR887	0.112748612	0.025669
gene	ENSG00000207615|MIR515-2	0.123916278	0.026092
gene	ENSG00000207616|MIR515-1	0.123916278	0.026092
gene	ENSG00000283289|MIR524	0.113863678	0.027262
gene	ENSG00000207735|MIR520D	2.23E-11	0.029247
gene	ENSG00000274111|MIR6777	5.057764358	0.029573
gene	ENSG00000207935|MIR204	0.099191158	0.029941
gene	ENSG00000207981|MIR519D	0.156595423	0.030665
gene	ENSG00000207767|MIR516A1	0.153533906	0.032513
gene	ENSG00000207620|MIR516A2	0.153612069	0.032579
gene	ENSG00000208027|MIR485	2.841368551	0.033311
gene	ENSG00000263987|MIR5698	5.24E-06	0.034019
gene	ENSG00000265154|MIR151B	0.14960269	0.034128
gene	ENSG00000276124|MIR6855	4.452141394	0.034439
gene	ENSG00000207585|MIR181D	0.045280444	0.035311
gene	ENSG00000198973|MIR375	0.02041764	0.035515
gene	ENSG00000211532|MIR526A2	0.007676977	0.035538
gene	ENSG00000283490|MIR518C	0.257563954	0.039272
gene	ENSG00000263629|MIR5586	0	0.040035
gene	ENSG00000284250|MIR6516	5.160604697	0.040961
gene	ENSG00000207629|MIR526A1	0.003018607	0.043683
gene	ENSG00000283685|MIR522	0.156935114	0.04413
gene	ENSG00000212051|MIR320C2	0.063984794	0.04522
gene	ENSG00000199107|MIR409	2.399349655	0.045242
gene	ENSG00000207838|MIR517C	0.158880411	0.047303
gene	ENSG00000207825|MIR519B	0.11005183	0.047597
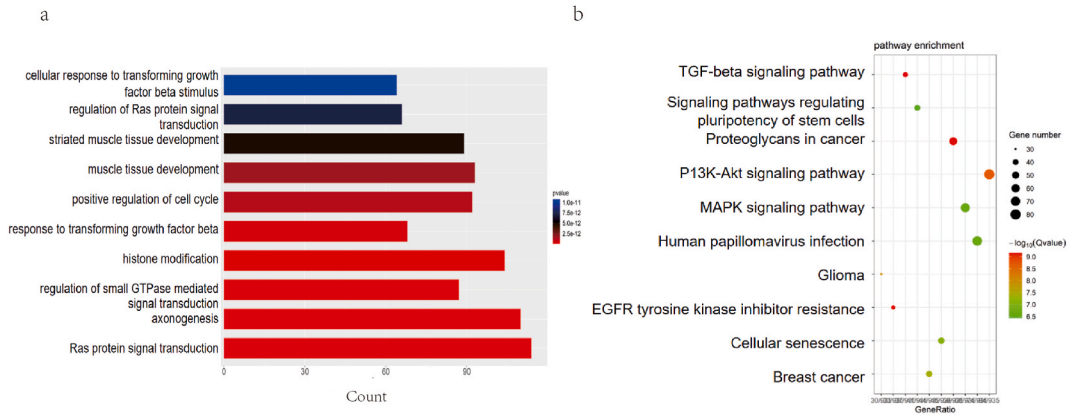
Fig. 3. Go analysis and KEGG pathway analysis. a:GO analysis of differentially expressed miRNAs for Biological Process (BP); b:KEGG pathway analysis for differentially expressed miRNAs.
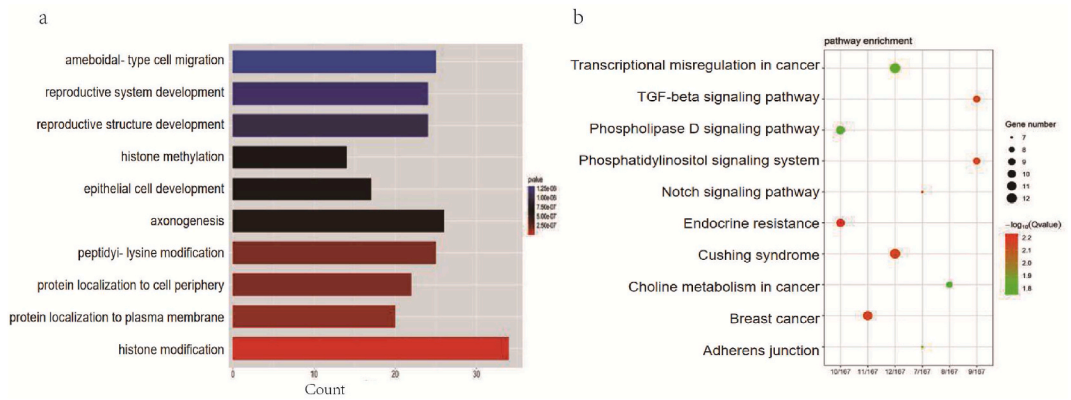
Fig. 4. GO and KEGG Pathway analysis of differentially expressed miRNA target genes. a:GO analysis for predicted target genes of differentially expressed miRNAs; b: KEGG analysis for predicted target genes of differentially expressed miRNAs.

3.5. Clinical validation of miRNAs expression

From the 65 significantly differentially expressed miRNAs identified, we selected 10 for subsequent clinical validation. These were chosen based on their *P*-values and previous literature implicating their involvement with PE. The selected miRNAs included: hsa-miR-
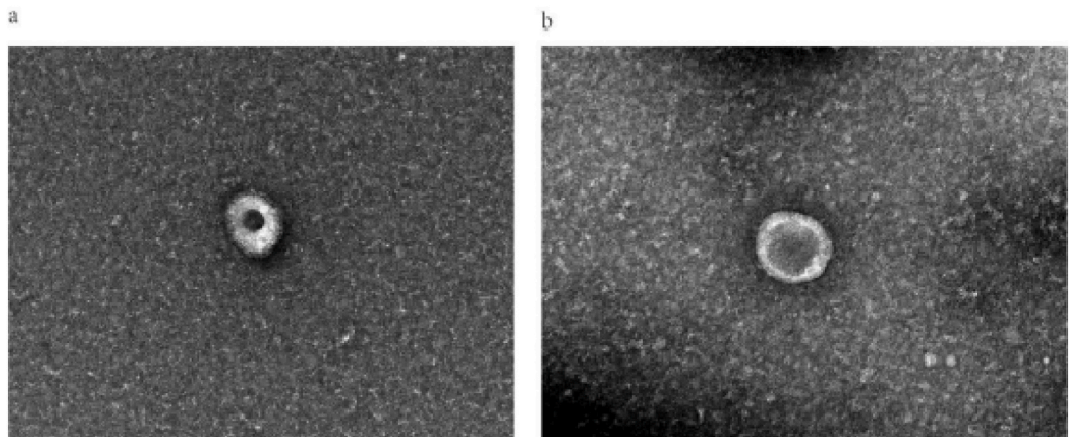
Fig. 5. Plasma-derived exosomes as observed under electron microscope (scale bar, 200 nm). a: normal people; b: PE patients.

7151–5p, hsa-miR-584–5p, hsa-miR-6721–5p, hsa-miR-520a-5p, hsa-miR-1301–3p, hsa-miR-520 h, hsa-miR-518 b, hsa-miR-204–5p, hsa-let-7c-5p, and hsa-miR-148 b-3p. Electron microscopy confirmed the exosomal morphology in collected samples from 20 normal controls and 20 PE patients (Fig. 5a and b).

Exosomal RNA was extracted, and qPCR was performed to assess the relative levels of the selected 10 miRNAs and external reference cel-miR-39 in the samples. Notably, hsa-miR-520a-5p was detected at low levels, and thus eliminated from further analysis. The qPCR results are shown in Fig. 6. In contrast to control, hsa-miR-7151–5p and hsa-miR-1301–3p were significantly up-regulated, while hsa-miR-148 b-3p was significantly down-regulated in PE patient exosomes. Other miRNA expressions appeared to be not significantly different between PE patients and controls.

Based on these discoveries, we identified hsa-miR-7151–5p as a candidate miRNA for future study because of its significant up-regulation in clinical sample validation and its low *P*-value in sequencing results. Using the Starbase database, we predicted 94 downstream genes of hsa-miR-7151–5p. The GO and KEGG annotation and enrichment analyses revealed that KCNQ10T1 and XIST might play important roles in PE regulation. Future validation studies could further investigate the relationship between KCNQ10T1 and PE.

4. Discussion

Pre-eclampsia (PE) is prevalent complication during pregnancy and a leading cause of maternal and neonatal deaths worldwide. This syndrome is primarily attributed to eclampsia and poses the most risk because of its poorly defined causative factors [17]. Our study aimed to identify potential molecular markers for early screening of this disease. In this study, we performed high-throughput sequencing of peripheral blood exosomes in both control and early-onset PE groups using Illumina HiSeq4000 sequencing platform. The data revealed significant differential expression of 65 exosomal miRNAs, showing a distinct miRNA profile in early-onset PE patients.

In order to understand the molecular mechanisms of how exosomal miRNAs modulate early-onset PE, we focused on ten predicted candidate miRNAs for validation in plasma exosomes derived from clinical samples. Through rigorous literature review and selection based on small *P*-value, seven out of these ten miRNAs were found to have explicit target genes involved in PE pathogenesis. For example, MIR584, which promotes HTR-8/SVneo cell migration by targeting *eNOS*, has been recognized as a potential novel thera-peutic target in PE [18]. Additionally, the placental-specific target genes of miR-6721, namely *IGF2R, PTCD2, SATB2, and PLAC8*, play roles in the trophoblast invasion and migration pathway. These genes, targeted by miRNA, encode proteins localized to the placenta and contributed to the pathogenesis of PE [19].

Furthermore, target genes of MIR520A, including *ACVR2B* and *IGF1R,* have also been associated with PE [20]. Reduced miR-1301 expression, inversely associated with maternal blood pressure, is observed in early-onset PE; its target gene is *LEP* [21]. Upregulated miR-520 h is associated with the risk of earlier onset of PE, with *ABCG2* as its target gene [22]. miR-518 b was found to regulate *EGR1*, affecting VEGF expression, migration and angiogenesis of HTR-8 SVneo cells [23]. Lastly, miR-204, a potential therapeutic target, may contribute to PE by inhibiting trophoblast invasion, involving *MMP9* [24]. No target genes have been reported for the other three miRNAs yet.

Our study showed that MIR7151 and MIR1301 were significantly upregulated in PE patients, consistent with the second-generation sequencing results. On the other hand, MIR148B was markedly downregulated in PE patients, conflicting with the sequencing data and hence excluded from further analysis. With *LEP* as known target gene for MIR1301 [21], we then turned our focus to MIR7151, whose target genes associated with the PE pathogenesis remain unreported. Target gene prediction software Starbase revealed 1669 MIR7151 target genes, and subsequent GO and KEGG enrichment analyses identified two significant downstream genes: *KCNQ10T1* and *XIST*. *KCNQ1OT1*, which negatively regulates miR-146a-3p to promote trophoblast cell proliferation migration and invasion via *CXCL12/CXCR4* signaling pathway, is potentially involved in PE pathogenesis [25].

We identified target genes for all differentially expressed miRNAs through the Starbase database. For instance, *TP53INP1* is a predicted target for has-miR-548 N, which is known to inhibit the migration and invasion of colorectal cancer cells [17]. Similarly, has-miR-1247 is able to protect rat cardiomyocytes from hypoxia/reoxygenation-induced injury by regulating *BCL2L11* [26]. These findings align with previous literature, emphasing the need for in-depth study of exosomal miRNAs' role in premature PE through targeted gene binding and involvement in biological pathways.

The GO and KEGG analyses revealed that these target genes are involved in biological functions such as GTPase-mediated signal regulation, histone modification, β-transforming growth factor regulatory processes, and regulation of trophoblast/endothelial cell apoptotic processes. Notably, MAPK and PI3K-Akt pathways, involved in trophoblast meiotic maturation and endothelial cell apoptosis regulation, respectively, along with *TNF-β*, closely associated with intravascular homeostasis, have been implicated in PE pathogenesis [7,12,27–29].

This study is limited by its small sample size of five PE patients and five healthy controls. In addition, due to time restrictions, we could not verify the interaction between MIR7151 and KCNQ10T1 and their impact on PE pathogenesis at a deeper level. Future experiments will examine the identified miRNAs at the cellular and animal levels for a more comprehensive understanding of the underlying mechanisms of PE.

5. Conclusions

In this study, through next generation sequencing, we discovered significant differences in miRNA expression within peripheral blood exosomes in women with early-onset PE. The subsequent GO and KEGG analyses of target genes for these differentially expressed
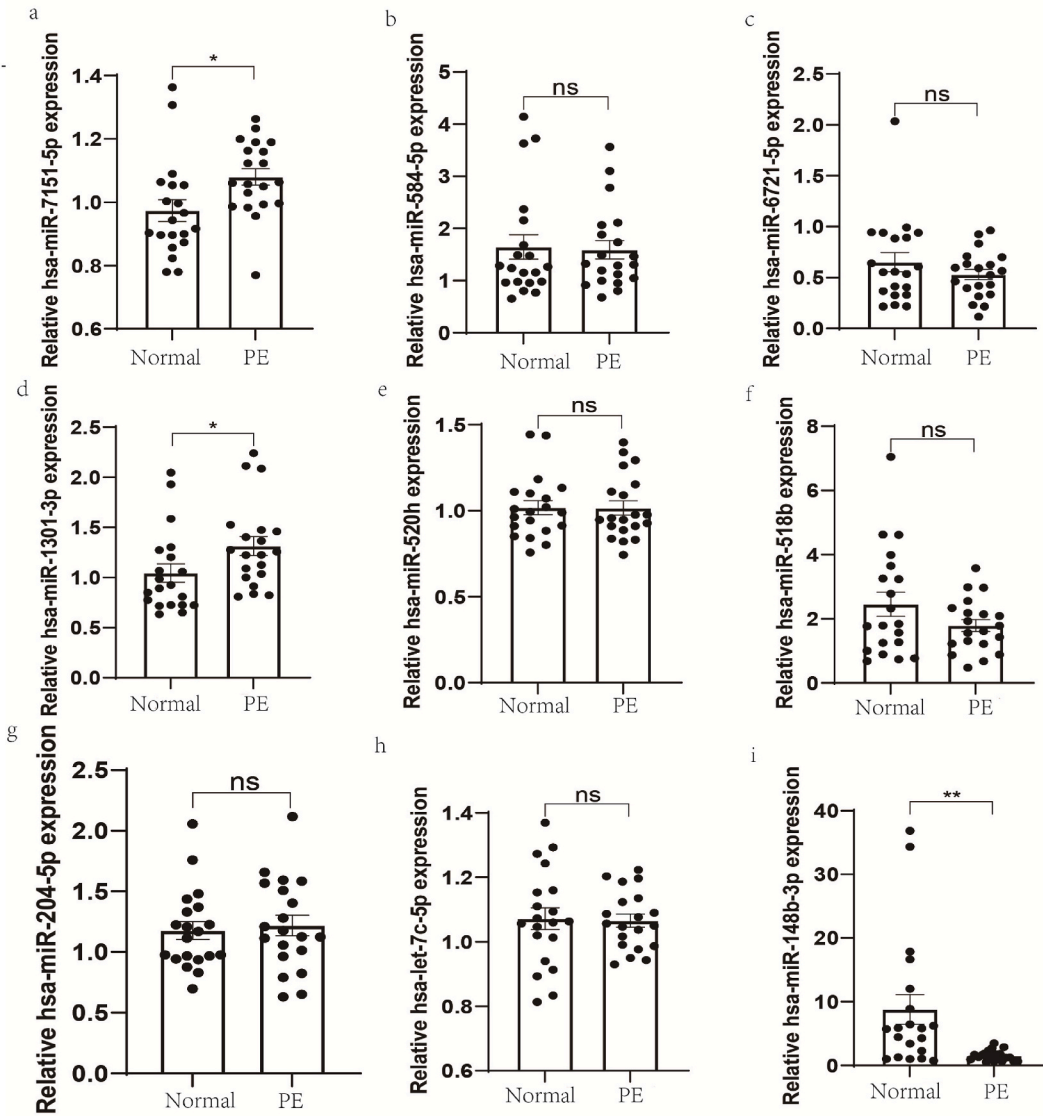
Fig. 6. Relative levels of miRNAs in plasma exosomes of normal people and PE patients by qPCR. a: Relative hsa-miR-7151–5p expression; b: Relative hsa-miR-584–5p expression; c: Relative hsa-miR-6721–5p expression; d: Relative hsa-miR-1301–3p expression; e: Relative hsa-miR-520 h expression; f: Relative hsa-miR-518 b expression; g: Relative hsa-miR-204–5p expression; h: Relative hsa-let-7c-5p expression; i: Relative hsa-miR-148 b-3p expression. Horizontal coordinates are normal and PE category samples, vertical coordinates are the relative expression of miRNAs. Ns: no significant differences.

miRNAs suggested potential involvement in the regulation of key signaling pathways such as TGF-β, PI3K- Akt, MAPK, tumor necrosis factor, and EGFR tyrosine kinase inhibitory pathways. These pathways are critical to the pathophysiological progression of PE. Our study notably revealed a specific miRNA, MIR7151, and its predicted target gene KCNQ10T1, both of which may have a vital role in regulating early-onset PE. This discovery enhances our understanding of the molecular mechanism driving the onset of early PE and provides novel insights and experimental foundations for the early diagnosis of PE.

Funding

Publication ethics

This study was approved by the Medical Ethics Committee of Jiaxing Maternal and Child Health Hospital (Approval No. 2019 (LUN)-6).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

Raw data were generated in the laboratory of Bio-X Institutes, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders (Ministry of Education), Shanghai Jiao Tong University, Shanghai. Data related to the study are not currently deposited in a publicly available repository. The data derived supporting the findings of this study are available from the corresponding author upon request.

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CRediT authorship contribution statement

Wuqian Wang: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Weihua Zhang:** Resources, Data curation. **Luan Chen:** Writing – review & editing, Formal analysis. **Xiaojia Wu:** Resources, Data curation. **Jianmei Gu:** Resources, Data curation. **Fan Yang:** Methodology, Formal analysis. **Bo Wang:** Methodology, Conceptualization. **Shengying Qin:** Supervision, Resources. **Ping Tang:** Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24543.

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