RESEARCH ARTICLE

Exosomal miRNAs as Potential Biomarkers for Preeclampsia: miR-1283 Has the Highest Expression, while miR-152-3p Has the Lowest Expression

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Abstract

BACKGROUND: Preeclampsia management is necessary, as it is one of the leading causes of death during pregnancy. Exosomal microRNAs (miRNAs) can serve as biomarkers for early detection, diagnosis, and prognosis of preeclampsia. NanoStrings is an effective method for identifying exosomal miRNA due to their high sensitivity and ability to work with small amounts of miRNA; however, the analysis using this method for determining preeclampsia biomarker is still limited. Therefore, this study was conducted to utilize the NanoStrings method in identifying preeclampsia biomarkers related to its underlying pathophysiology.

METHODS: This study involved 12 pregnant women at 20–40 weeks of gestation, including 6 preeclampsia women and 6 normotension women. The miRNAs from plasma exosomes were processed using NanoStrings method with NanoString nCounter SPRINT Profiler. Enrichment analysis of The Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were performed to examine the pathophysiological pathways of preeclampsia, using the DIANA–miRPath v3.0.

RESULTS: Forty-eight miRNAs were downregulated and 7 were upregulated (miR-1283, miR-613, miR-520a-3p, miR-3185, miR-556-3p, miR-1973, and miR-598-3p) in women with preeclampsia. The highest expression was observed in miR-1283 (log fold-change: 3.69) and the most lowest expression was in miR-152-3p (log fold-change: 1.41). Enrichment analysis showed that the most upregulated miRNAs pathways was estrogen signaling pathway, and the most downregulated was Hippo signaling pathways.

CONCLUSION: miR-1283 has the highest expression, while and miR-152-3p has the lowest expression in preeclampsia women. These miRNAs are shown to be linked to specific pathways, shedding light on the pathophysiology of preeclampsia, and may serve as promising biomarkers.

KEYWORDS: exosomes, biomarker, miRNAs, pathophysiology, preeclampsia, pregnancy

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Introduction

Preeclampsia is a placenta-based syndrome that causes morbidity, long-term impairment, and mortality during pregnancy and the postpartum period. It contributes to approximately 2–8% of all maternal fatalities, with rates as high as 14% in developing nations.(1) In Indonesia, preeclampsia is a leading cause of death, with 801 of the 3,572 total fatality cases (22.42%) in 2022 were attributable to hypertension in pregnancy.(2) Therefore, management of preeclampsia becomes important.

MicroRNAs (miRNAs) are single-stranded, medium to long (20–24 nt) noncoding RNAs that control gene expression. miRNAs play various roles as regulators of physiological processes, and their dysregulation has been linked to the pathophysiology of many diseases, including preeclampsia.(3) The number of miRNAs expressed in placental tissue and their levels increase during pregnancy and placental development.(4) miRNAs play an essential role in the biological functions of trophoblasts, such as trophoblast differentiation, invasion, migration, proliferation, apoptosis, angiogenesis, cellular metabolism (5), lipid metabolism, nervous system development, and immune and inflammatory responses.(3) miRNAs in the placenta are released into the bloodstream via exosomes, microvesicles, and apoptotic bodies. These miRNAs have been identified in the blood and plasma of mothers.(6) Plasma exosomes miRNAs are advantageous for identifying pathological changes in diseases because they are stable and unaffected by RNase, pH, and even incubation temperature. (7) Thus, identifying specific plasma exosomal miRNAs may help to reveal the pathophysiology of preeclampsia and could potentially yield biomarkers to help diagnose preeclampsia or monitor its severity during management of the condition.(8)

Exosomal miRNA has been identified in preeclampsia using next generation sequencing (NGS) technology. However, there are some shortcomings in using NGS, such as their specific role in preeclampsia and the challenges associated with their analysis due to the low abundance. Therefore, more precise and rapid analysis strategies are demanded by many. NanoStrings technology addresses these requirements by offering digital precision, high sensitivity, and reproducibility without the need for amplification. (9,10) It enables the simultaneous analysis of multiple targets with minimal RNA samples, providing advantages over other transcriptomic analyses such as quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarrays, or NGS.(11)

Various methods of miRNA analysis have yielded diverse results, suggesting that the pathophysiology of preeclampsia may vary across populations. This variation could be linked to environmental exposures, race, residency, ethnicity, lifestyle, and individual patient characteristics.(12) Since the analysis using NanoStrings method is still limited; therefore, this study was conducted to examine the profile of miRNAs to determine which exosomal miRNAs were associated with preeclampsia and which pathophysiological pathways were predominantly involved in the occurrence of preeclampsia, using the said bioinformatic analysis methods. The obtained information may form a basis for the management of preeclampsia.

Methods

Study Design and Subjects

Consecutive pregnant women (at 20–40 weeks of gestation) were enrolled in this study and were divided into two groups: 6 subjects with preeclampsia and 6 subjects with normotension. Subjects were recruited from Prof. Dr. Margono Soekarjo Hospital, Purwokerto. Preeclampsia was defined as the onset of hypertension (systolic blood pressure [BP] ≥140 mmHg and diastolic BP ≥90 mmHg at two measurements taken at an interval of at least 4 hours) and proteinuria (albumin:creatinine ratio ≥30 mg/mol; protein:creatinine ratio, ≥300 mg/24 hours; or two or more positive dipstick results) after the 20th week of gestation in a previously normotensive woman. Severe preeclampsia was defined as at least one of the following signs: systolic BP ≥160 mm or diastolic BP ≥110 mmHg; pulmonary oedema; renal abnormalities (serum creatinine >1 mg/dL); uteroplacental dysfunction; cerebral symptoms (such as persistent headaches and neurological symptoms); visual disturbances; or abnormal liver function, enzymes, or platelet counts <150,000/mL. Subjects with hypertension (BP ≥140/90) without proteinuria, but who met one of the above criteria, were classified as having severe preeclampsia.(13) Subjects with multiple pregnancies and any previous medical conditions, such as autoimmune diseases, diabetes mellitus, renal diseases, cardiovascular diseases, or infectious diseases were excluded from the study. All subjects had signed informed consent as required by the Declaration of Helsinki. The study protocol was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital (No. KE-FK/1049/EC/2021).

Exosome Isolation

Blood samples for miRNA analysis were obtained from subjects using vacutainer tubes (EDTA) during the hospital visit. The blood was centrifuged at 3,500 × g for 10 min to collect plasma, and the samples were then stored at −80°C for subsequent exosome extraction. Sample preparation involved removal of cells and debris from the plasma sample after defrosting, by centrifugation at 2,000 × g for 20 min at room temperature. The clear plasma-containing supernatant was transferred to a fresh tube without disturbing the pellet.

These tubes were then centrifuged at 10,000 × g for 20 min at room temperature to eliminate debris. Without disturbing the pellet, the supernatant containing the clarified plasma was transferred to a new tube for the exosome isolation.

The clarified plasma and 0.5 mL of phosphate buffered saline (PBS) were combined in the tube and mixed thoroughly using a vortex mixer. As much as 0.05 volumes of proteinase K was then added to the sample. After the sample was vortexed, the tube was incubated for 10 min at 37°C. Next, 0.2 volumes of the exosome precipitation reagent was added to the samples (total volume = plasma + PBS), and the samples were mixed by vortexing or inverting. The samples were then incubated for 30 min at 2–8°C and centrifuged at 10,000 × g for 5 min at room temperature. The supernatant was then removed with a pipette and discarded, before the pellet containing the exosomes was used for further extraction. To isolate exosomes and extract total RNA and to separate RNA from proteins, a Total Exosome Isolation Kit (Cat. No. 4484450; Invitrogen, Waltham, MA, USA) and a Total Exosome RNA and Protein Isolation Kit (Cat. No. 4478545; Thermo Fisher Scientific, Waltham, MA, USA) were used following the manufacturer's instructions. The obtained exosomal pellet was then resuspended in the exosome resuspension buffer. Finally, a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) was used to measure the total RNA concentration.

miRNA Testing with the NanoString nCounter System

Exosomal RNA was analyzed in the two sample groups using a NanoString nCounter SPRINT Profiler (NanoString Technologies, Seattle, WA, USA). Quick-RNA Miniprep Kit (Cat. No. R1055; Zymo Research, Sunnyvale, CA, USA) were used to extract total RNA. The genomic DNA contamination of RNA was removed using DNase and RNAClean XP (Cat. No. A63987; Beckman Coulter Diagnostics, Brea, CA, USA). The concentration of the extracted RNA samples was assessed using a NanoDrop 2000 Spectrophotometer and TapeStation (Agilent Technologies, Santa Clara, CA, USA). The nCounter Digital Analyzer recorded the reporter probe counts for every sample, and we used nSolver Software v4.0 (NanoString Technologies) and the ROSALIND platform (https://www.rosalind.bio/) for further analysis.

Before data normalisation, the following solver parameters were used to evaluate the nCounter data imaging quality control metrics: binding density, imaging, positive control limit of detection, and positive control linearity. The binding densities of the samples varied from 0.13–0.15, while the optimal range for the nCounter SPRINT system was 0.1–1.8 spots per square micron. The normalisation factor modified the variations in the analyte quality and quantity among the samples. When different degradation states were considered and the input variation was eliminated through normalisation, the acceptable values would fall into the default range of 0.1–10.

Statistical Analysis

The nSolver Analysis Software v4.0.7 (NanoString Technologies) was used to analyse the data and graphics. For comparison, ROSALIND was used to compute the fold-changes and p-values using the t-test method. The Benjamini–Hochberg technique was used to obtain the adjusted p-value and determine the false discovery rate (FDR). The miRNA ratio was compared between the groups using nSolver analytic software and the ROSALIND platform. The selection criteria for miRNA ratios were an arbitrary |fold-change| ≥1.5 and FDR ≤0.05 on the $\log_2$ scale. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were comprehensively analyzed. Analysis using the Descriptive Intermediate Attributed Notation for Ada (DIANA)–miRPath (v3.0) and microT-CDS (v5.0) algorithms (gene union interaction $p<0.05$ and microT threshold 0.8 as a boundary) were then performed. GraphPad Prism 9 (GraphPad, La Jolla, CA, USA) was used to create and analyzed all graphics.

Results

Clinical Characteristics of Subjects

A comparison of the characteristics of subjects in the preeclampsia and normotensive groups showed that age, gestational age, parity, body mass index (BMI), gestational weight, and haemoglobin levels did not differ significantly (Table 1). As expected, BP was higher in subjects with preeclampsia than in those with normotension ($p<0.05$). Proteinuria was higher and foetal weight was lower in those with preeclampsia than in those with normotension (both $p<0.05$).

Expression of miRNA in Preeclampsia and Normotension Subjects

Figure 1 showed a heatmap of miRNA expression in subjects with preeclampsia as compared to miRNA expression in those subjects with normotension. Seven miRNAs (miR-1283, miR-613, miR-520a-3p, miR-3185, miR-556-3p, miR-1973, and miR-598-3p) were found to be upregulated, and 48 were downregulated in subjects with preeclampsia.

Table 1. Clinical characteristics of participants.

Variable	Normotensive Group	Preeclampsia Group	*p*-value
Age (years)	29.33±2.06	33.33±6.86	0.221
Gestational age (weeks)	33.83±2.99	34.50±3.20	0.718
Parity	0.50±0.54	1.50±1.05	0.065
BMI (kg/m^2)	21.70±2.36	23.53±3.12	0.448
Gestational weight gain (kg)	13.28±4.45	12.66±3.38	0.793
Systolic BP (mmHg)	112.5±12.14	164.00±19.62	0.001*
Diastolic BP(mmHg)	73.17±6.49	98.83±8.72	0.001*
Proteinuria (g/dL)	0	220±224.95	0.037*
Haemoglobin (g/dL)	10.98±0.59	11.45±2.33	0.646
Foetal weight (g)	3185±542.45	2121.67±777.57	0.020*
RNA exosome concentration (ng/µL)	11.30 (6.1–16.50)	11.75 (4.6–18.60)	0.361

p-value was analyzed with t-test, *significant different if $p<0.05$.

The highest expression was observed in miR-1283 with log fold-change of 3.69, followed by miR-613 with log fold-change of 1.35.

The 10 miRNAs that have the lowest expression among 38 miRNAs were miR-152-3p, miR-4451, miR-329-5p, miR-195-5p, miR-15b-5p, miR-151b, miR-541-3p, miR-320d, miR-605-5p and miR-664b-5p. The miR-152-3p (fold-change: 1.41) and miR-4451 (fold-change: 1.36) were observed to have the lowest expression (Figure 2).

KEGG Pathway Analysis Related to Pathophysiology of Preeclampsia

The KEGG enrichment analysis indicated that the 7 upregulated miRNAs were involved in various pathways, including amino acid metabolism, signaling pathways (estrogen, transforming growth factor (TGF)-β, ErbB, and mammalian target of rapamycin (mTOR)), cellular processes (gap junction), organismal systems (axon guidance, retrograde endocannabinoid signaling, and adrenergic signaling in cardiomyocytes), and cancer biology (glioma and proteoglycans in cancer). Notably, the estrogen signaling pathway was closely linked to the upregulated miRNAs ($p<0.00001$). Moreover, pathways regulating proteoglycans in cancer, axon guidance, and estrogen signaling exhibit the highest number of target genes influenced by microRNAs. Importantly, estrogen signaling and gap junctions demonstrated significant interactions with microRNAs (Figure 3).

The KEGG analysis of the 48 downregulated miRNAs revealed their involvement in a wide range of critical pathways and processes, encompassing signaling pathways, metabolic pathways (fatty acid biosynthesis, fatty acid metabolism and amino acid metabolism), cellular processes (signaling pathways regulating pluripotency of stem cells), organismal systems, and cancer biology. Notably, the Hippo signaling pathway emerged as particularly significant among the downregulated miRNAs, with a remarkably low *p*-value ($p<0.00001$). Furthermore, pathways in cancer, proteoglycans in cancer, and the Hippo signaling pathway exhibited the most gene targets and interactions with miRNAs (Figure 4).

Discussion

In this study, the miRNA profile of plasma exosomes was compared in individuals with preeclampsia and those with normotension to identify a biomarker related to its pathophysiology. We identified 48 downregulated and seven upregulated miRNAs in women with preeclampsia. The hsa-miR-1283 has the highest expression, with a log fold-change of 3.69. miR-1283 is specified in pregnancy and part of C19MC, the largest known human microRNA cluster and is mainly expressed in placenta and undifferentiated cells releasing miRNAs into the maternal circulation in placenta-derived exosomes.(14)

These results confirmed those of previous studies that found that miR-1283 expression is elevated in preeclampsia. (15) This miRNA is related to endoplasmic reticulum (ER) stress via activating transcription factor 4 (ATF4) targets in the protein kinase R-like ER kinase (PERK) pathway. ATF4 is the primary regulator of autophagy and apoptotic processes.(16) miRNA-1283 is part of the mitogen-activated protein kinase (MAPK) pathway that targets the transcription

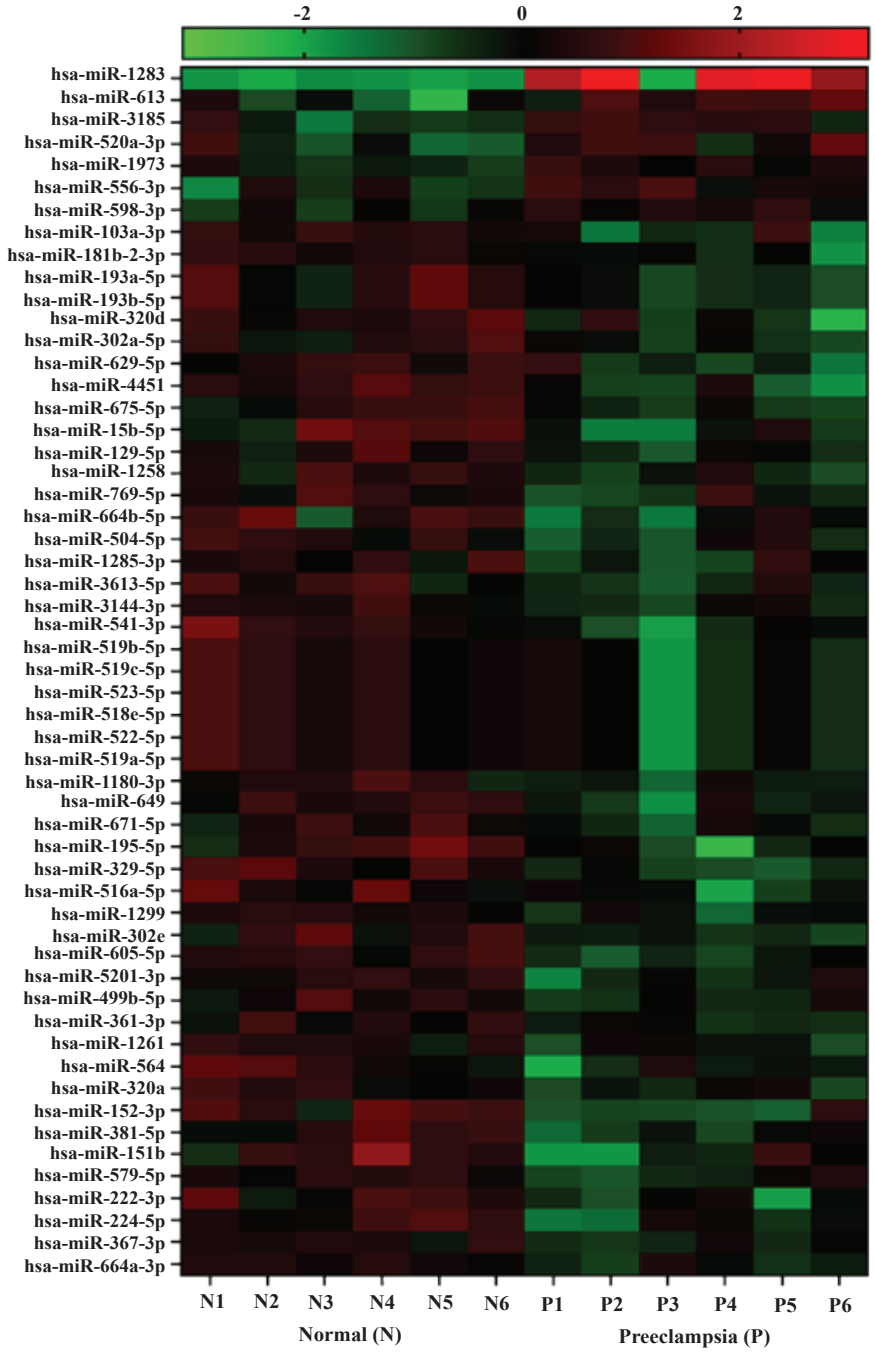

Figure 1. MiRNA expression profiles of participants in the preeclampsia and normotensive groups. The heatmap represents differentially expressed miRNAs in participants with preeclampsia (n=6) and normotension (n=6). MiRNA profiles are clustered from green to red, representing relative miRNA expression. The bar indicates relative expression level from low (green) to high (red). The groups are shown in columns, and miRNAs are shown in rows.

factor cAMP-response element binding protein (CREB). CREB is activated in response to oxidative stress, hypoxia, ER stress, and amino acid deficiencies. It controls several cellular responses, including cell survival, proliferation, and differentiation.(17,18) The ER stress affects trophoblastic invasion of the placenta.(19) Another study showed that hsa-miR-1283 regulates TGF-β2 expression.(20) TGF-β2 is part of TGF- β signaling pathway plays a crucial role in regulating various cellular processes in placentae, including trophoblast implantation, differentiation, apoptosis, and immune response. TGF-β2 also helps maintain immune tolerance at the interface between the mother and foetus by changing immune cell activity. Previous studies have shown that TGF signaling pathways, both TGF-β1 and TGF-β2, have significant changes of expression between preeclampsia and normal pregnancy.(21,22) Despite a previous study showing a decrease in miR-1283 levels at 36 weeks gestation in preeclampsia, it's important to note that this difference can be attributed to the fact that the sample used in that study only included term preeclampsia cases analyzed through microarray analysis. In contrast, current study encompassed a wider range of gestational ages, from

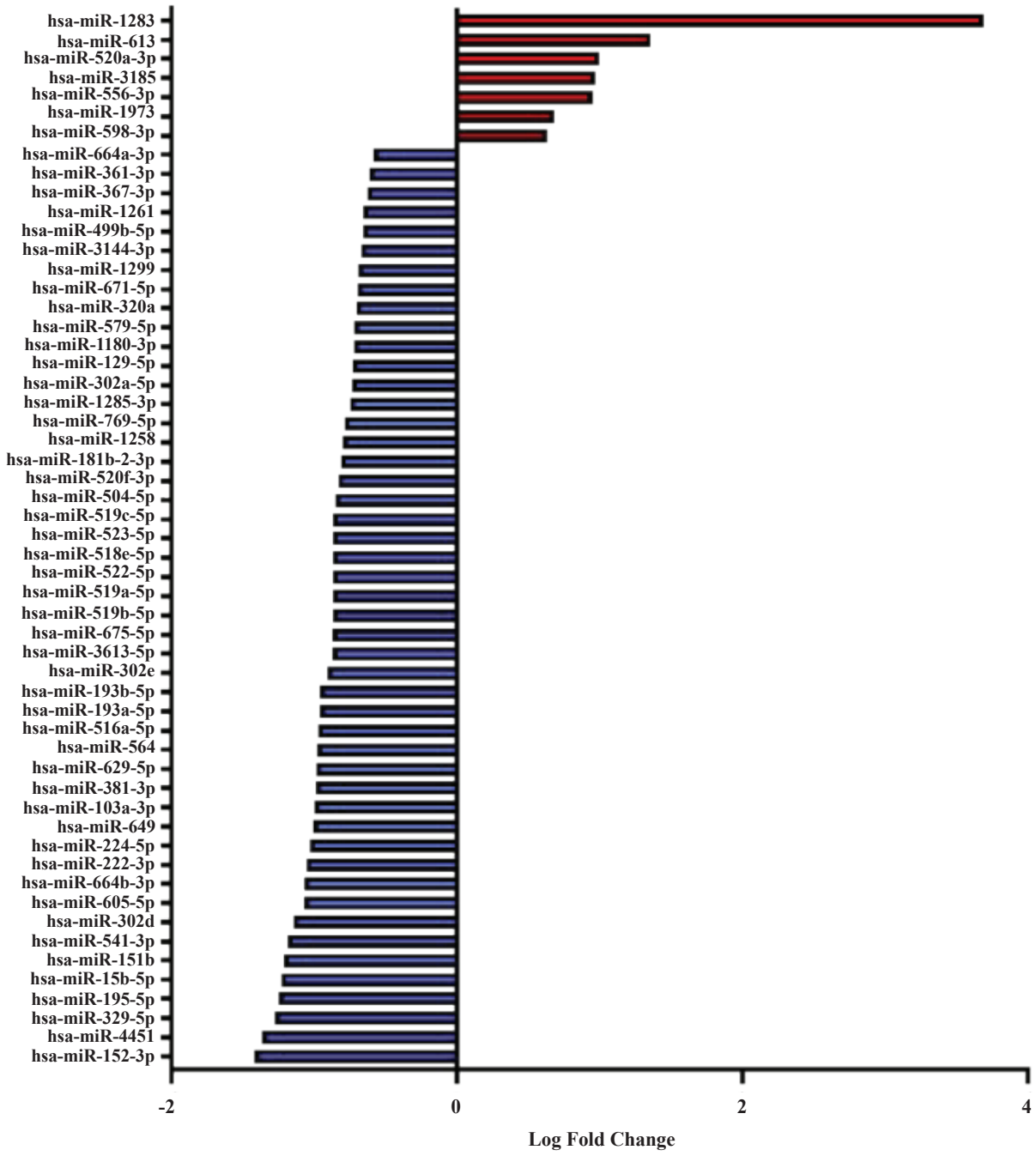

Figure 2. Fold-changes of miRNAs with upregulated and downregulated expression. Red bar represents upregulated expression, and Blue bar represents downregulated expression.

20 to 40 weeks. The high expression of hsa-miR-1283 in our study indicates its potential as a promising biomarker for preeclampsia within the population. Nevertheless, further evidence is necessary to provide comprehensive support for this conclusion.

Other miRNAs that were upregulated were also identified in this study, which are also the first reported upregulations in preeclampsia. These included the following: hsa-miR-613 that suppresses the proliferation, invasion, and angiogenesis of glioma tumours via vascular endothelial growth factor (VEGF) (23); hsa-miR-3185 which is associated with autophagic processes via autophagy-related protein (ATG)5 and ATG7 targets in pregnancy with intrahepatic cholestasis (24); hsa-miR-556-3p that shows a different expression in cases with preterm labour than in those with normal pregnancies; hsa-miR-1973 which is increased in cases of Down syndrome (25) and is associated with a reduction of apoptosis in tumours (26); and hsa-miR-

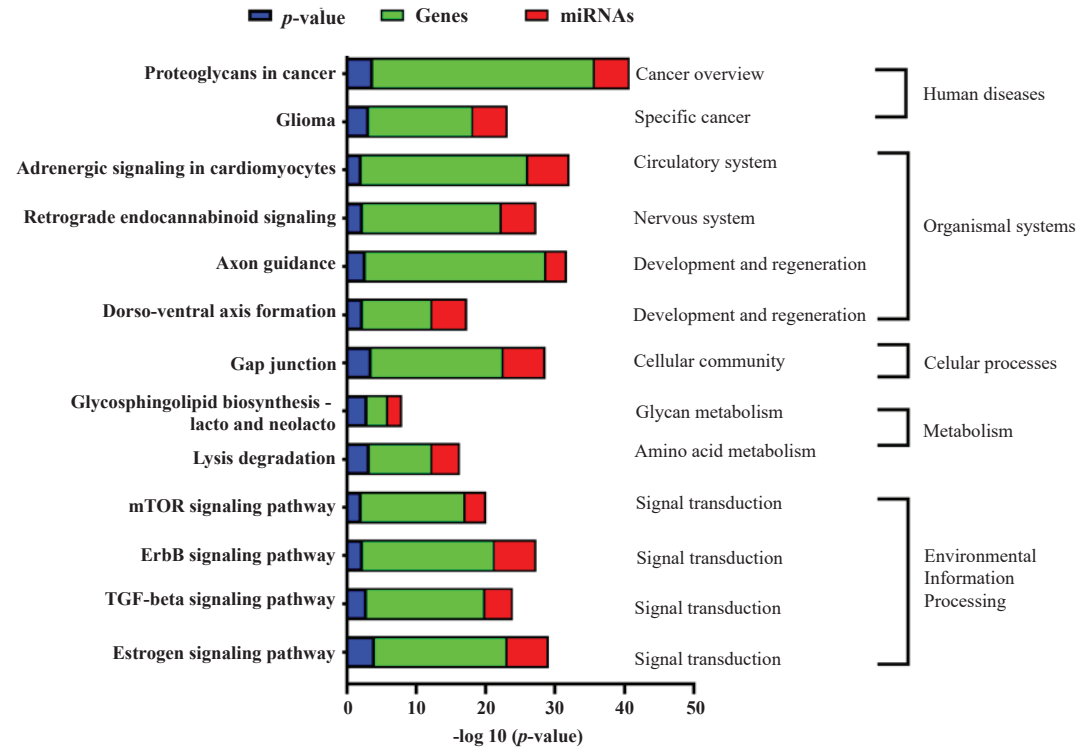

Figure 3. Enrichment analysis in the KEGG signaling pathways of upregulated miRNAs. The blue box represents p-value, green box represents the number of genes, and the red box represents the number of interactions of miRNAs.

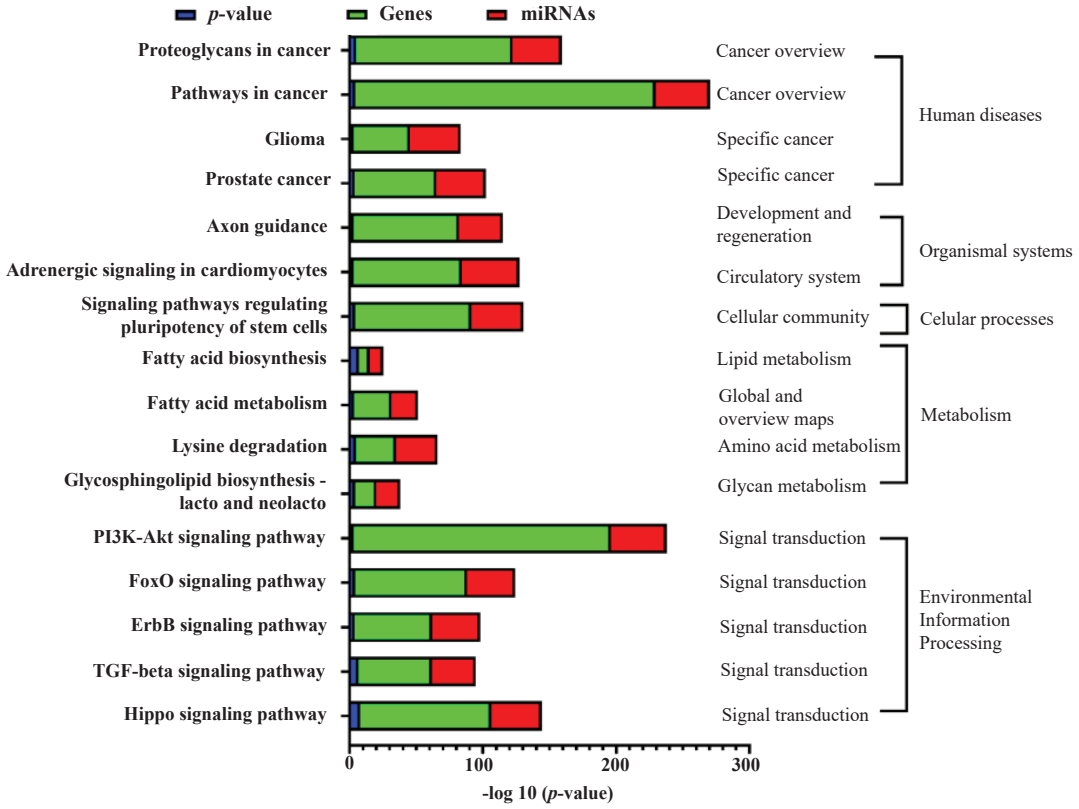

Figure 4. Enrichment analysis in the KEGG signaling pathways of downregulated miRNAs. The blue box represents p-value, green box represents the number of genes, and the red box represents the number of interactions of miRNAs.

598-3p which shows differences in expression in patients with polycystic ovary syndrome patients (27).

In this study, 48 downregulated miRNAs were also identified. The most downregulated miRNAs was hsa-miR-152-3p with log fold-change of 1.41. The downregulated expressions of miR-152-3p and miR-15b-5p were consistent with the results of a previous study.(28,29) miR-152 is known to have an inhibitory effect on interleukin (IL)-12, IL-6, and tumour necrosis factor (TNF)-α, and is an immune system enhancer acting by upregulating natural killer cell-mediated cytolysis of host cells.(30) The miR-152 family regulates proliferation, differentiation, and apoptosis.(31) These expression results align with those of previous studies that show the downregulation of this miRNA in preeclampsia. (32) Other studies have shown that this miRNA is increased in preeclampsia, is associated with VEGF, and promotes apoptosis in trophoblast cells by inhibiting the Bcl-2 which also influenced by miRNA Bax expression.(33,34) miR-152 decreases the expression of DNA methyltransferases (DNMT), which alters the methylation of fatty acid binding protein 4 (FABP4), resulting in the abnormal expression of genes involved in metabolic and immunological processes. (35) This suggests that miR-152 is a microRNA that is closely related to the mechanism of preeclampsia, so this miR has the potential to be one of the interesting markers to be explored more deeply.

A KEGG functional enrichment analysis was performed to explore the potential molecular mechanisms in this context further. The estrogen signaling pathway was the most significant pathway involving the upregulated miRNAs. This pathway has recently shown promising results as a therapeutic target for preeclampsia treatment. Estrogen metabolism significantly reduces preeclampsia by reducing the expression of the estrogenic G protein-coupled receptor 30 (GPR30) in placental trophoblasts and by upregulating angiopoietin-like 4 (ANGPTL4) expression.(36) GPR30 activation protects the placenta against hypoxia–reoxygenation damage by enhancing proliferation via the PI3K/AKT and endothelial nitric oxide synthase (eNOS) as the main source of vascular nitric oxide (NO). The upregulation of NO roles in pregnancy was due to the increase of estrogen level. Disruption in NO production leads to an imbalance of reactive oxygen species (ROS) and NO which then causes vasoconstriction, hence, hypertension.(37,38)

The estrogen signaling pathway interacts with the MAPK and PI3K signaling pathways, which are also involved in pathophysiology of preeclampsia. Target genes in the estrogen signaling pathway are related to the cell cycle, promotion of apoptosis, cell adhesion membrane proteins, membrane components, and cytoplasmic signaling cascades. The TGF-β and mTOR signaling pathways were two other significant pathways identified as being involved in preeclampsia; the finding of this study was consistent with those of previous studies.(39)

The Hippo signaling pathway was the most significant pathway involved in the downregulated miRNAs. This pathway is related to the TGF-β and Wnt signaling pathways, which were also significant in this study. Transcriptional coactivator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP) regulate the Hippo signaling pathway. Therefore, precise regulation of the levels and positioning of these elements is crucial for initial developmental processes and for maintaining tissue stability, healing, and regrowth. YAP is involved in regulation of caudal-related homeobox transcription factor 2 (CDX2), which affects the process of trophoblast invasion and apoptosis in preeclampsia.(40) These results were consistent with those of other studies, in which the Hippo signaling pathway was one of the most significant pathways in terms of miRNA downregulation in preeclampsia.(9)

This study is subject to several limitations, including the small sample size. Variations in subjects' characteristics may have impacted miRNA expression, despite no significant differences in demographic characteristics between the two study groups. It is important to consider the type of preeclampsia, whether early- or late-onset, as this could influence the miRNA profile, disease severity, and related complications, necessitating further investigations. Moreover, factors such as concentration of exosome extraction may also have implications for miRNA profiling results. Future investigations will involve the validation of these miRNAs as biomarkers of the severity, subtype, and prognosis of preeclampsia.

Conclusion

Some miRNAs are identified as promising biomarkers of preeclampsia, such as miR-1283 which show the highest expression and miR-152-3p which show the lowest expression in preeclampsia women. These miRNAs are mainly related to the processes of proliferation, invasion, apoptosis, and angiogenesis, acting through changes in signal transduction in the Hippo and estrogen signaling pathways. The results of this study provided evidence that exosomal miRNAs may influence the pathophysiology of preeclampsia.

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Authors Contribution

HS, SF, DRH, and HP contributed to the design and conceptualisation of the manuscript. HS and IG contributed to the implementation and analysis of results. HS contributed to the data collection and wrote the first draft of the manuscript. All the authors have read and approved the final manuscript.

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