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Plasma CircRNAs for First Trimester Prediction of Preeclampsia and Potential Biomarkers

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KEYWORDS Circular RNAs. Hsa_circ_0046677. Hsa_circ_00429703. Preeclampsia. RNA-Sequencing

ABSTRACT This study investigated expression profiles and mechanisms of circular RNAs on preeclampsia patients between 7-14 weeks. RNA sequencing demonstrated 12,579 circRNAs (7,684 upregulated and 4,895 downregulated) expressed differentially in 8 pairs of plasma samples from preeclampsia patients and healthy controls. Predicted 15 upregulated and 9 downregulated circRNAs then were assessed through qRT-PCR in 50 preeclampsia patients and 30 controls. Differentially expressed circRNAs in preeclampsia patients and controls were analyzed by RNA sequencing and gene ontology, Kyoto Encyclopedia of Genes and Genomes and circRNA-miRNA-mRNA network analyzed data. Hsa_circ_0046677 and hsa_circ_0029703 were markedly increased in preeclampsia patients. Receiver operator characteristic curve analysis indicated the area under the curve was 0.083 for hsa_circ_0046677 and 0.965 for hsa_circ_00429703 while the sensitivity and specificity of these two genes were 78 percent, 88 percent and 83 percent, 93 percent, respectively. Hsa_circ_0046677 and hsa_circ_00429703 had enormous potentials for diagnosing preeclampsia of pregnant women in the first trimester.

INTRODUCTION

Preeclampsia is a special disease in pregnancy containing systemic pathophysiological changes (Gyselaers 2020), which results in increased morbidity and mortality of maternal and neonatal worldwide (Lemoine et al. 2019; Starling 2020). PE was defined as a new-onset hypertension and a sign of maternal organ dysfunction in proteinuria, elevated aminotransferase, pulmonary edema, nervous system, blood system, digestive system and other abnormal performances after 20 weeks of gestation (Brown et al. 2018; Webster et al. 2019). Presently, no effective treatment or prevention option is available except termination of pregnancy (Burton et al. 2019; Phipps et al. 2019). The etiology and pathogenesis of PE are unclear, but so far, PE is described as a two-stage phenomenon (Staff 2019). The first stage is the asymptomatic phase during the first trimester of pregnancy, characterized by deficient invasion of trophoblast and inadequate uterine spiral arteries remodeling resulting in poor placental perfusion, abnormal placentation or utero-placental perfusion, which causes systemic maternal syndrome by increasing inflammatory responses and endothelial dysfunctions (Sato 2020). Insufficient placental blood flow perfusion will lead to is-

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chemia, hypoxia and oxidative stress, which will aggravate the placental vascular dysfunction (Erlandsson et al. 2016), and then lead to the second stage-the clinical symptom stage (Ridder et al. 2019), that is, the maternal systemic reaction of the mother to abnormal placenta (Jena et al. 2020). In summary, the development of PE was determined at the time of placental implantation. Therefore, the first three months of pregnancy had the validity to predict and prevent pregnancy complications (Poon et al. 2018). Early intervention when necessary has great importance to prevent the long-term cardiovascular events of PE patients and their children.

Recently, many studies have demonstrated that circRNA, an emerging non-coding RNA (Azari et al. 2021; Memczak et al. 2013), has become the latest participant in PE related gene network. The differentially expressed circRNA in the maternal-fetal interface may be an important epigenetic regulator mediating PE pathophysiological process (Wen et al. 2020). CircRNA is a closed circular structure formed by 5' and 3' splice sites through reverse splicing (Huang and Shan 2015), which is not easy to be degraded by nucleic acid exonuclease. The structure makes circRNAs have 3 times longer half-life than linear RNAs showing high stability (Azari et al. 2021). In addition, circRNA is specific to tissues or diseases or specific stages of development (Kristensen et al. 2019). Types or expressions of circRNA change widely in different tissues or at different stages of development (a certain stage of disease progression) within the same tissue (Maass et al. 2017). CircRNA is widely found in various organisms (Memczak et al. 2013) and strongly participates in the pathophysiology of many disorders (Filardi et al. 2020; Jakobi et al. 2020; Prestes et al. 2020; Zhang et al. 2020). Biological functions of circRNA include the sponge adsorption function of miRNAs, which modulates the occurrence and development of diseases through adsorbing and binding miRNA and mediating expressions of miRNAs and their targets (Misir et al. 2020; Pereira et al. 2020). Some circRNAs also contain RNA protein binding sites, acting as protein sponges or baits to participate in regulating gene expressions (Huang et al. 2020). Studies have shown that circRNA with RNA polymerase all complex combination regulates gene transcription and protein translation indicating that circRNA has the function of protein translation (Dergunova et al. 2021, Villa et al. 2020). CircRNA has the potentiality to be a disease-specific biomarker based on its unique structural characteristics and biological functions (Reijnders et al. 2019). Therefore, researchers determined to analyze differentially expressed circRNA in plasma samples of patients and functions of circRNAs in samples would be examined.

Objectives

This present study aimed at examining circRNAs that expressed differentially in plasmas of PE patients and potentials of hsa_circ_0046677 and hsa_circ_0029703 for diagnosing PE.

METHODOLOGY

Patients and Plasma Collection

The subjects from the prospective study cohort of hypertensive disorders in pregnancy were established in this research group, study population from January 2018 to June 2019 were selected from 19 every community health service center of Tianjin first prenatal care of pregnant women. All participants signed informed consent based on the declaration of Helsinki. The research got the permission from the Ethical Committee of the Affiliated Hospital of the Armed Police Logistics College. 5ml of peripheral blood samples from pregnant women during the initial pregnancy examination were collected, then centrifuged (3000rpm, 10 min at 25^0C), the plasma, serum and blood cells were separated and stored in the biobank at -80^0C until use. Plasma samples from 8 paired PE patients and healthy controls of gestational age were screened for RNA-Seq and qRT-PCR validation was performed in plasma from 50 PE patients and 30 healthy pregnant women in the first trimester. Researchers obtained circRNA profiling by RNA-Seq in peripheral blood plasma of PE patients and matched healthy pregnant women at 7-14 weeks of gestation, and then differentially expressed candidate circRNAs were screened out for qRT PCR validation and evaluated the predictive value of circRNA in early pregnancy in PE patients. Finally, a network map of circRNA miRNA mRNA was structured to better investigate potential mechanisms of circRNAs on the pathogenesis of PE and the potential risk of

maternal and child cardiovascular disease in the next two generations.

RNA Sequencing Analyzing and RNA Libraries Preparation

Cloud-Seq Biotech (Shanghai, China) was applied for RNA high-throughput sequencing. TRIzol reagent (TaKaRa, Dalian, China) was first used to segregate the total RNA of 16 plasma samples. Total blood samples of plasma stored at -70^{0}C were first tawed at 4^{0}C and centrifugated for 10min at 12,000xg to remove impurities. Thereafter, 250μl whole blood samples were transferred into 1.5ml centrifuge tube and mixed with 750μl TRIzol reagent (TaKaRa, Dalian, China) and 20μl glacial acetic acid (Sigma-Aldrich, USA) by shaking violently. Samples were cultivated for 5min at 30^{0}C and added with 0.2ml chloroform (Sigma-Aldrich) and centrifugated for 15min at 12,000xg at 4^{0}C. After centrifugation, RNA was allocated to aqueous phase. Later, each sample was added with 1ml TRIzol and 0.5ml Isopropyl alcohol (Sigma-Aldrich) and incubated for 10min at 30^{0}C followed by centrifugation at 12,000xg, 10min at 4^{0}C. Next, the supernatant was removed and each 1ml TRIzol reagent homogenized sample was added with 1ml 75% ethanol (Sigma-Aldrich). Afterward, RNA sediment was dissolved and stored at -70^{0}C. According to supplier instructions, the NanoDrop ND-1000 was applied to qualify RNAs and the degree of degradation was examined by denatured agar-gel electrophoresis (Invitrogen, USA). Using NEBNext rRNA Depletion Kit (New England Biolabs, Inc., Massachusetts, USA) and NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England Biolabs, Inc., Massachusetts, USA), the ribosomal RNA was removed from samples and circRNAs were enriched to construct the RNA sequencing library. BioAnalyzer 2100 System (Agilent Technologies, USA) was used for the quality control and quantification of library and illumina Hiseq 4000 (Illumina, USA) was used to harvest paired end reads quality controlled by Q30 for library sequencing with 150bp paired end reads. After 3' adaptor-trimming, low-quality reads were removed by cutadapt software (v1.9.3). Finally, data standardization were conducted by edgeR software (V3.16.5) and circRNA differential expression were screened (Baik et al. 2020). The GO and KEGG pathways were applied to analyze enrichments and potential functions of differentially expressed circRNAs.

qRT-PCR Validation

15 promoted and 9 low expressed circRNAs were picked out for qRT PCR validation in plasma of PE patients and healthy-pregnant women. After RNA extraction, circRNA specific primer design was performed using Primer Premier 5.0 software, validated by Pubmed/Primer BLAST, and the primer sequences were finally synthesized from Beijing Liuhe BGI Technology Co., LTD (Table 1). Relative expressions of circRNAs in plasma were calculated using $2^{-\Delta\Delta ct}$ method.

Statistical Analysis

SPSS 22.0 software (IBM, USA) and the GraphPad 8.0 software (GraphPad, USA) were conducted for statistical analysis. Quantitative data were expressed as meanstandard deviation (SD). Student's *t* test was chosen for analyzing differences between two groups. Receiver operating characteristics (ROC) curve analysis was applied for validating the predictive diagnostic values of circRNAs in PE patients of the first trimester. *P*-value 0.05 was significant statistically.

RESULTS

Subject Characteristics

Study population characteristics are listed in Table 2. There were signiûcant differences between PE and controls in GA at blood draw, white blood cell, NLR, infant birthweight/height and blood pressure. As expected, white blood cell, NLR, SBP, DBP and MAP were prominently higher, while neonatal birthweight and neonatal height were significantly lower in PE group than the controls.

Differential Expression of circRNA Spectrum

RNA-Seq was performed on the peripheral blood of 8 PE patients and 8 matched healthy pregnant women at 7-14 weeks of gestation. A total of 12,579 circRNAs were expressed differentially (Fig. 1), of which 7,684 were promoted and 4,895 were inhibited. Clustering heat map (A) refers to circRNA differentially expressed and classified by RNA-

Table 1: circRNAs and internal references for qRT-PCR validation

Name	*circRNA ID*	*Forward primer (5'-3')*	*Reverse primer (5'-3')*
hsa_circ_0046677	chr18:670692-671451+	GATTGCGCACATC ACGGG	CATAGAACTGGCA GAGGGCA
hsa_circ_0131266	chr6:163899812-163956157+	TCACTGTGGAAGAT GCTCAGA	CCTTTGCCTCGG ACCATGA
hsa_circ_0061396	chr21:30701808-30702014+	CGCTGTCGCAAGAGA AAACTT	TGTTCTGGAGTAAG CTTGTGC
hsa_circ_0115056	chr20:34304662-34320057-	GCTTCGTGGGATCTT TGAGC	CTGTCTCTACTTCGC TTCCGT
hsa_circ_0070207	chr4:79791937-79800045+	CAGGGTGGAATCCTT TTGGAGA	TACTCTATGCTGCT GTGGCG
hsa_circ_0002164	chr18:23632588-23658124-	CACAGAGCATGCCAG TACAGA	TACTCTATGCTGCT GGTAAA
hsa_circ_0138318	chr9:15199859-15211395-	CAGAAAGCTGCACTC ACGTT	GTCCTGTTGCTCG AAGGTCA
hsa_circ_0008346	chr15:41648237-41669502+	ACCATGGGGGCAAT CTAAAGAA	AGGTACCTTTGAATCT CTGTTACT
hsa_circ_0006952	chr18:48581151-48593557+	GCACCTGGAGATGC TGTTCAT	ATGTCCTTCAGTG GACAACG
hsa_circ_0120761	chr2:64199300-64211153-	TTGTCCTCCTAAAG ATACCTTCG	ACATCACTGCTGCTT CCTTGA
hsa_circ_0102912	chr14:92537279-92548810-	GGGACCTATCAGGA CAGAGTT	CCTGAGCCATCATT TGCTTCT
hsa_circ_0017041	chr1:235316021-235318427-	TGTTGGGAATTTGC CTGTTACAT	TTCTGCCCTTGTTTCT GGTGAA
hsa_circ_0029703	chr13:21955573-21965993-	GGAGCTGAAGAAGG CATCGT	GCTGAAAAGTGAGAG GACGCT
hsa_circ_0007538	chr1:186359843-186360923+	GAATGGACTTACAC GCTGGG	CTTCCAATCTGCTGG TCTTGC
hsa_circ_0002553	chr14:102661275-102676199+	TCACTGCTTGTCAG GAGGGAT	CTGTGGCTGTTAGG TGGTTGA
hsa_circ_0092415	chr10:103190102-103221815+	TGTAATAATGGCGA ACCCCCT	CTGGGTTATACAGGC ATCGCA
hsa_circ_0123459	chr3:28520305-28533664+	AAGACTGGAAATGTG CTGCCTA	AGAGTAGACATGC TTCTTGTAGTGC
hsa_circ_0064725	chr3:33418765-33420233+	ATCCTGATAACCGA CAGCACA	TGGCAATTCCGAGC TAAAAGT
hsa_circ_0008011	chr11:62406455-62407203-	CCCCGATACCGTGT ACCAGA	GCTTCTATCCACAG CAAGGGT
hsa_circ_0047078	chr18:19140820-19155738-	ACAGCATCTGCTTT TCCACAAC	TAGACTCGATGTCT CAGCCAC
hsa_circ_0113246	chr1:3765172-3768985-	GCTTGCCTGAATATT TTGCACC	AAGCCGTCTTCGT GTCCAG
hsa_circ_0062726	chr22:29439287-29440878+	GGCTTTCTTCGTC GTGGTCT	CATACACCACCGGC CTCTTG
hsa_circ_0098338	chr12:30818154-30822216-	AGGACCAAATGGAGC TGTTTC	TCTTCTTGCCACAGCT CTTCAT
hsa_circ_0100288	chr13:33247353-33284243+	AGCAGAAGCTGCAC TACAAA	ACATGCTCTGACAAA TCGCTG
β-actin	—-	CCTGTACGCCAACA CAGTGC	ATACTCCTGCTTGCT GATCC

Seq analysis (up-regulated or down-regulated). Scatter plots (B) were drawn using the mean values of circRNA expression in PE group and control group. Volcanogram (C) used to express fold changes and *P* values was applied to describe the differentially expressed circRNA.

A: Clustering heat map displayed differentially expressed circRNAs generated by RNA-Seq. Each column represented peripheral blood samples from PE patients or healthy pregnant women, and each row represented a circRNA. Red represented up-regulated circRNAs and green represented down-

Table 2: Study population characteristics

Characteristics	*PE (n=55)*	*Control (n=30)*	*t value*	*P value*
Age, year	30.2 ± 2.9	30.4 ± 2.4	-0.334	0.739
GA at blood draw, wks	10.1 ± 1.9	11.2 ± 1.5	-2.780	0.007*
BMI, kg/m2	24.81 ± 3.86	24.08 ± 5.39	0.706	0.483
White blood cell, ×109/L	8.65 ± 2.06	7.69 ± 1.82	2.101	0.039*
Platelet, ×109/L	252.06 ± 70.30	233.70 ± 44.60	1.282	0.204
NLR	3.2 ± 0.8	2.6 ± 0.6	3.462	0.001*
Fasting blood-glucose, mmol/L	4.93 ± 0.91	4.62 ± 0.38	1.824	0.072
Blood urea nitrogen, mmol/L	3.13 ± 0.64	3.27 ± 0.81	-0.871	0.387
Creatinine, μmol/L	56.73 ± 22.44	55.23 ± 11.82	0.337	0.737
Uric Acid, μmol/L	217.52 ± 55.10	220.13 ± 48.18	-0.214	0.831
Total protein, g/L	69.08 ± 3.74	68.41 ± 3.74	0.769	0.444
Albumin, g/L	42.29 ± 3.31	42.36 ± 2.63	-0.091	0.928
ALT, U/L	22.57 ± 15.22	19.24 ± 19.96	0.840	0.403
AST, U/L	19.73 ± 8.72	16.43 ± 6.14	1.817	0.073
Neonatal birthweight, g	2591 ±482	3455 ±411	-8.191	0.000*
Neonatal height, cm	48 ± 3	49 ± 2	-3.049	0.003*
SBP at first pregnancy examination, mmHg	113 ± 12	107 ± 11	2.217	0.03*
DBP at first pregnancy examination, mmHg	73 ± 9	69 ± 7	2.097	0.039*
MAP at first pregnancy examination, mmHg	86 ± 9	82 ± 8	2.274	0.026*
SBP at admission, mmHg	156 ± 13	117 ± 7	14.937	0.000*
DBP at admission, mmHg	101 ± 11	72 ± 8	12.895	0.000*
MAP at admission, mmHg	120 ± 11	87 ± 7	15.149	0.000*
SBP at follow-up, mmHg	129 ± 14	117 ± 8	4.147	0.000*
DBP at follow-up, mmHg	86 ± 12	74 ± 6	5.025	0.000*
MAP at follow-up, mmHg	100 ± 12	88 ± 4	5.051	0.000*

Abbreviations: GA, gestational age; BMI, Body mass index; NLR, neutrophil-to-lymphocyte ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SBP, systemic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. Continuous variables are displayed as mean±SD, and *t*-test was to measure comparison of means. *signiûcant at $P<0.05$.

regulated circRNAs. Expressions of 15 promoted circRNAs and 9 suppressed circRNAs were screened for qRT-PCR validation. B: Scattergram: The mean values of circRNA expressions in plasma of PE and normal pregnant women were used to draw the scattergram. The red region and the green region respectively represented high and low expressed circRNAs with the absolute value of Fold Change (FC) ≥2. C: Volcanogram: Differential multiple changes and P values were used to describe circRNAs expressed differentially in plasma between PE and normal controls. The two vertical green lines to the left and right respectively represented down-regulated and up-regulated circRNAs from Log2 (|FC| ≥2) and the horizontal green lines above represented differentially expressed circRNAs from Log10 (P≤0.05). The red square represented differentially expressed circRNAs with the absolute value of the default Fold Change (FC) ≥2 times and P≤0.05.

GO and KEGG Pathway Analysis

Functions and pathways of differentially expressed circRNAs were examined using GO and KEGG. GO is one of several ontological languages to describe the functions of genes and their products. The GO database provides a battery of terms for describing the features of genes and related products. These terms are made up of three parts, including biological process (BP), that is, ordered combinations of molecules resulting in wider range of biological functions; Cell components (CC) are used to describe locations, macromolecular complexes and subcellular structures; and molecular function (MF), which is applied for describing the functions and products of individual genes. Gene products may have molecular biological functions, biological pathways and cellular component roles, respectively. Of course, they may have multiple properties in one way or another. In this study,

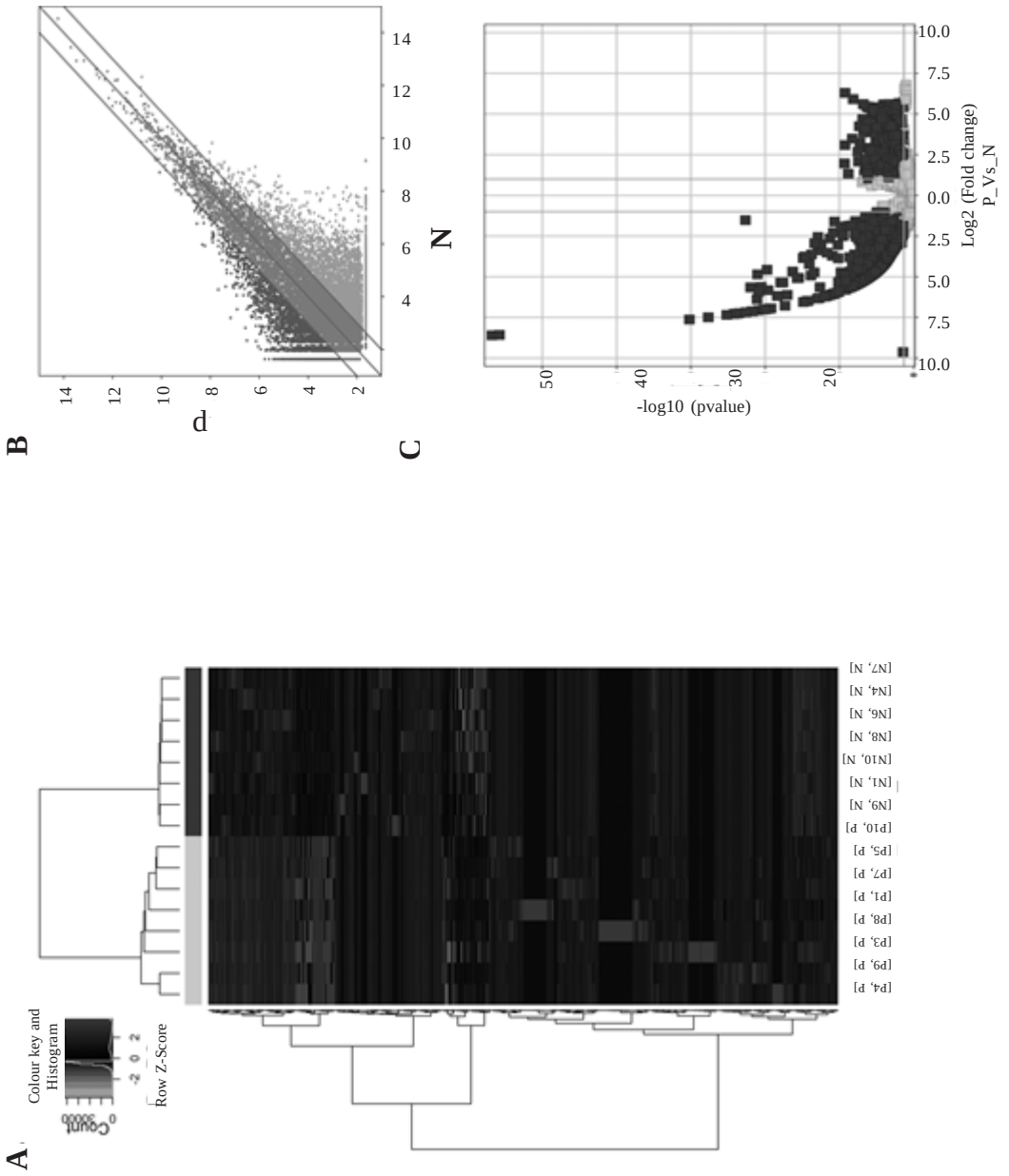


Fig. 1. CircRNAs were expressed differentially in plasma between PE patients and normal pregnant women
Source: Authors

functions of these circRNAs were annotated and speculated through GO function analysis of the host genes, specifically, the functional analysis of the circRNA enrichment pathway in the top 10 in the GO analysis. The pie chart intuitively showed the enrichment amount of circRNA source genes in the GO pathway in terms of BP, CC and MF, and indirectly inferred the function of differentially expressed circRNA in PE (Fig. 2 A, B). Bar chart described the significance of circRNA enrichment in each pathway based on the enrichment degree, that was, the higher the enrichment degree was, the more significant the GO term was (Fig. 3 A, B). In the top 10 terms of the up-regulated circRNAs, "organelle organization" and "cellular macromolecule metabolic process" were the top 2 in BP; "intracellular" and "intracellular part" were the top 2 in CC; "protein binding" and "binding" were the top 2 in MF. While, in the top 10 terms of the down-regulated circRNAs, "organelle organization" and "single-organism organelle organization" were the top 2 in BP; "intracellular part" and "intracellular" were the top 2 in CC; "protein binding" and "binding" were the top 2 in MF.

KEGG is a database to analyze gene function and genome information systematically, helping researchers to integrate gene and expression information. KEGG databases contain complete and partial genome sequences, and more advanced functional information contains information on graphical cellular biochemical processes including metabolism, signaling, cell cycle, membrane transport and homologous conserved sub-pathways. KEGG provides integrated metabolic pathways including carbohydrate, nucleoside, amino acid metabolism, and biodegradation of organic compounds. It not only offers all possible metabolic pathways, but provides comprehensive annotations of enzymes catalyzing each step of the reaction. KEGG is a powerful tool to research metabolism and its network in vivo. In this study, KEGG analyzed the metabolic pathways of these circRNAs by locating associated gene-related pathways to elucidate potential biological functions in the pathophysiological process of PE (Fig. 3 C, D). 84 pathways connected with functions of differentially expressed circRNAs in the PE group were defined by the KEGG analysis (Fig. 4-A, B, C, D). And "ESTROGEN SIGNALING PATHWAY", "SPHINGOLIPID SIGNALING PATHWAY", "CHEMOKINE SIGNALING PATHWAY" and "RAP1 SIGNALING PATHWAY" were involved in regulating the expression of ERK among 84 up-regulated pathways. Therefore, ERK was a pivotal factor in the pathophysiological mechanism of PE. Previous studies have demonstrated the accelerated effect on the proliferation of trophoblasts in PE rats through MAPK/ERK pathway (Wang et al. 2020).

CircRNA-miRNA-mRNA Interaction Network

Bioinformatics analysis has revealed that some circRNAs could sponge miRNA to promote expression levels of related mRNAs through the adsorption or the binding of miRNA. The circRNA-miRNA-mRNA interaction network was shown by Cytoscape software (Fig. 5). The interaction network showed that circRNA had extensive and close interaction with related miRNAs and mRNAs in PE.

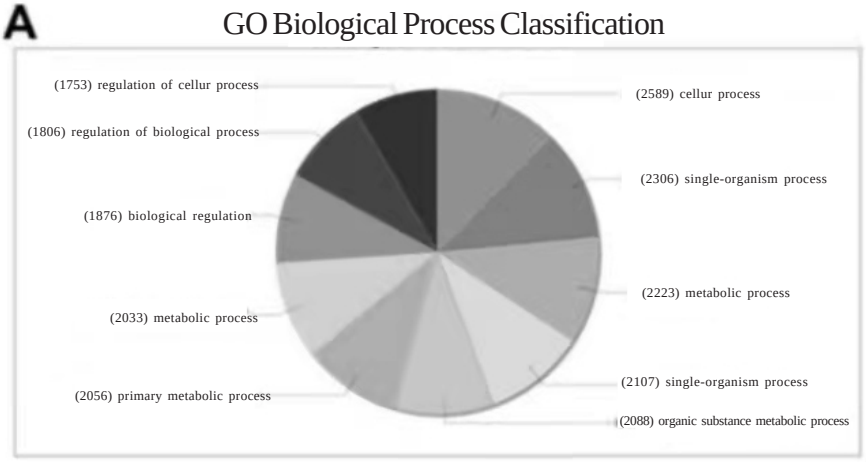
CircRNA: orange and round; miRNA: red and triangular; mRNA(gene): wathet and square.

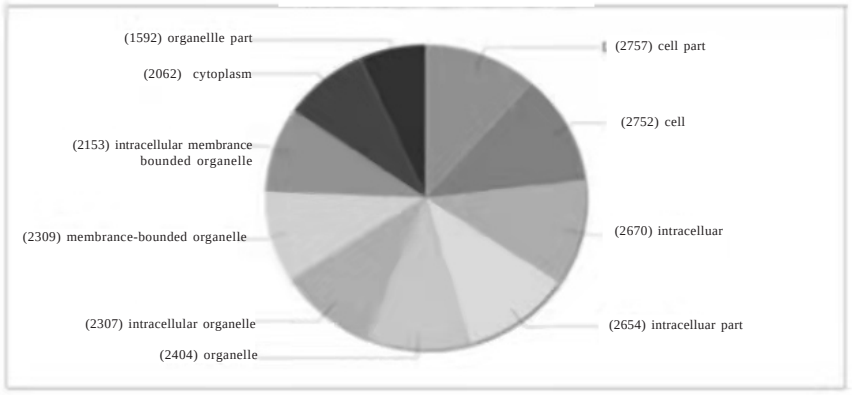
Validation of qRT-PCR

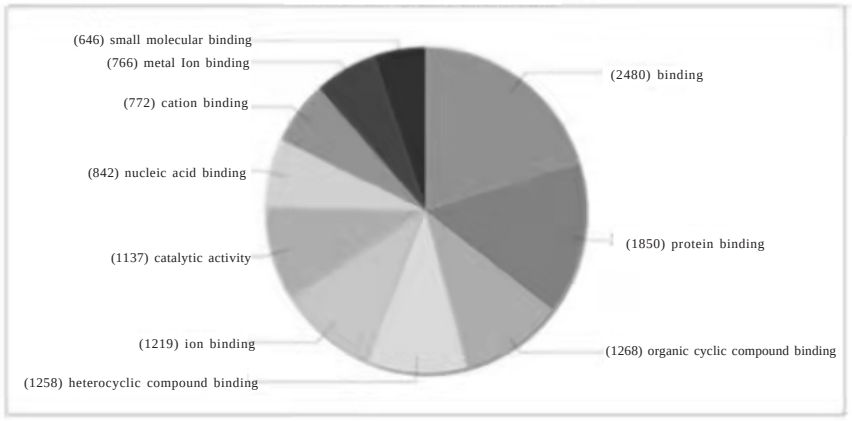
To verify the differential expressions of the candidate circRNAs, qRT-PCR was conducted in 50 PE and 30 control plasma samples. Researchers selected 24 circRNAs based on the results of RNA-Seq. Among the 24 differentially expressed circRNAs, 2 circRNAs were the most highly expressed in PE plasma, while other 2 circRNAs were significantly lower in PE plasma than the control samples. As shown in the Figure 6 (A, B), the expressions of hsa_circ_0046677, hsa_circ_00429703, hsa_circ_0047078 and hsa_circ_0062726 were consistent with their levels as tested by the RNA sequencing.

Differentially expressed circRNAs in preeclampsia plasma compared with the normal controls (A, B). Hsa_circ_0046677 and hsa_circ_0029703 were significantly increased, while hsa_circ_0047078 and hsa_circ_0062726 were dramatically downregulated in PE plasma versus the normal control. *P0.05, **P0.001. The plasma expression levels of hsa_circ_0046677 and hsa_circ_0029703 in early pregnancy women had high predictive values for PE patients (C, D). C: The sensitivity and specificity of the expression level of plasma hsa_circ_0046677 in early pregnancy were 78 percent and 83 percent, respectively; D: The sensitivity and specificity of expressions of plasma

A


GO Biological Process Classification
(1753) regulation of cellur process
(1806) regulation of biological process
(1876) biological regulation
(2033) metabolic process
(2056) primary metabolic process
(2589) cellur process
(2306) single-organism process
(2223) metabolic process
(2107) single-organism process
(2088) organic substance metabolic process


GO Cellular Component Classification
(1592) organellle part
(2062) cytoplasm
(2153) intracellular membrance bounded organelle
(2309) membrance-bounded organelle
(2307) intracellular organelle
(2404) organelle
(2757) cell part
(2752) cell
(2670) intracelluar
(2654) intracelluar part


GO Molecular Function Classification
(646) small molecular binding
(766) metal Ion binding
(772) cation binding
(842) nucleic acid binding
(1137) catalytic activity
(1219) ion binding
(1258) heterocyclic compound binding
(2480) binding
(1850) protein binding
(1268) organic cyclic compound binding

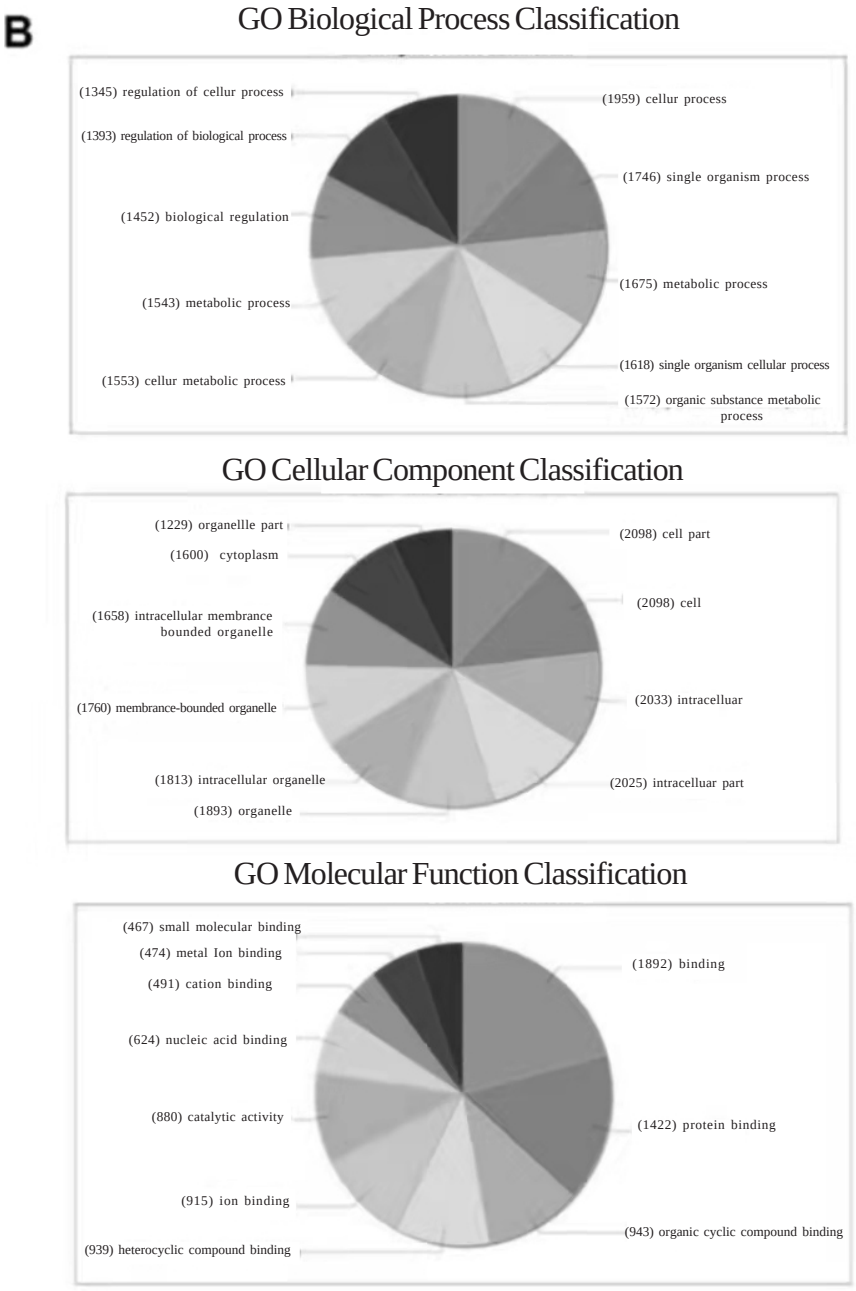


Fig. 2. Pie charts were used to describe the enrichment of circRNAs increased or inhibited in the GO pathway for BF, CC and MF (top 10). A-upregulated circRNAs; B-downregulated circRNAs
Source: Authors

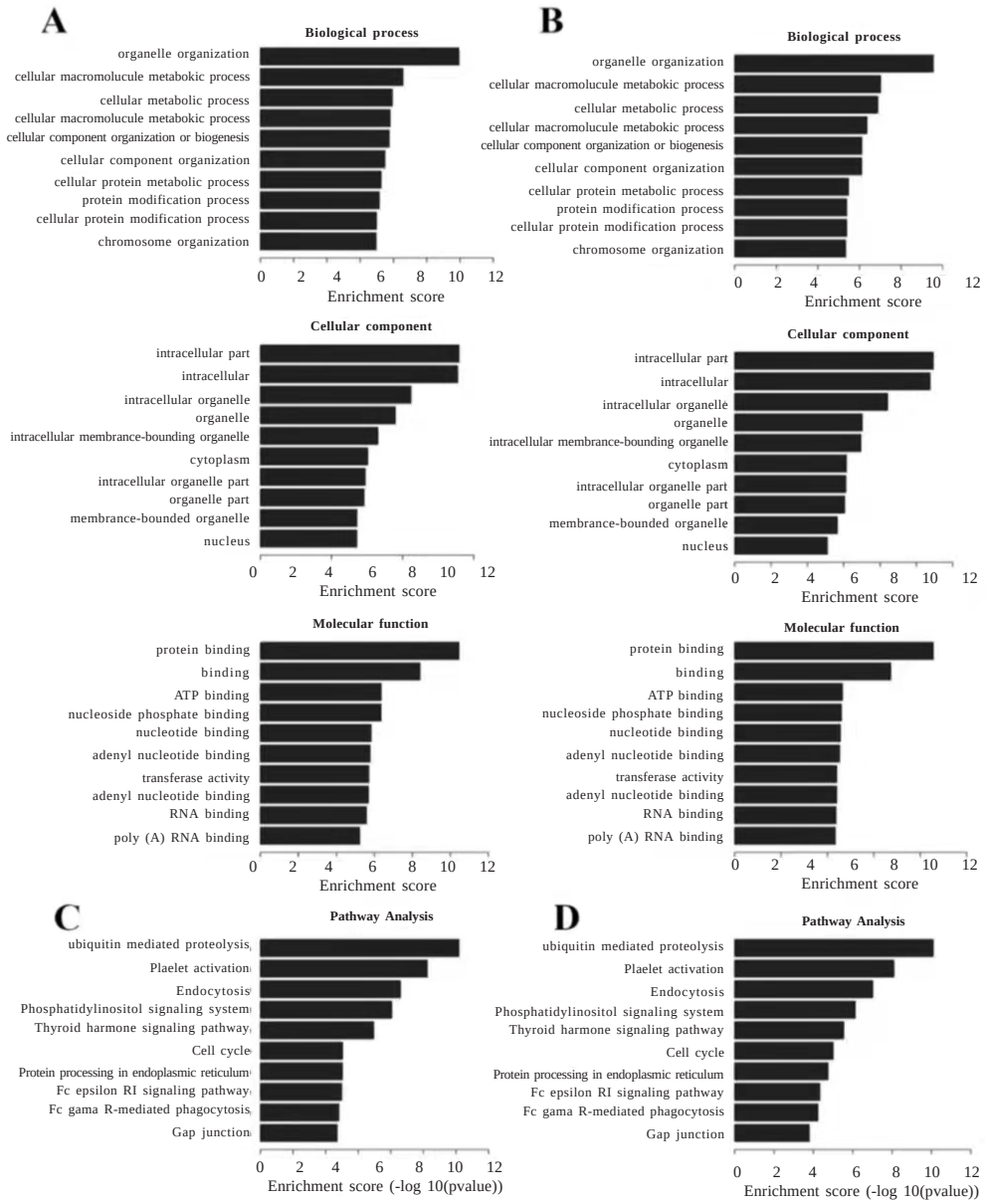
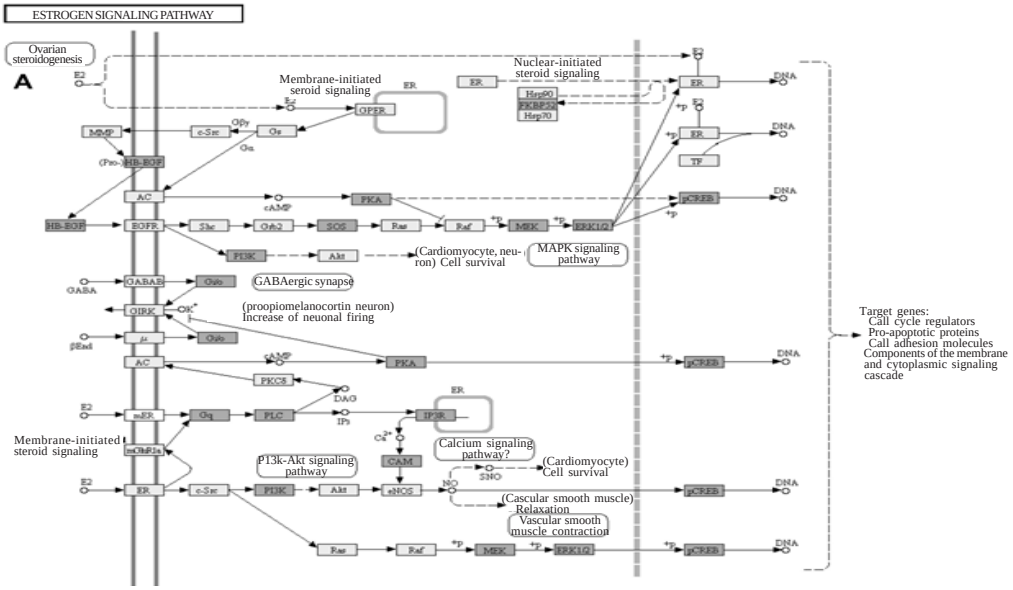
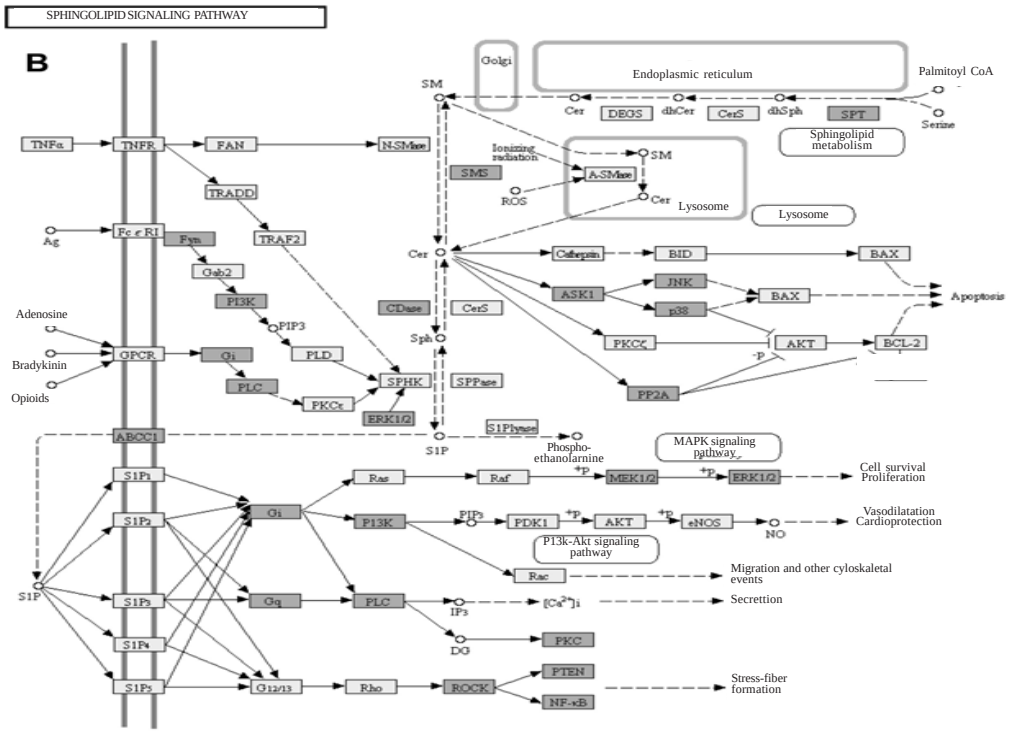


Fig. 3. GO analysis and KEGG pathway analyzed top 10 enrichment scores of differentially expressed circRNAs. The X-axis represented the GO term enrichment fraction (degree of enrichment), and the Y-axis represented the GO term (A, B).
A-GO annotation analysis of upregulated circRNA;
B-GO annotation analysis of downregulated circRNA. The enrichment score was calculated as –log10 (P-value). KEGG pathway analyzed enrichments of differentially expressed circRNAs with top 10 enrichment score (C, D).
C-KEGG pathway analysis of upregulated circRNA;
D-KEGG pathway analysis of down-regulated circRNA
Source: Authors


ESTROGEN SIGNALING PATHWAY
A
Ovarian steroidogenesis
Membrane-initiated seroid signaling
Nuclear-initiated steroid signaling
MAPK signaling pathway
(Cardiomyocyte, neuron) Cell survival
GABAergic synapse
(proopiomelanocortin neuron) Increase of neuonal firing
Target genes:
Cell cycle regulators
Pro-apoptotic proteins
Cell adhesion molecules
Components of the membrane and cytoplasmic signaling cascade
Membrane-initiated steroid signaling
P13k-Akt signaling pathway
Calcium signaling pathway?
(Cardiomyocyte) Cell survival
(Cascular smooth muscle) Relaxation
Vascular smooth muscle contraction


SPHINGOLIPID SIGNALING PATHWAY
B
Golgi
Endoplasmic reticulum
Palmitoyl CoA
Serine
Sphingolipid metabolism
Lysosome
Apoptosis
Adenosine
Bradykinin
Opioids
Phospho-ethanolamine
MAPK signaling pathway
Cell survival Proliferation
Vasodilatation Cardioprotection
P13k-Akt signaling pathway
Migration and other cyloskaletal events
Secrettion
Stress-fiber formation

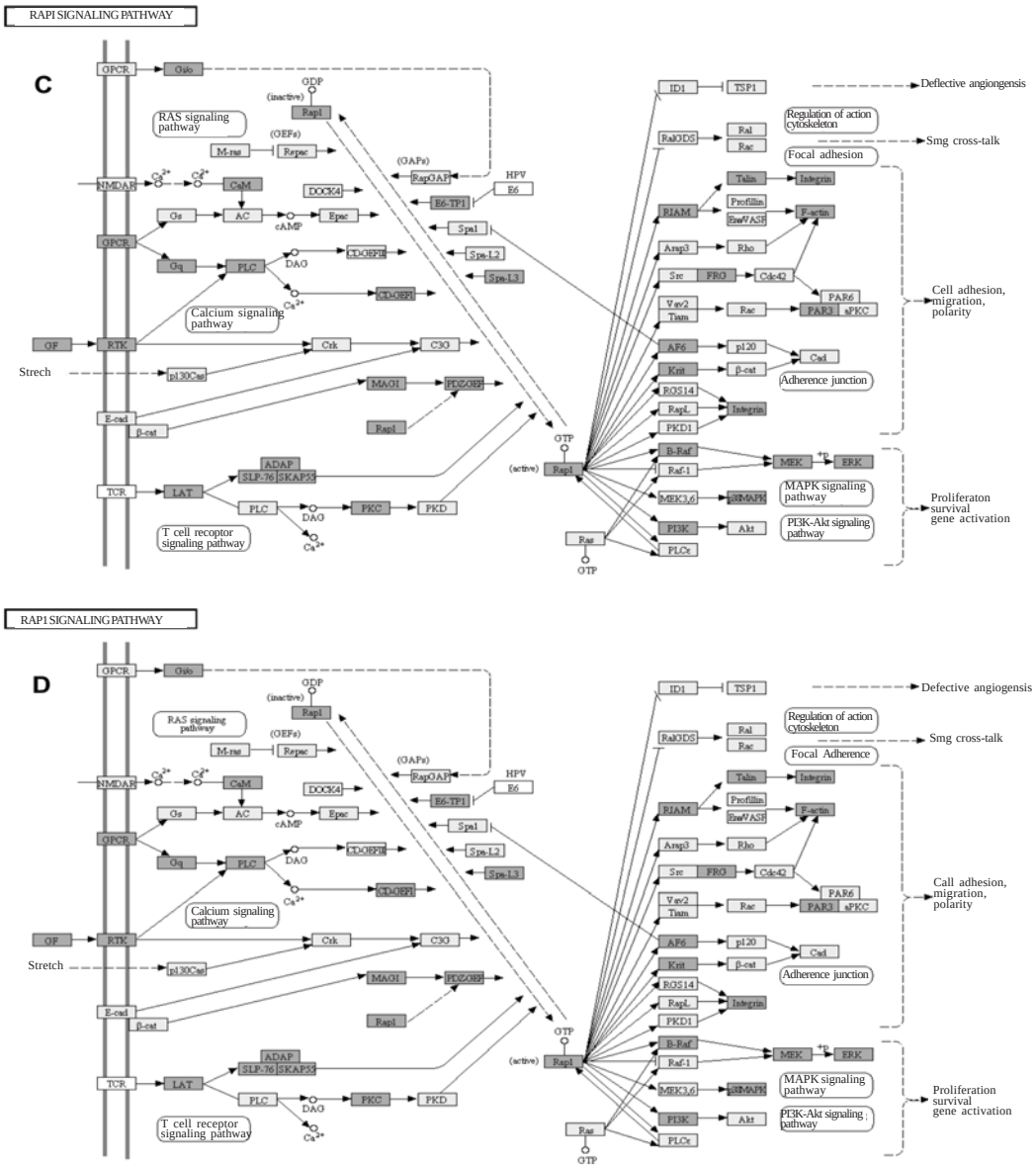


Fig. 4. KEGG pathway analysis displayed related signaling pathways to upregulated circRNAs in PE.
The KEGG analysis displayed 84 signaling pathways associated with the upregulated circRNAs in PE (A, B, C, D).
A-ESTROGEN SIGNALING PATHWAY,
B-SPHINGOLIPID SIGNALING PATHWAY,
C-CHEMOKINE SIGNALING PATHWAY and
D-RAP1 SIGNALING PATHWAY were relevant to expressions of ERK, which played an important role in PE.
Source: Authors

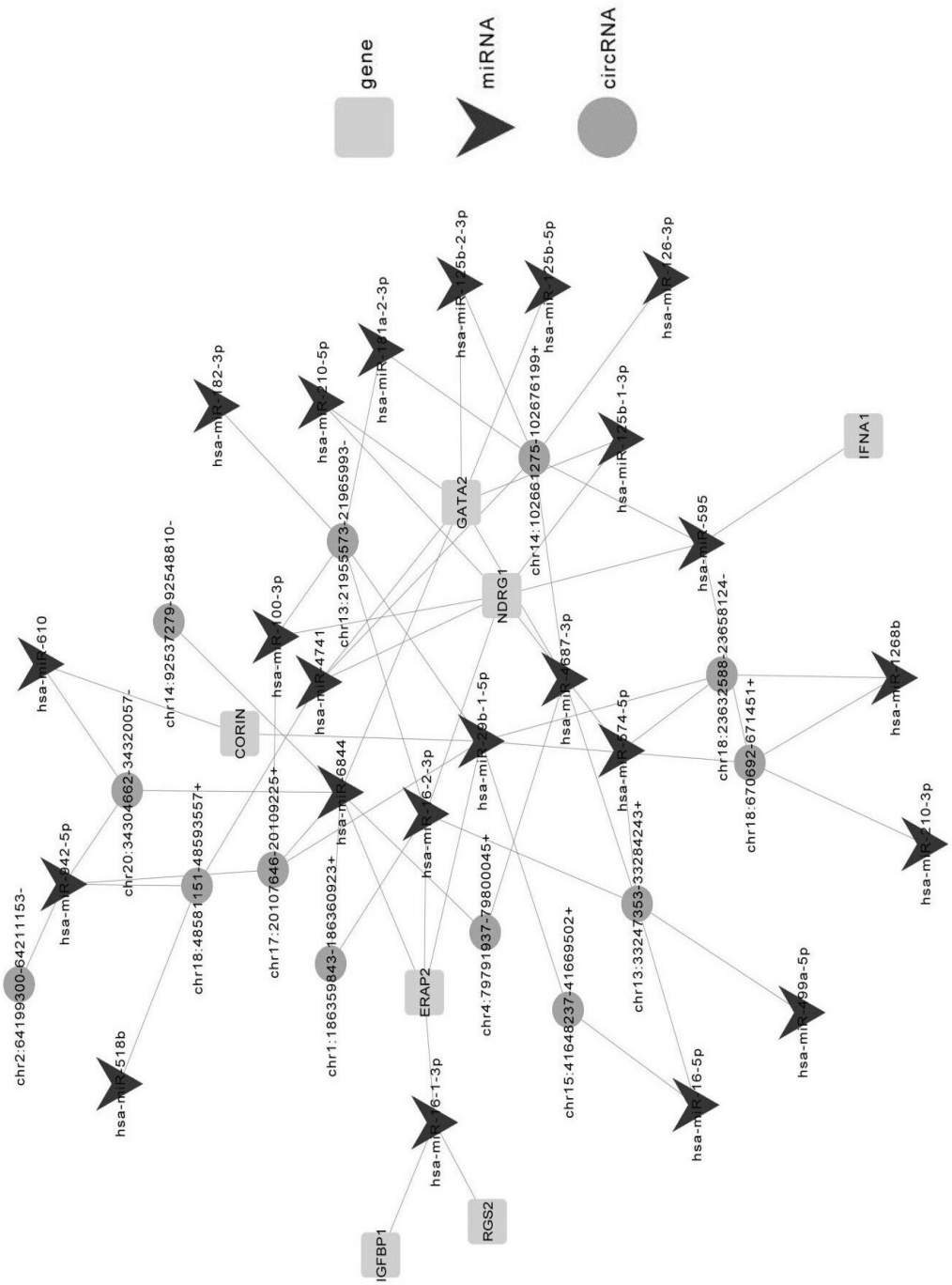


Fig. 5. CircRNA-miRNA-mRNA-network analysis in PE were displayed
Source: Authors

hsa_circ_0029703 in early pregnancy were 88 percent and 93 percent, respectively.

Receiver Operating Characteristic Curve Analysis, ROC Curve Analysis

The predictive or diagnostic power of circRNA for PE patients was evaluated using ROC curve analysis. The closer the curve is to the upper left corner, the greater the AUC, and the higher the accuracy of the diagnosis or prediction. After qRT-PCR validation, the plasma expressions of hsa_circ_0046677 and hsa_circ_0029703 were distinctly increased in PE pregnant women in early pregnancy. ROC curve analysis was applied to validate the predictive diagnostic value of them. The AUC of hsa_circ_0046677 was 0.883, as shown in Figure 6-C. And 95 percent confidence interval (CI) was 0.807 ~ 0.958 and the cut-off value was 8.098. The AUC of PE predicted by hsa_circ_0029703 was 0.965, as shown in Figure 6-D. And 95 percent CI was 0.931 ~1.000 and the cut-off value was 40.365. ROC curve analysis showed that these two circRNAs had potential predictive values in PE patients of the first trimester. The best predictive value of hsa_circ_ 0046677 and hsa_circ_ 0029703 in plasma during the first three months of pregnant women were 8.098 and 40.365, respectively.

DISCUSSION

There is no unified standard for biomarkers of PE, and the current research is still in the exploratory stage (Fishel Bartal and Sibai 2020). In recent years, genomics, epigenetics, transcriptome, proteomics and metabolomics have been increasingly applied to the etiological study of PE. However, due to the complicated and confusing pathogenesis of PE, the "switch" to start the pathogenesis of PE is still a "mystery" (Benny et al. 2020). For the first time, researchers performed RNA-Seq analysis in the plasma of women in the first trimester of pregnancy, and differentially expressed circRNAs were evaluated by large-scale qRT-PCR. The identification of biomarkers that predicts PE occurrence before clinical symptoms is critical for early intervention, which can reduce the morbidity of cardiovascular adverse events in both mother and child generations (Nzelu et al. 2018). Previous studies have shown that the occurrence of PE during pregnancy not only causes the system damage to the body, but also induces long-term hypertension, cardiovascular diseases, diabetes and an increased risk of metabolic abnormalities diseases. Additionally, PE brings adverse effects on offspring health, including cardiovascular, metabolic and immune, mental neurological and cognitive concerns (Fox et al. 2019). To sum up, early screening and diagnosis of PE in the first three months of pregnancy through non-invasive testing are essential for the long-term health of both mothers and children. Meanwhile, the research provided a theoretical basis for the construction of early warning model of PE. The pathophysiological mechanism of PE is complex, and subtle pathophysiological changes have occurred in the first three months of pregnancy, but the changes at this stage are not sufficient to lead to clinical symptoms. Previous studies have shown that the development of PE is predetermined from the moment of placental implantation, which involves many changes (Ridder et al. 2019), including inadequate invasion of trophoblast and inadequate spiral artery remodeling (Yu et al. 2018), ischemia and hypoxia (Qu and Khalil 2020), oxidative stress (Mannaerts et al. 2018), immune inflammatory response (Cheng et al. 2019) and other physiological changes. In this study, the clinical biochemical indexes of PE patients were compared to normal controls in the first trimester and concluded that there were significant differences in WBC, NLR and blood pressure in PE group compared with healthy control group, and previous studies also showed that (Kang et al. 2020; Wang et al. 2019; Zheng et al. 2019), WBC and NLR were markers of systemic inflammation, especially NLR could work as an indicator for the underlying inflammatory components and predict the onset of PE.

As an emerging non-coding RNA, circRNA is widely found in the blood samples with characteristics of stability, universality and specificity. In this study, RNA-Seq analysis was conducted on the plasma of PE patients and healthy pregnant women in the first trimester to obtain differentially expressed circRNAs for large-scale verification. It was concluded that hsa_circ_0046677, hsa_circ_00429703, hsa_circ_0047078 and hsa_circ_0062726 could be used as plasma biomarkers to predict PE. ROC curve analysis of highly expressed hsa_circ_0046677 and hsa_circ_ 00429703 showed that these two circRNAs had potential predictive diag-

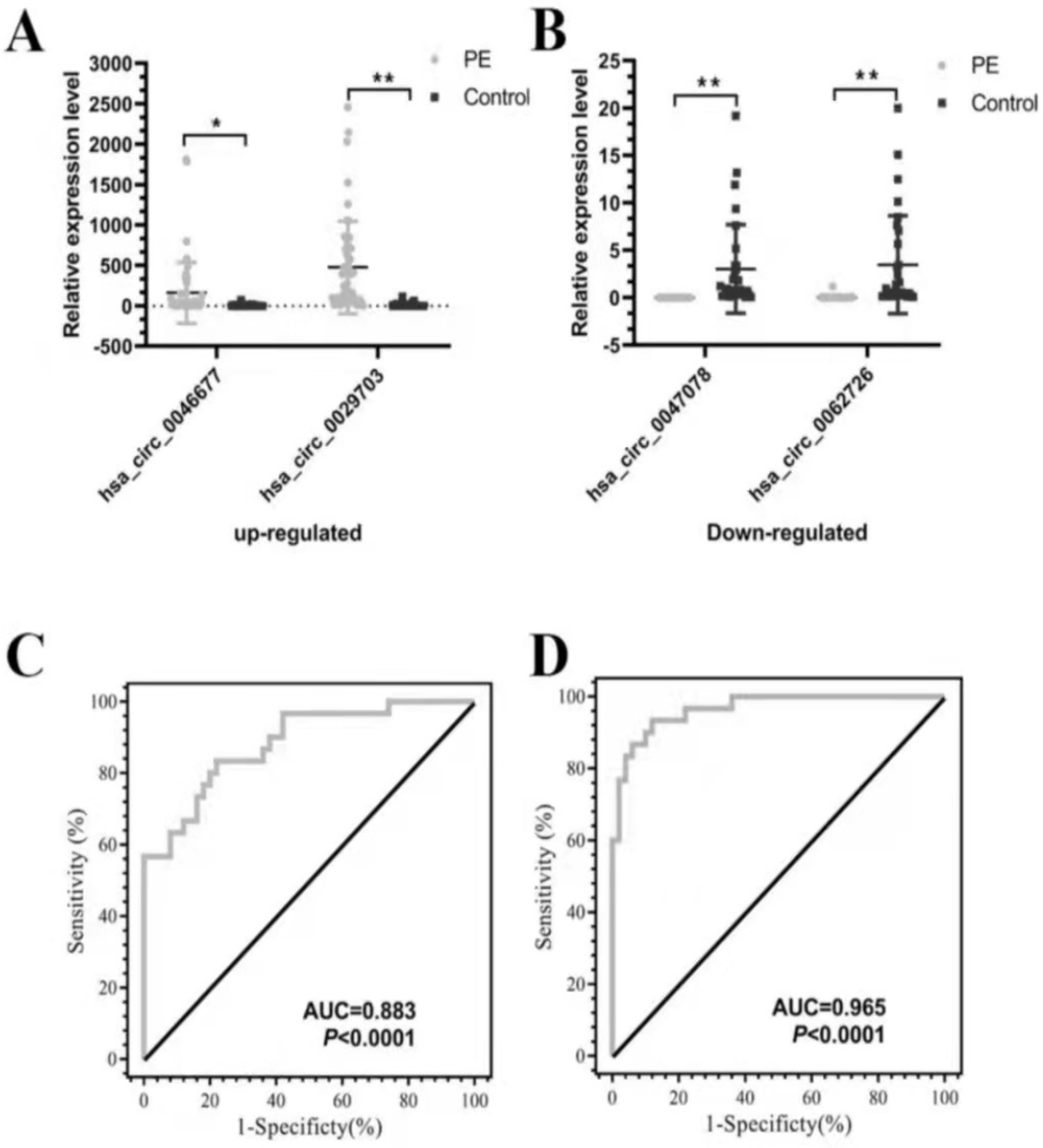


Fig. 6. Hsa_circ_0046677 and hsa_circ_00429703 were highly expressed in PE while hsa_circ_0047078 and hsa_circ_0062726 were downregulated
Source: Authors

nostic values for PE. Meanwhile, the interaction network of circRNA-miRNA-mRNA also supported the involvement of both hsa_circ_0046677 and hsa_circ_00429703 in the pathogenesis of PE, hsa_circ_0046677 (chr18: 670692-671451+) and hsa_circ_00429703 (chr13: 21955573 21965993) in the binding of corresponding miRNAs through the miRNA sponge mechanism. For instance, hsa_circ_0046677 targeted hsa_miR_595 and hsa_miR_574-5P participating in regulation of NDRG1, which inhibited PE angiogenesis and reduced trophoblast invasion capacity (Dai et al. 2020; Fu et al. 2017), and hsa_circ_00429703 participated in regulating ERAP2 by targeting hsa_ miR_16-2-3p and hsa_miR_296-1-5p, which acted on the corresponding target genes to participate in the pathogenesis of PE.

CONCLUSION

In this study, researchers obtained differential expressions of circRNAs between PE patients and normal controls. And researchers also indicated that hsa_circ_0046677 and hsa_circ_00429703 were promising biomarkers for PE patients by the relative verification test. This discovery might provide new targets for diagnosing PE. However, this study was limited and more evidence about cell functions is needed for a better understanding.

RECOMMENDATIONS

12,579 differentially expressed circRNAs were discovered, in which 7,684 were promoted with 4,895 downregulated. GO analysis confirmed expressions of 24 circRNAs (15 upregulated and 9 downregulated) in peripheral blood of PE while KEGG signaling pathway analysis revealed that ERK was involved in the mechanism of PE. Thereafter, circRNA-miRNA-mRNA interaction network verified that hsa_circ_0046677 and hsa_circ_00429703 were greatly increased while hsa_circ_0047078 and hsa_circ_0062726 were suppressed in PE. Hsa_circ_0046677 and hsa_circ_00429703 were then demonstrated as promising circRNAs to predict progression of PE. This study examined new circRNAs and analyzed functions of them in PE in vivo, which might provide promising molecular ways to treat PE.

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ABBREVIATION LIST

ALT: alanine aminotransferase; CI: confidence interval; MAP: mean arterial pressure; NLR: neutrophil-to-lymphocyte ratio; ROC: receiver operating characteristic; qRT-qPCR: quantitative reverse transcription polymerase chain reaction; PE: preeclampsia; RNA-Seq: RNA sequencing; GO: gene ontology; AUC: area under the curve; GA: gestational age; BMI: Body mass index; SBP: systemic blood pressure; FC: Fold Change; BP: biological process; CC: Cell components; AST: aspartate aminotransferase; MF: molecular function; MAPK: mitogen-activated protein kinase; KEGG: Kyoto Encyclopedia of Genes and Genomes; ERK: extracellular signal-regulated kinase; DBP: diastolic blood pressure; WBC: white blood cell.

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CONFLICT OF INTERESTS

All authors declare no conflict of interest

ETHIC APPROVAL

Experiments on human animals are not included in this article

DATA AVAILIABILTY

All data can be requested from the corresponding author

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