Profiles of circular RNAs in human blood and their potential roles in preeclampsia

Keywords
blood, ceRNA, circRNAs, preeclampsia, expression profile, immunity response

Abstract
Introduction
To identify circular RNAs (circRNAs) expression profiles in the blood of preeclampsia (PE) and healthy pregnant women, further clarify the possible mechanisms of circRNAs involved in the pathogenesis of PE.

Material and methods
Methods: Whole blood samples were collected from 5 paired PE and healthy pregnant women, the differentially expressed circRNAs (DE-circRNAs) were investigated by using high-throughput sequencing. Bioinformatics was performed to evaluate the sequencing results and obtain insights into possible mechanisms, such as GO and KEGG pathway analyses. Then, six DE-circRNAs were chosen and validated by quantitative real-time PCR (RT-qPCR) in an enlarged sample size. Their diagnostic values were analyzed by the receiver operating characteristic (ROC) curve. The establishment and analysis of the circRNA-miRNA-mRNA network were made for the validated DE-circRNAs.

Results
Results: A total of 139 DE-circRNAs between PE and controls were revealed. Of them, 18 circRNAs were upregulated, and 31 circRNAs were downregulated (fold change >2 and P-value < 0.05). Three circRNAs (has-circ-0007717, has-circ-0006460, and has-circ-0093055) were higher in both blood of early-onset and late-onset PE patients. The ROC analysis showed area under the curve values of has-circ-0007717, has-circ-0006460, and has-circ-0093055 were 0.64 (P=0.11), 0.72 (P=0.01), and 0.72 (P=0.01), respectively. Then, the circRNA-miRNA-mRNA competing for endogenous RNAs (ceRNAs) network comprised 2 circRNAs, 154 miRNAs, and 6 mRNAs. KEGG analysis of these mRNAs included immunity response-related pathways and cellular senescence.

Conclusions
Conclusion: CeRNA regulatory network indicated that the DE-circRNAs might participate in the processes of cell immunity response.

Explanation letter
Dear Editor:
Thank you for your decision letter and the five reviewers’ comments concerning our manuscript [AMS-13474-2021-01], entitled: Profiles of circular RNAs in human blood and their potential roles in preeclampsia. Those comments are of great value and help for improving our manuscript. We have studied these comments carefully and have made corrections that we hope to meet with approval; the revised portions are marked in red in the revised manuscript. In addition, we have cited 2 papers published in the Archives of Medical Science in the revised manuscript. The main corrections are in the revised manuscript, and the point-by-point response to the reviewers’ comments are as follow:

Review 1:
The article entitled ‘Profiles of circular RNAs in human blood and their potential roles in preeclampsia.’
The issue of this article is interesting and the article was well written.

Abstract
1. In abstract the authors state that ‘Whole blood samples were collected from 5 paired PE and healthy pregnant women’. On the contrary a total of 51 pregnant women (33 cases with PE patients, including 10 with early onset PE and 23 with late onset PE, and 18 healthy pregnant women without hypertension) from the West China Second University Hospital, Sichuan University, were enrolled in Methods. Please clarify this.

Response: Thanks for your question. We are sorry that our statements confused you. Whole blood samples for high-throughput sequencing and bioinformatics analyses were collected from 5 paired PE and healthy pregnant women, the subsequent series experiments were carried out in more samples. In addition, the samples were revised to 47 pregnant women (30 cases with PE patients, including 10 with early-onset PE and 20 with late-onset PE, and 17 healthy pregnant women without PE) according to the other reviewer’s suggestion. We have revised related content as follow:

Line 38: six DE-circRNAs were chosen and validated by quantitative real-time PCR (RT-qPCR) in an enlarged sample size.

Line 113: Five pairs of samples were randomly selected for the RNA extraction and sequencing.

Line 128: All subsequent bioinformatics analyses were from these five pair samples.

Line 131: Total RNA was extracted for 30 PE samples and 17 control samples without PE.

2. Please delete ‘Profiles of circRNAs of blood were investigated using high-throughput sequencing, and their potential regulatory mechanisms in PE was revealed.’ In Conclusion.

Response: Thanks for your suggestion. We have deleted this sentence in the revised manuscript.

3. Please specify abbreviations when first used. (CeRNA)

Response: Thanks for your suggestion. We have added an abbreviation for ceRNAs as competing for endogenous RNAs in line 46.

Introduction
1. Please delete the sentence ‘A circRNA-miRNA-mRNA network was constructed and found that hsa_circRNA_104515 and hsa_circRNA_100291 might function as ceRNAs to play critical roles in hepatocellular carcinoma [16]. Competitive interactions between circ_001422 and miR-195-5p increased FGF2 expression while activating the PI3K/Akt signaling, further promotes the progression and metastasis of osteosarcoma [17]’

Response: Thanks for your suggestion. We have deleted these sentences.

2. Please add the objective of this study as a last sentence.

Response: Thanks for your suggestion. We have added the objective of this study as the last sentence.

Line 88-90: This study aims to use the ceRNA network to strengthen the understanding of the pathogenesis of PE, and reveal the possible molecular mechanisms.

Methods
1. Please add reference for ‘The patients were diagnosed with PE according to the following criteria: new onset of hypertension after 20 weeks of gestation, systolic blood pressure (BP) ≥140 mmHg or diastolic BP ≥90 mmHg on two occasions at least 4 hours apart, 24-hour proteinuria ≥ 0.3 g.’

Response: Thanks for your suggestion. We have added the reference in line 100: As described in a previous study (16).


2. Please add the inclusion and exclusion criteria

Response: Thanks for your suggestion. The inclusion and exclusion criteria have been inserted as follows:

Line 100-106: The patients were diagnosed with PE according to the following criteria: new onset of hypertension after 20 weeks of gestation, systolic blood pressure (BP) ≥140 mmHg or diastolic BP ≥90 mmHg on two occasions at least 4 hours apart, 24-hour proteinuria ≥ 0.3 g. According to the gestational age at diagnosis and/or delivery, PE has been classified into early-onset PE (<34
gestational weeks) and late-onset PE (>34 gestational weeks). The pregnant women without PE were included as controls. Control subjects took the normal blood pressure measurements on the day of enrollment.

Line 106-108: Exclusion criteria including multiple pregnancies and transplanted organs, other pregnancy complications (e.g., chronic hypertension and diabetes mellitus), other complications (e.g., renal diseases, oncological diseases and autoimmune diseases), and any known fetal anomalies, were excluded.

3. Please add reference/references for ‘identification and quantification of circRNAs, Functional enrichment analysis, CeRNA network analysis’
Response: Thanks for your suggestion. We have added the related references as follows:

Results
1. Please add the scientific rationale for the selection ‘Four upregulated and two downregulated circRNAs (Table 1) were selected based on the highest expression, fold change value > 2, and P-value < 0.05 for validation by RT-qPCR with divergent primers’.
Response: Thanks for your suggestion. Refer to the other study [20], the selection of significantly altered genes (Table 1) was based on the highest expression, fold change value > 2, and P-value < 0.05, and these genes were validated by RT-qPCR with divergent primers.

2. Are there any differences between women with early onset PE and with late onset PE.
Response: Thanks for your question. We define this in the methods section line 102-104: According to the gestational age at diagnosis and/or delivery, PE has been classified into early-onset PE (<34 gestational weeks) and late-onset PE (>34 gestational weeks).

Discussion
1. Please add reference for Among them, cardiovascular complications are reported to as a common complication of PE.
Response: Thanks for your suggestion. We have added the reference.

2. Please delete first paragraph ‘As we know, PE is a serious and common pregnancy complication, and it is believed that many 198 biological processes (immune maladaptation, shallow placental implantation, reduced trophoblast invasion, 199 placental ischemia, and oxidative stress, etc.) were associated with the etiology and the pathogenesis of PE 200 [3]. However, its mechanism underlying PE remains still unclear. It is generally recognized that the placenta 201 development was involved in all the symptoms of PE, while in some cases of PE occurs in the postpartum 202 phase when the placenta has been removed [20]. Therefore, certain molecules in blood also play an 203 important role in the development of PE. Inflammatory cytokines such as IL-6 and IL-10 have consistently 204 been
present at higher serum levels in women with PE than in normal pregnant women [21, 22]. Downregulation of CD163 in monocytes, lower concentrations of IL-10 and soluble CD163 in the plasma indicates improper modulation of the systemic inflammatory response [23]."

Response: Thanks for your suggestion. We have deleted this paragraph.

3. Please delete the sentence 'With the rapid development of the second generation of high-throughput sequencing and bioinformatics, more functional circRNAs in human diseases have been identified [24, 25]' Response: Thanks for your suggestion. We have deleted this sentence.

4. Please add the limitations and strengths of this study
Response: Thanks for your suggestion. We have added limitations in the revised manuscript, as follows:
Line 274-282: There are several limitations to the study. (1) The characteristic features of pregnant women are different in these groups, not homogeneous. The expression of genes may be influenced by gestational age, especially. Hence, the validation with a larger sample size may contribute to improving the power of results and supporting the use of these circRNAs (hsa_circ_0007717, hsa_circ_0006660, and hsa_circ_0093055) for finding the molecular mechanisms that drive the progression of PE. (2) Possible mechanisms for these circRNAs were only predicted using bioinformatics, the further research for functional verification and investigation of downstream signaling molecules would be performed in the subsequent study. (3) The diagnostic values of hsa_circ_0093055 did not show very good results, which needs to be confirmed by a follow-up study with larger sample sizes.

5. Please add the clinical importance of the study results.
Response: Thanks for your suggestion. We have added the clinical importance of the study results in Conclusion.
Line 290-292: This study provides profiles of circular RNAs in human circulation and new insights into the pathogenesis of PE. It is of great significance to find the molecular mechanisms that drive the progression of PE, which may give more effective clinical therapeutic schedules.

Review 2:
Well written article, in a very interesting topic.
Response: Thank you very much!

Review 3:
The authors present a manuscript which aims to determine the profiles of circular RNAs in peripheral circulation of women with preeclampsia. Although it is a novel study which offers interesting data, several corrections should be made to achieve better comprehension. First of all, the manuscript contains many grammatical and typographical errors and, thus, the whole manuscript should be edited by a professional in English language. Second, the introduction part should be condensed to one page. Third, the authors should mention about the factors that limit the power of their study and the potential clinical implications of their findings in the discussion part.
Response: Thanks for your suggestion. First, the manuscript was edited by a professional in English language, and we hope our language problems have been corrected. Second, we have deleted some content in the introduction part, and it has been condensed to one page. Third, we have added the limitations of this study in the Discussion, as follows:
Line 274-282: There are several limitations to the study. (1) The characteristic features of pregnant women are different in these groups, not homogeneous. The expression of genes may be influenced by gestational age, especially. Hence, the validation with a larger sample size may contribute to improving the power of results and supporting the use of these circRNAs (hsa_circ_0007717, hsa_circ_0006660, and hsa_circ_0093055) for finding the molecular mechanisms that drive the progression of PE. (2) Possible mechanisms for these circRNAs were only predicted using bioinformatics, the further research for functional verification and investigation of downstream signaling molecules would be performed in the subsequent study. (3) The diagnostic values of hsa_circ_0093055 did not show excellent results, which needs to be confirmed by a follow-up study with larger sample sizes.
Review 4:
Dear Author,
Interesting and well thought out work. However, the working method is not adequately explained. If necessary explanations are added to the working method, it will provide a good perspective.
The changes I have suggested for your article are listed below:
Key words
“immunity response” can be added to keywords.
Response: Thanks for your suggestion. “immunity response” has been added into keywords.

Introduction
The purpose of the research should be added at the end of the introduction section. The problem should be clearly stated and research hypotheses should be written.
Response: Thanks for your suggestion. The purpose of the research has been added at the end of the introduction section. In addition, the research procedures were presented in the last paragraph of the introduction, and we hope these could state the problem clearly.
Line 88-90: This study aims to use the ceRNA network to strengthen the understanding of the pathogenesis of PE, and reveal the possible molecular mechanisms.

Materials and methods
The number of pregnant women included in the sample groups is quitely variable. Why were these numbers (33 cases with PE patients, including 10 with early onset PE and 23 with late onset PE, and 18 healthy pregnant women without hypertension) selected for sampling?
Has a method been followed for selection?
Why weren’t equal numbers of samples taken from all groups?
Response: Thanks for your above questions. The samples were collected just according to inclusion and exclusion criteria. Pregnant women with PE often have other pregnancy complications included in the exclusion criteria. Besides, expectant treatment is usually given to pregnant women once detected. Thus, sample collection is limited. Considering our sample size is small, we did not use related statistical methods to estimate the sample and did not equal numbers of samples. This study aims to preliminarily use the ceRNA network to reveal the possible molecular mechanisms, and we will conduct subsequent investigations into specific molecular mechanisms in larger sample sizes.
The gestational week is very important for preeclampsia. 10 were reported as early-onset PE and 23 as late-onset PE. but how many weeks pregnant? not open.
How many weeks pregnant were healthy pregnant women? Is it the same week as the gestational week of women with preeclampsia?
The characteristic features of pregnant women are different groups, not homogeneous, that is, heterogeneous. So how accurate can the comparison be? Interpreting the results of the study in this situation would be very difficult.
Response: Thanks for your above questions. The gestational weeks of pregnant women were shown in Table 1. Indeed, gestational week of each group was different. This study is only a preliminary result to indicate several circRNAs may be involved in the mechanism of PE, and we will confirm the relevant mechanism in a larger sample size in the future study. Thus, we present this as a limitation of the study.
Between what dates was the study carried out? should be written.
Response: Thanks for your question. We have added the dates in line 96: The samples were collected from October 2020 to July 2021.

The characteristics of the healthy pregnant should also be written.
Response: Thanks for your suggestion. We have added the characteristics of the healthy pregnant in line 104-106:
The pregnant women without PE were included as controls. Control subjects took the normal blood pressure measurements on the day of enrollment. The exclusion criteria of controls are the same as for PE.

How did you test the normal distribution of the data?
Response: Thanks for your question. The Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. We have added this in the revised manuscript (line 160).

RT-qPCR
Were the instruments used in the laboratory calibrated? When was it last done?
Response: Thanks for your question. The instruments are customarily calibrated every two months. Thus the accuracy of instrument is adequate.

Result
Table.1: According to the study aim ("This study aimed to identify circular RNAs (circRNAs) expression profiles in the blood of preeclampsia (PE) and healthy pregnant women, further clarify the possible mechanisms of circRNAs involved in the pathogenesis of PE."), the characteristics of both groups in Table 1 are not homogeneous. It will affect blood sample. How reliable are these data in this direction?
Response: Thanks for your question. This study aims to preliminarily use the ceRNA network to reveal the possible molecular mechanisms, and we will confirm the relevant mechanism in a larger sample size in the future study. We have added this as a limitation of the study.

There is also a pregnant woman with diabetes and you added her as a healthy pregnant woman. this will make a difference in complications and cellular activities. Both healthy and pregnant women with preeclampsia should not be included in the groups.
Response: Thanks for your suggestion. We have ruled out the pregnant woman with diabetes, and statistics and comparisons were made again, including the tables and figures.

References
• The 13th reference is incorrect, it should be corrected.
• The 41st reference is incorrect, it should be corrected.
Response: Thanks for your suggestion. The references have been corrected as follows:

Article (PDF)
Review 5:
(this review has file attachment)
The study is interesting, of potential clinical value. The manuscript is well written. It has, however, limitations. Some points have to be clarified (see above). Other limitations have to be at least discussed and pointed out. The results have to be validated in a second larger population. Experimental data are necessary to confirm the data generated by the authors analysis.
Response: Thanks for your suggestion. We have added the limitations in the revised manuscript (line 274-282). Experimental data were uploaded to the cloud disk, and please click the link: https://www.jianguoyun.com/p/Ddz_6NsQjom6CRiBnqIE
More point-by-point responses are shown in the attachment.

We deeply appreciate the editor’s and reviewers’ valuable input and hope that the final revised manuscript will meet with your approval.
Once again, thank you very much for your comments and suggestions.

Thanks
With best regards
Yours sincerely
Tao Wang, M.D.
Department of Obstetrics and Gynecology,
West China Second University Hospital, Sichuan University
Profiles of circular RNAs in human blood and their potential roles in preeclampsia

Min Liu\textsuperscript{2}, Xiaolei Luo\textsuperscript{1}, Linbo Gao\textsuperscript{1}, Mengdan Shi\textsuperscript{2}, Rong Zhou\textsuperscript{2}, Tao Wang\textsuperscript{1,*}

\textsuperscript{1}Center for Translational Medicine, Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, Chengdu 610041, P. R. China;

\textsuperscript{2}Department of Obstetrics and Gynecology, Center for Translational Medicine, Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu 610041, P. R. China.

*Correspondence to Tao Wang, Center for Translational Medicine, Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, Chengdu 610041, P. R. China.

Telephone/fax number: 86-28-8550-3604

Email: taowang@scu.edu.cn
Abstract

Objectives: To identify circular RNAs (circRNAs) expression profiles in the blood of preeclampsia (PE) and healthy pregnant women, further clarify the possible mechanisms of circRNAs involved in the pathogenesis of PE.

Methods: Whole blood samples were collected from 5 paired PE and healthy pregnant women, the differentially expressed circRNAs (DE-circRNAs) were investigated by using high-throughput sequencing. Bioinformatics was performed to evaluate the sequencing results and obtain insights into possible mechanisms, such as GO and KEGG pathway analyses. Then, six DE-circRNAs were chosen and validated by quantitative real-time PCR (RT-qPCR) in an enlarged sample size. Their diagnostic values were analyzed by the receiver operating characteristic (ROC) curve. The establishment and analysis of the circRNA-miRNA-mRNA network were made for the validated DE-circRNAs.

Results: A total of 139 DE-circRNAs between PE and controls were revealed. Of them, 18 circRNAs were upregulated, and 31 circRNAs were downregulated (fold change >2 and P-value < 0.05). Three circRNAs (has-circ-0007717, has-circ-0006460, and has-circ-0093055) were higher in both blood of early-onset and late-onset PE patients. The ROC analysis showed area under the curve values of has-circ-0007717, has-circ-0006460, and has-circ-0093055 were 0.64 (P=0.11), 0.72 (P=0.01), and 0.72 (P=0.01), respectively.

Then, the circRNA-miRNA-mRNA competing for endogenous RNAs (ceRNAs) network comprised 2 circRNAs, 154 miRNAs, and 6 mRNAs. KEGG analysis of these mRNAs included immunity response-related pathways and cellular senescence.

Conclusion: CeRNA regulatory network indicated that the DE-circRNAs might participate in the processes of cell immunity response.

Keywords: preeclampsia, circRNAs, expression profile, ceRNA, blood, immunity response
Introduction

Preeclampsia (PE) is a pregnancy-specific disorder associated with new-onset hypertension after 20 weeks of gestation and is one of the main causes of maternal and perinatal mortality and morbidity worldwide, often accompanied by proteinuria or other organ damage such as to the kidney, brain or liver, it also may present in some women in the absence of proteinuria [1]. PE is classified into subtypes of early (delivered before 34 weeks) and late-onset (delivered after 34 weeks) according to the time of diagnosis [2]. Many factors such as defective deep placentation, oxidative stress, endothelial dysfunction, and intravascular inflammation contribute to it [3], but the etiology and pathogenesis are far from being completely clarified. Among them, immune responses play a central role [4]. For example, NK cells interact with trophoblast cells and endothelial cells involved in remodeling the spiral arteries during early pregnancy [5]. In pregnant women with preeclampsia, the changes in circulating Tregs are reflected by high proportions of Th17 cells and the decreased IL-35 levels accompanied by elevated IL-17A levels [6].

Circular RNAs (circRNAs), defined as covalently closed circular RNAs, have recently considered being correlated with various pathological processes of cancer, diabetes, and neurodegenerative disorders, etc. [7, 8]. Compared with other RNAs, the abundance of circRNAs is relatively low, but stability is highly stable due to the circular configuration. Qian et al. first conducted a circRNA microarray and compared placentas from PE and healthy pregnancies and found 301 differentially expressed circRNAs might be involved in the development of PE through acting as microRNA (miRNA) sponges [9]. Subsequently, an increasing number of studies have demonstrated the involvement of circRNAs in the pathogenesis of PE using high-throughput sequencing and bioinformatics techniques [10, 11]. Still, it can't be ruled out that some novel circRNAs may be missed due to tissue-specific characteristics and database updates.

CircRNAs can interact with other RNAs and proteins, acting as miRNA sponges to regulate the transcription; they also are translated into proteins in rare cases [12]. Also, the miRNA response elements shared by circRNA and mRNA competitively bind to miRNAs, thereby influencing the regulatory function of miRNAs and gene expression [13]. Studies often focus on competing for endogenous RNAs (ceRNAs) such as long non-coding RNA, circRNAs, and mRNAs to understand the pathogenesis of diseases [14, 15].

In this study, the differential expression profile of circRNAs in healthy and PE pregnant women were demonstrated by high-throughput sequencing and predicted the potential functions by GO and KEGG pathway analyses. Moreover, the differential expression mRNAs and predicted the miRNAs targeted by...
circRNAs to generate a circRNA-miRNA-mRNA network were gained. This study aims to use the ceRNA network to strengthen the understanding of the pathogenesis of PE and reveal the possible molecular mechanisms.

Materials and methods

Sample collection and RNA extraction

A total of 47 pregnant women (30 cases with PE patients, including 10 with early-onset PE and 20 with late-onset PE, and 17 “healthy” pregnant women without hypertension) from the West China Second University Hospital, Sichuan University, were enrolled. The samples were collected from October 2021 to July 2021. All participants give informed consent before participation. The Institutional Committee approved the study protocol for the Protection of Human Subjects (the Institutional Review Board of West China Second University Hospital, Sichuan University, permit number 2019030).

As described in a previous study [16], the patients were diagnosed with PE according to the following criteria: new onset of hypertension after 20 weeks of gestation, systolic blood pressure (BP) ≥140 mmHg or diastolic BP ≥90 mmHg on two occasions at least 4 hours apart, 24-hour proteinuria ≥ 0.3 g. According to the gestational age at diagnosis and/or delivery, PE has been classified into early-onset PE (<34 gestational weeks) and late-onset PE (>34 gestational weeks). The pregnant women without PE were included as controls. Control subjects took the normal blood pressure measurements on the day of enrollment. Exclusion criteria including multiple pregnancies and transplanted organs, other pregnancy complications (e.g., chronic hypertension and diabetes mellitus), other complications (e.g., renal diseases, oncological diseases and autoimmune diseases), and any known fetal anomalies were excluded. The clinical characteristics are shown in Table 1. Before delivery, the whole blood samples (3.0-5.0 mL) were collected in a sterile EDTA-containing vacutainer tube and cut carefully into pieces. Then, we froze samples at -80 °C immediately after adding 1 mL TRIzol reagent.

RNA extraction and sequencing

Five pairs of samples were randomly selected for RNA extraction and sequencing. Total RNA was extracted by using mirVana miRNA Isolation Kit following its protocol and quantified with a NanoDrop ND 2000 spectrophotometer. According to the manufacturer's instructions, the libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold. Then these libraries were sequenced on the Illumina
sequencing platform (HiSeqTM 2500 or other platforms), and 150 bp/125 bp paired-end reads were generated.

**Identification and quantification of human circRNAs**

We first used sortmerna software to remove the residual rRNA [17], then conducted quality testing through FASTQC software to ensure that high-quality reads obtained [18] that have been filtered and tested strictly are called clean reads, the expression patterns of circRNAs and mRNAs were obtained. CIRI software is highly sensitive and can reduce false positives through multiple screening, which is the authoritative software for circRNA prediction [19]. Match to the circBase and circpedia database, CIRI got Single CircBed after predicting and merging samples and then counted the number of junctions reads of each predicted circRNA in different samples. DESeq software was performed to analyze differential expression among the samples and to set the fold-change of $\geq 2$, and P-values of $<0.05$ were identified as significantly differentially expressed transcripts. All subsequent bioinformatics analyses were from these five pair samples.

**Real-time quantitative PCR (RT-qPCR) assay**

Total RNA was extracted for 30 PE samples and 17 control samples without PE, and was subjected to cDNA synthesis using the Prime Script RT reagent Kit (Takara, Dalian, China) following the manufacturer’s instructions. RT-qPCR was performed in the Light Cycle 480 Real-Time PCR Detection System (Roche, Germany) using qPCR SYBR Green Master (Vazyme, Nanjing, China). Refer to the other study [20], four upregulated and two downregulated circRNAs (Table 2) were selected based on the fold change value $> 2$, P-value $<0.05$, and expression levels for validation by qRT-PCR. The divergent primer pairs were designed using circPrimer software and Primer 5.0 for target circRNAs, the primer sequences used are shown in Table 1. Relative expression of circRNAs was analyzed using the $2^{\Delta\Delta Ct}$ method with normalization to $\beta$-actin expression and used as a quantitative control.

**Functional enrichment analysis**

The differentially expressed circRNAs were subjected to gene ontology (GO) to illustrate the genetic regulatory networks according to the cellular component, biological process, and molecular function aspects (http://www.geneontology.org) [21]. Likewise, KOBAS software (KEGG Orthology-Based Annotation System) was performed to make a KEGG pathway enrichment analysis of the networks [22].

**CeRNA network analysis**
Based on the validated DE-circRNAs verified by RT-qPCR, we further constructed a circRNA-miRNA-mRNA interaction network to explore the validated ceRNA mechanisms [23]. MiRNA-bound circRNAs can be identified using miRNA target gene prediction (miRanda database), on account of multiple miRNA binding sites of circRNAs. The threshold parameter was set as described previously: S ≥ 150, ΔG ≤ −30 kcal/mol and strict 5’ seed pairing [24]. Subsequently, shared pairs of miRNA-mRNA and miRNA-circRNA were used to predict ceRNA score using MuTaME [25] according to the following formula:

\[
\text{ceRNA_score} = \frac{\text{MRE_for_share_miRNA}}{\text{MRE_for_circRNA_miRNA}}
\]

with P-value was calculated using the Benjamini and Hochberg’s approach for controlling the false discovery rate. The positively correlated pairs of circRNA-mRNA based on ceRNA score principle were then considered as the true ceRNAs. Based on established co-expression data, deregulated ceRNA networks and circRNA, or miRNA interactions of interest were mapped using Cytoscape software (V. 3.7.1). The functions of these circRNAs were clarified according to the functional annotations of circRNA target mRNAs.

Statistical analysis

All statistical analysis was performed with SPSS 19 (SPSS Science Inc., Chicago, Illinois) and GraphPad Prism (Version 6.0, GraphPad Software, La Jolla, CA). The Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. The differences between the two groups were evaluated by independent t-test, and nonparametric Mann-Whitney t-tests were used for analyses while the data was not normally distributed. Data are shown by means ± SEM or interquartile range (IQR). P < 0.05 was presented as a statistical significance. The diagnostic value of these DE-circRNAs was determined by using receiver-operating characteristics (ROC) curves.

Results

Profiles of DE-circRNAs in the blood of women with PE and healthy pregnancy

Compared with the information of human circRNAs in circBase (http://www.circbase.org/) utilizing sequence data, 688,310 circRNAs in the blood were identified. When matched to the circBase and circpediae database, 25,886 circRNAs of these circRNAs were annotated, and 2722 circRNAs were newly discovered (Figure 1A). Analysis of high-throughput sequencing data revealed 139 DE-circRNAs between PE and healthy pregnant women. The results showed that 18 circRNAs were upregulated and 31 circRNAs were downregulated (fold change >1 and P-value < 0.05), as shown in heat maps and volcano plots (Figure
Functional analysis of DE-circRNAs

GO annotation analysis and the KEGG pathway were used to gain insights into the functions of DE-circRNAs. As shown in Figure 2A, enriched GO terms of the vital PE-related circRNAs were presented according to biological processes, cellular components, and molecular functions. For biological processes, DE-circRNAs were markedly enriched in terms such as biological regulation, immune system process, metabolic process, and so on. For molecular function, the vital PE-related circRNAs were mainly associated with binding, catalytic activity, and protein binding transcription factor activity. Meanwhile, the most enriched KEGG pathways included transport and catabolism, membrane transport, cell growth and death. Human diseases enrichment result shows that they might involve in infectious disease, cancer, cardiovascular disease, etc. (Figure 2B). Among them, cardiovascular complications are reported to as a common complication of PE [26].

Validation of DE-circRNA expression by RT-qPCR

Four upregulated and two downregulated circRNAs (Table 1) were selected based on the highest expression, fold change value > 2, and P-value < 0.05 for validation by RT-qPCR with divergent primers. The results revealed that the expression levels of circRNAs (has-circ-0007717, has-circ-0006460, and has-circ-0093055) in the blood were significantly higher in both subtype groups of PE patients than those in healthy pregnant women; the differential expression levels of other circRNAs were not significant (Figure 3A-3F). Moreover, the expression levels of circRNAs did not differ significantly between the subtype groups. Then, the ROC curve analysis was performed to detect the diagnostic values of these DE-circRNAs for PE. The results of area under the curve (AUC) showed that the values of has-circ-0007717, has-circ-0006460, and has-circ-0093055 were 0.64 (P=0.11), 0.72 (P=0.01), and 0.72 (P=0.01), respectively (Figure 3G-3I).

The threshold that provided maximal sensitivity and specificity for the diagnosis of PE was determined as the cut-off value. As shown in Table 3, the sensitivity and specificity of hsa_circ_0093055 was the highest, at 73.33% and 64.71%, respectively. Moreover, the positive predict value and negative predict value hsa_circ_0093055 were the highest, at 78.57% and 63.16%, respectively.
CircRNA-miRNA-mRNA interaction analysis

The three validated DE-circRNAs (has-circ-0007717, has-circ-0006460, and has-circ-0093055) were chosen for further ceRNA network analysis. The miranda was used to predict the binding between these miRNA-circRNA sequences, and a total of 2567 miRNA-circRNA relationship pairs were obtained, the top 100 of them were presented (Figure S1A). Subsequently, we constructed the networks for circRNA-mRNA through co-expression analysis (Figure S1B). As shown in Figure 4A, a global ceRNA network was constructed.

A hypergeometric distribution test was used to calculate the enrichment significance of these mRNAs regulated by circRNA in GO or KEGG entries as functional annotation of circRNA. For biological processes, the GO terms were found to be markedly enriched in terms such as regulation of immune response, regulation of dendritic spine development, and negative regulation of tumor necrosis factor-mediated signaling pathway, and so on (Figure 4B). To get the pathways affected by our validated circRNAs, we performed a KEGG analysis of the six mRNAs in the network. The result indicated that antigen processing and presentation, natural killer cell-mediated cytotoxicity, and cellular senescence might involve and have been reported to participate in the occurrence of PE (Figure 4C).

Discussion

It is well accepted that circRNAs are involved in many obstetric complications by function prediction, including fetal growth restriction and infection during pregnancy [27]. Also, a few researchers have focused on the relationship between circRNAs and PE. Studies revealed that the expression profile of circRNAs and their potential regulatory targets in the placentas of PE [10, 28, 29]. For example, a total of 180 DE-circRNAs were detected, hsa_circ_0011460 may serve as a potential therapeutic target for patients with PE with severe features [29]. Tong et al. conducted an RNA sequencing analysis to compare gene expression between the decidua of PE and normal pregnancy and found 293 key PE-related genes [30]. However, most of these studies were carried out in the placental tissue of PE. Fewer studies involve analysis of peripheral blood samples from PE patients to explore the related pathological mechanisms. In this study, we performed high-throughput sequencing to discover 139 DE-circRNAs in circulation from PE and normal controls; their biological functions and the possible mechanism of PE were predicted by GO and KEGG analyses. DE-circRNAs were found to be markedly enriched in biological processes such as biological regulation,
immune system process, metabolic process and so on. Meanwhile, human diseases enrichment result shows that they might involve in infectious disease, cancer, cardiovascular disease, etc. The cardiovascular complications are known to usually associated with PE. These suggested that DE-circRNAs may be involved in the pathogenesis of PE.

Six circRNAs were selected to confirm the results of the RNA sequencing by RT-qPCR. Consistent with the sequencing results that the expression of these circRNAs (hsa_circ_0007717, hsa_circ_0006460, and hsa_circ_0093055) was significantly increased in PE. It indicates that our sequencing data can truly reflect the expression of genes in the blood of PE patients. Moreover, the expression levels of circRNAs were not affected by gestational weeks at delivery and was closely associated with this disease. Ping et al. constructed the ceRNAs network based on all DE-circRNAs and determined that hsa_circRNA_13301 was the only one upregulated circRNA in ceRNA being targeted by four miRNAs in the blood of PE patients [11]. Distinctively, we selected the three validated circRNAs to performed further circRNA-miRNA-mRNA interaction networks and bioinformatics analysis. It's worth noting that the GO terms enriched in immune response and negative regulation of tumor necrosis factor (TNF)-mediated signaling pathway, and the KEGG analysis showed the antigen processing and presentation and natural killer (NK) cell-mediated cytotoxicity might involve in the occurrence of PE. As we know, maternal adaptation to fetal (paternal alloantigens) is vital in the embryonic implantation stage. During a normal pregnancy, immune cells either assist in required gestational processes or show immunoregulatory roles and are partially inactivated [31]. The critical role of NK cells in the pathogenesis of PE has been shown in numerous studies. Compared to healthy pregnancies, lower numbers and functional changes of NK cells were found in pregnant women with PE [32, 33]. Generally, Natural cytotoxicity receptors (NCRs) are the major receptors involved in NK cytotoxicity and function the recognition and lysis of tumor cells by NK cells. A study reported that pregnant women with PE present immunological abnormalities of NCRs on peripheral blood NK cells during pregnancy [34]. In terms of NK cell cytokine production, the decreased expression of NKP46+ NK cells in women with PE may be responsible for the high production of TNF-α in women with PE [35]. TNF-α blockade improves NK cell activation, hypertension, and mitochondrial oxidative stress in a preeclamptic rat model [36]. Overall, has-circ-0006460 and has-circ-0093055 may trigger a disorder in maternal immunological response in pregnant women with PE.

CeRNA hypothesis firstly proposed that regulatory networks working predict and manipulate through
miRNA competition [37]. The expression of mRNA and post-transcriptional gene control are regulated by circRNAs [38]; thus, the functional analysis of circRNA-targeted mRNAs may help us deeply understand the role of circRNAs in the pathogenesis of PE. Hence, the function of these six mRNA in the network was analyzed. As a newly identified secretory protein, the coiled-coil domain containing 3 (CCDC3) is expressed in adipose tissues and vascular endothelial cells. It reported that CCDC3 represses TNF-α-induced pro-inflammatory response in endothelial cells [39], and this process is closely related to the development of PE. KIR2DL4 is the framework of killer cell immunoglobulin-like receptors (KIRs) that implicate the interaction between dNK and extravillous trophoblast in vascular remodeling and its higher expression induces a higher risk of PE [40]. Similarly, SDK1 and OLFML3 also reflect immune function [41, 42]. MOK belongs to the MAP kinase superfamily to treat various inflammatory conditions [43]. Therefore, these mRNA further reflect that has-circ-0006460 and has-circ-0093055 could mediate the occurrence of PE through improper immune regulation.

There are several limitations to the study. (1) The characteristic features of pregnant women are different in these groups, not homogeneous. The expression of genes may be influenced by gestational age, especially. Hence, the validation with a larger sample size may contribute to improving the power of results and supporting the use of these circRNAs (hsa_circ_0007717, hsa_circ_0006460, and hsa_circ_0093055) for finding the molecular mechanisms that drive the progression of PE. (2) Possible mechanisms for these circRNAs were only predicted using bioinformatics, the further research for functional verification and investigation of downstream signaling molecules would be performed in the subsequent study. (3) The diagnostic values of hsa_circ_0093055 did not show excellent results, which needs to be confirmed by a follow-up study with larger sample sizes.

Conclusion

This study demonstrates the existence of dysregulated circRNAs in the blood of pregnant women with PE and healthy. The higher expression levels of circRNAs (has-circ-0007717, has-circ-0006460, and has-circ-0093055) were verified by RT-qPCR. Then, we focus on these circRNAs to perform circRNA-miRNA-mRNA interaction network and bioinformatic analysis. The results revealed that has-circ-0006460 and has-circ-0093055 might trigger a disorder in maternal immunological response in pregnant women with PE. This study provides profiles of circular RNAs in human circulation and new insights into the pathogenesis...
It is of great significance to find the molecular mechanisms that drive the progression of PE, which may give more effective clinical therapeutic schedules.

Declarations

Authors' contributions
Tao Wang designed the project; Min Liu and Linbo Gao analyzed data and experiments; Tao Wang evaluated and interpreted the results; Rong Zhou and Mengdan Shi collected samples; Min Liu and Tao Wang prepared, reviewed and finalized the draft manuscript. Finally, all authors approved the final version for journal submission.

Ethics approval
All participants give informed consent before participation. All the experiments were carried out following the Institutional Committee approved the study protocol for the Protection of Human Subjects (the Institutional Review Board of West China Second University Hospital, Sichuan University, permit number 2019030).

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Availability of data and material
Supplementary material is available on the publisher's website. Contact the corresponding author for all raw data.

Conflicts of interest
The authors declare no conflicts of interest.

Acknowledgments
None

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and challenges in the field. Oncogene 37(5), 555-565.


Figure Legends

Figure 1. Differentially expressed (DE) circRNAs in the blood of women with preeclampsia (PE) and healthy pregnant women (CTR). (A) The represented circRNAs were compared with the circRNAs in the circBase and circpediea database. (B) Heat map presentation of the expression profiles of the DE-circRNAs. The red reveals an increase and the blue reveals a decrease in expression level when compared PE with CTR. (C) The volcano plot displayed DE-circRNAs. Red dots indicated significantly up-regulated circRNAs, and green dots indicated significant down-regulated, gray dots indicated circRNAs with no significant difference.

Figure 2. Functional analysis of differential expressed (DE) circRNAs by Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. (A) GO terms enriched in the DE-circRNAs. (B) KEGG pathways enriched in the DE-circRNAs. PE: preeclampsia, CTR: normal controls.

Figure 3. Relative expression of the DE-circRNAs in blood from women with early-onset
preeclampsia (EOPE), late-onset preeclampsia (LOPE), and normal pregnancy (CTR) detected by RT-qPCR. (A-E) Relative expression of the DE-circRNAs was determined by RT-qPCR. (G-I) ROC analysis of PE patients based on has-circ-0007717, has-circ-0006460, and has-circ-0093055 expression.

The presented values are the means ± SEM or interquartile range (IQR), *P < 0.05, **P < 0.01, ns present no differences.

Figure 4. CeRNA network and functional analysis of validated DE-circRNA. (A) The ceRNA network was based on circRNA/miRNA and miRNA/mRNA interactions. This ceRNA network concerned includes has-circ-0007717, has-circ-0006460, and has-circ-0093055. The edges represent sequence matching, and circRNAs connect the expression of mRNAs through miRNAs. Round nodes (orange) represent circRNAs, quadrate nodes (light green) represent miRNAs, and triangle nodes (red) represent correlated mRNAs. The size of the node indicates the number of interactions. (B, C) GO terms and top 30 KEGG pathways enriched in the mRNAs regulated by circRNAs.

Figure S1. The ceRNA interactome. (A) The network for differential circRNA-mRNA interactions. Red points represent circRNAs and blue arrows represent mRNAs. (B) The network for predicted circRNA-miRNA interactions. Red points represent circRNAs and blue arrows represent miRNAs.
Table 1. Clinical characteristics of the study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal term pregnancy (n=17)</th>
<th>Early onset preeclampsia (n=10)</th>
<th>Late onset preeclampsia (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.35±1.03 (23–39)</td>
<td>31.9±0.79 (27–36)</td>
<td>32.75±1.87 (27–41)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.57±0.61 (22.31–30.67)</td>
<td>29.95±1.61 (20.83–36.33)*</td>
<td>30.51±1.87 (24.46–36.33)**</td>
</tr>
<tr>
<td>Primigravida</td>
<td>8 (47.1%)</td>
<td>6 (60.0%)</td>
<td>14 (70.0%)</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>38.99±0.27 (37–41)</td>
<td>30.06±4.77 (14.14–33.71)**</td>
<td>36.31±2 (34.14–40.86)**</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>117.06±1.19 (107–119)</td>
<td>155.6±16.94 (122–165)**</td>
<td>151.4±8.6 (126–170)*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>74±1.56 (61–88)</td>
<td>98.9±11.63 (75–114)**</td>
<td>98.2±5.76 (78–121)**</td>
</tr>
<tr>
<td>24-h urinary protein (g)</td>
<td>/</td>
<td>2.71±2.3 (0.357–7.903)**</td>
<td>1.32±0.45 (0.3–4.723)**</td>
</tr>
</tbody>
</table>

BMI indicates body mass index.
Comparisons between groups were performed using Student’s t-test, and results are presented as mean ± SD. *P<0.05, **P<0.01, healthy vs. preeclamptic pregnant women.

Table 2. Divergent primers of circRNAs for quantitative real-time PCR.

<table>
<thead>
<tr>
<th>circBase_ID</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>hsa_circ_0007717</td>
<td>CAGGTCCAGAGGGAAGATGA</td>
<td>GGACCTTGTGGAACCTG</td>
</tr>
<tr>
<td>hsa_circ_0093055</td>
<td>TTTTAGTCCTCCTGCGCTCA</td>
<td>CAATCCCGGTGCTCGAGC</td>
</tr>
<tr>
<td>hsa_circ_0006460</td>
<td>TGAGGGCTCCAGCTTGATC</td>
<td>GATGTTGCGCTGGATTCTTG</td>
</tr>
<tr>
<td>hsa_circ_0094867</td>
<td>GACACCACCAAGGTCAAACA</td>
<td>CCTGAATGCTGGTCTG</td>
</tr>
<tr>
<td>hsa_circ_0000048</td>
<td>CAATCTCTGGGACTGACTC</td>
<td>ACTCTGCGTGGGTATGA</td>
</tr>
<tr>
<td>hsa_circ_0009175</td>
<td>CCACTGCAACATTACCTCCG</td>
<td>CTATACAGAGGGCAAGC</td>
</tr>
<tr>
<td>β-actin</td>
<td>CAGTACGTTGCTATCCAGGC</td>
<td>CTCTTAATGTCACGACGAT</td>
</tr>
</tbody>
</table>

The genome version is hg38.

Table 3. The diagnostic value of validated DE-circRNAs for PE.

<table>
<thead>
<tr>
<th></th>
<th>hsa_circ_0007717</th>
<th>hsa_circ_0093055</th>
<th>hsa_circ_0006460</th>
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<tbody>
<tr>
<td>Sensitivity (Se) (%)</td>
<td>56.67</td>
<td>73.33</td>
<td>76.67</td>
</tr>
<tr>
<td>Specificity (Sp) (%)</td>
<td>82.35</td>
<td>64.71</td>
<td>70.59</td>
</tr>
<tr>
<td>Positive predictive value (PPV) (%)</td>
<td>85</td>
<td>78.57</td>
<td>73.33</td>
</tr>
<tr>
<td>Negative predictive value (NPV) (%)</td>
<td>51.85</td>
<td>63.16</td>
<td>52.94</td>
</tr>
</tbody>
</table>
Figure 1
Figure 4
Figure S1