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Global gene expression analysis of cell-free RNA in amniotic fluid from women destined to develop preeclampsia

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Abstract

Preeclampsia (PE) is a disorder specific to pregnancy characterized by new-onset hypertension and proteinuria after 20 weeks of gestation. There is no definite treatment for PE except delivery of the placenta. The purpose of this study was to elucidate the biological pathways involved in the development of PE and to discover a novel biomarker for PE by performing global gene expression analysis of amniotic fluid cell-free RNA.

The participants were recruited from the Department of Obstetrics and Gynecology of CHA Gangnam Medical Center (Seoul, Korea) between March 2014 and February 2015. Eight samples were collected from 8 subjects at second trimester who were later diagnosed with PE. From the amniotic fluid samples, cell-free RNA extraction was performed and gene expression was analyzed using the GeneChip PrimeView Array. Transcriptome data previously analyzed by our group from 9 euploid mid-trimester amniotic fluid samples were used as the control for comparative analysis. Functional analysis of the probe sets was performed using the online Database for Annotation, Visualization, and Integrated Discovery (DAVID) toolkit 6.7.

We identified 1841 differentially expressed genes (DEGs) between the PE group and the control. Of these, 1557 genes were upregulated in the PE group, while 284 genes were upregulated in the control. The functional annotation of DEGs identified specific enriched functions such as "transport," "signal transduction," and "stress response." Functional annotation clustering with enriched genes in the PE group revealed that translation-related genes, cell–cell adhesion genes, and immune-related genes were enriched. KEGG pathway analysis showed that several biological pathways, including the ribosome pathway and various immune pathways, were dysregulated. Several genes, including *RPS29, IGF-2*, and *UBC*, were significantly upregulated in PE, up to tenfold.

This study provides the first genome-wide expression analysis of amniotic fluid cell-free RNA in PE. The results showed that gene expression involving the ribosome pathway and immunologic pathways are dysregulated in PE. Our results will aid in understanding the underlying pathogenesis of PE.

Abbreviations: AF-cf-RNA = amniotic fluid cell-free RNA, AF-cf-transcriptome = amniotic fluid cell-free transcriptome, DAVID = The online Database for Annotation, Visualization, and Integrated Discovery, DEGs = differentially expressed genes, GO = Gene ontology, KEGG = The Kyoto Encyclopedia of Genes and Genomes, OMIM = The Online Mendelian Inheritance in Man, PE = preeclampsia, RPs = ribosomal proteins, rRNAs = ribosomal RNAs, TTTS = twin–twin transfusion syndrome.

Keywords: amniotic fluid, fetal development, microarray analysis, preeclampsia, transcriptome

1. Introduction

Preeclampsia (PE) is a disorder specific to pregnancy characterized by new-onset hypertension and proteinuria after 20 weeks of gestation. PE adversely affects multiple organs, including the liver, kidney, lung, and brain, and can lead to serious complications if left untreated. This systematic disorder affects 3% to 5% of all pregnancies and remains one of the main causes of maternal and perinatal morbidity and mortality.[1]

The treatment of PE is symptomatic, and the purpose of treatment is to maintain the pregnancy until fetal lung maturation

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completes.[2] The ultimate treatment for PE is preterm delivery of the placenta. Therefore, the placenta is crucial for the development of PE. Multiple pathogenic mechanisms have been implicated in PE. However, further understanding of the pathogenesis of PE is required to prevent and predict the development of PE.

Amniotic fluid cell-free RNA (AF-cf-RNA) has been reported to reflect fetal organ development and amniotic fluid (AF) composition.[3] Several studies have been performed to elucidate novel pathogenesis for fetal aneuploidy by using AF-cf-RNA.[4–6] Slonim et al[6] compared the differences in gene expression of AF supernatant samples and suggested the importance of oxidative stress and ion transport in the trisomy 21 fetuses. In trisomy 18, AF cell-free transcriptome (AF-cf-transcriptome) analysis revealed that genes involved in adrenal development are significantly downregulated, which may explain the pre- and postnatal growth restriction of trisomy 18.[4] In addition, gene expression involving neurological and cardiovascular pathways in AFs are significantly different between twin–twin transfusion syndrome (TTTS) recipients and singleton controls.[7] This result suggests that AF-cf-transcriptome reflects the long-term complications of TTTS survivors.

However, there has been no report analyzing the AF transcriptome in PE. Therefore, this study was performed to characterize the AF-cf-transcriptome during second trimester pregnancy destined to develop PE and to provide novel biological pathways involved in the pathogenesis of PE by performing global gene expression analysis using AF-cf-RNA.

2. Materials and methods

AF samples were obtained from 82 pregnant women who underwent amniocentesis for fetal chromosomal analysis after finding a soft marker for Down syndrome through ultrasonography screening in the Department of Obstetrics and Gynecology, CHA Gangnam Medical Center (Seoul, Korea) between March 2014 and May 2015. This study was reviewed and approved by the institutional review board of CHA Gangnam Medical Center in 2014 (GCI-14–11). All participants provided written informed consent to participate in the study before AF was obtained. Initially, 82 participants were enrolled and 68 subjects receiving early second trimester amniocentesis before 22 gestational weeks were included. Among these, 16 participants were diagnosed with PE later in the pregnancy. Diagnosis of PE was based on the general diagnostic criteria of PE, such as new-onset hypertension with proteinuria after 20 gestational weeks (systolic blood pressure of 140 mm Hg or higher, or diastolic blood pressure of 90 mm Hg or higher on 2 occasions at least 4 hours apart and urine dipstick test of 1+ or higher). We excluded 4 participants with twin pregnancy, 2 with gestational diabetes in later pregnancy, 1 subject who was diagnosed with Turner syndrome, and 1 who failed to follow-up. Karyotyping confirmed that all 8 fetuses were euploid. For comparative analysis, we used gene expression datasets from mid-trimester euploid AF samples that were produced in our previous studies.[8] We calculated appropriate sample size with the model that is provided by the department of bioinformatics and computational biology of MD Anderson Cancer Center https://bioinformatics.mdanderson.org/main/SampleSizes:Overview#Sample_Sizes). Using this model, we set the number of differentially expressed genes (DEGs): 2000; acceptable number of false positives:1; desired fold differences: 3; desired power 0.8; standard deviation 0.7. With per-gene value of alpha is 0.0050, the result of the computation was 8 in each group.

2.1. Cell-free RNA extraction

AF was centrifuged at $350 \times g$ for 10 minutes at room temperature to remove amniocytes. The supernatants were stored at −80°C. The cell-free RNA (cfRNA) was extracted from 5 to 10 mL of the AF supernatant. To extract cfRNA, the QIAamp Circulating Nucleic Acid (Qiagen, Germany) kit with an on-column DNase digestion step to remove genomic DNA was used according to the manufacturer's instructions. The RNA was purified and concentrated with the RNeasy MinElute Cleanup kit (Qiagen, Germany) and eluted with RNase-free water. The UV absorbance at 260 nm (A260 value) was measured, and samples with A260:A280 ratios greater than 1.8 were stored at −80°C for further analyses.

2.2. Microarray

Gene expression was analyzed with the GeneChip PrimeView array (Affymeterix, Santa Clara, CA). Biotinylated aRNA was prepared according to the standard Affymetrix protocol (Expression Analysis Technical Manual, 2001; Affymetrix) from 100 ng total RNA using the GeneChip 3'IVT Express Kit (Affymetrix). Following fragmentation, 12 μg of aRNA was hybridized for 16 hours at 45°C on a GeneChip Human array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed with Robust Multi-array Analysis (RMA) using the Affymetrix default analysis settings and global scaling as a normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized and log-transformed intensity values were then analyzed using GeneSpring 12.5 (Agilent Technologies, CA).

2.3. Data analysis

The data were imported into the GeneSpring GX 7.3 software (Agilent Technologies) for filtering and basic statistical analysis. Signals were considered "detected" when their value was larger than the median value of the control probe signal. We identified DEGs in PE, control, and both samples if they had a Benjamini–Hochberg adjusted P value $< .05$. Genes were considered significantly differentially expressed in both PE and control if they had a fold-change of at least 1.5 according to Welch t test. Unsupervised hierarchical clustering analyses and principal component analysis were performed to visualize the overall expression characteristics of all samples used in the study. Biological function and pathway analyses were performed using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) toolkit 6.8, an ontology-based webtool (http://david.abcc.ncifcrf.gov/). DAVID provides typical batch annotation and gene ontology (GO) term enrichment was ascertained by computing the EASE score (modified Fisher exact test) for each GO classification. We uploaded the gene lists defined as DEG for each group using the official gene symbols to identify enriched gene ontologies for gene expression, specific tissue expression, and functional pathway analysis. The biological functions of selected genes were analyzed using information from the GO database,[9] the Kyoto Encyclopedia of Genes and Genomes (KEGG),[10] and the Online Mendelian Inheritance in Man (OMIM) database.[11]

3. Results

In the study, we evaluated DEGs in the AF-cf-transcriptome between samples from PE and control. Table 1 presents the characteristics of the participants. The median age, median

Table 1

Subject characteristics.

ID	Age	Prepregnant BMI	Parity	G. age at amniocentesis	Indication of amniocentesis	G. age at PE diagnosis	G. Age at delivery	Mode of delivery	Indication for C/S	Fetal sex	Birth weight, g	Apgar score 1 min	Apgar score 5 min	Others
P1	45	20.7	0	22+4	AMA	34+5	35+0	C/S	Preeclampsia, breech	46, XY	2220	6	7	IVF, severe form
P2	27	20.5	0	26+5	IUGR	28+2	28+2	C/S	Preeclampsia	46,XY	590	6	7	Severe form
P3	34	19.8	2	17+0	Positive for Down	31+3	31+4	C/S	Previous C/S	46,XY	1380	5	6	Severe form
P4	29	18.4	0	16+2	Positive for Down	36+1	38+1	C/S	Preeclampsia	46,XY	2920	8	9	Severe form
P5	37	22.1	0	17+0	Positive for Down	37+4	37+4	C/S	Previous myomectomy	46,XY	2660	8	9	IVF, severe form
P6	37	19.8	2	16+4	Positive for Down	30+6	31+0	C/S	Preeclampsia	46,XY	1300	6	7	Severe form
P7	31	21.7	3	17+4	Positive for Edward	35+1	36+5	VD	—	46,XY	2200	8	9	Severe form
P8	32	21.8	1	17+3	Positive for Down	33+1	33+5	C/S	Previous C/S	46,XY	2140	7	9	Severe form
C1	38			17+3	AMA		39+4	VD		46,XY	3700	8	9	
C2	36			17+3	Single umbilical artery		37+6	C/S	Breech	46,XX	3290	8	9	
C3	40			18+6	AMA					46,XX				
C4	31			17+6	Positive for Down					46,XX				
C5	34			17+2	Bad OB history					46,XX				
C6	39			17+2	AMA		39+4	C/S	Breech	46,XX	3600	8	9	
C7	31			17+5	Positive for Down		40+4	VD		46,XX	3490	8	9	
C8	36			16+1	AMA		41+0	VD		46,XX	3710	8	9	
C9	35			17+5	Positive for Down		36+1	VD		46,XY	3010	8	9	

AMA = advanced maternal age, BMI = body mass index, C/S = cesarean section, G.age = gestational age, IUGR = intrauterine growth restriction, IVF = in vitro fertilization, OB = obstetric, VD = vaginal delivery.

gestational ages of delivery, and median birth weight of babies in the PE group were 33 years (27–45 years), 34 + 3 weeks (28 + 2–38 + 1 weeks), and 2170 g (590–2920 g), respectively. On the contrary, the median age, median gestational ages, and median birth weight of babies in the control group were 36 years (31–40 years), 39 + 4 weeks (36 + 1–41 + 0 weeks), and 3545 g (3010–3710 g), respectively.

There were 1841 genes that were significantly differentially regulated in PE compared with the control; 1557 genes were upregulated in PE and 284 were downregulated. Hierarchical clustering analyses were performed to visualize the overall expression characteristics of all samples used in the study (Fig. 1A). The changes between PE and control were distinct at a global level (Fig. 1B). The analyses revealed that the overall patterns of gene expression in PE were distinctly different from those in the control.

Functional classification of DEGs using DAVID showed that genes associated with stress response, signal transduction, and cellular transport are differentially expressed in PE (Table 2). It is important to identify dysregulated biological process to suggest potential underlying mechanisms of PE. KEGG pathway analysis with 1557 upregulated genes in PE provided insight into the aberrantly regulated signaling pathways, including the ribosomal pathway, viral myocarditis, rheumatoid arthritis, intestinal immune network for IgA production, graft-versus-host disease, Leishmaniasis, allograft rejection, asthma, and antigen processing and presentation (Table 3). Functional annotation clustering analysis with upregulated genes in PE using DAVID showed that several functional annotation groups, such as ribosome and protein translation, cell–cell adhesion, and immune response, were significantly enriched.

We analyzed general annotations of upregulated genes in PE and identified several chromosomes, including chromosomes 1, 12, 16, 17,19, 20, and 22. To provide potential candidates for predictive biomarkers of PE, we selected 7 upregulated genes and 3 downregulated genes based on fold-change value in PE (Table 4). Most of the upregulated genes were associated with the ribosome pathway.

4. Discussion

AF is a complex and dynamic solution that provides valuable information on fetal health status, such as fetal karyotyping, fetal lung maturity testing, and diagnosis of intra-amniotic infection. The presence of cell-free NAs (cf-NAs) in AF has been reported.[12] Although several studies have examined how the fetal status affects the expression of cf-NAs in the AF, as well as used AF-cf-NAs for discovering novel biomarkers for complications during pregnancy, including fetal aneuploidy, TTTS, and maternal obesity,[4–6,13,14] there has been no study on the pathogenesis of PE using AF-cf-NAs. In the present study, we analyzed the differences in global gene expression in the AF-cf-RNAs between PE and normal pregnancy to provide valuable information for the pathogenesis of PE. Our results showed that immune-related pathways and the ribosome pathway were enriched in the early second trimester AF-cf-transcriptome of PE.

The current proposed model for the pathophysiology of PE is a placental dysfunction, which results from defective deep placentation.[1] Several causative factors of defective placentation have been suggested, including maternal-fetal genotype incompatibility, preconception exposure to a paternal antigen that disrupts pregnancy-induced immunomodulation, and abnormal trophoblast decidual interaction. For successful pregnancy, embryo

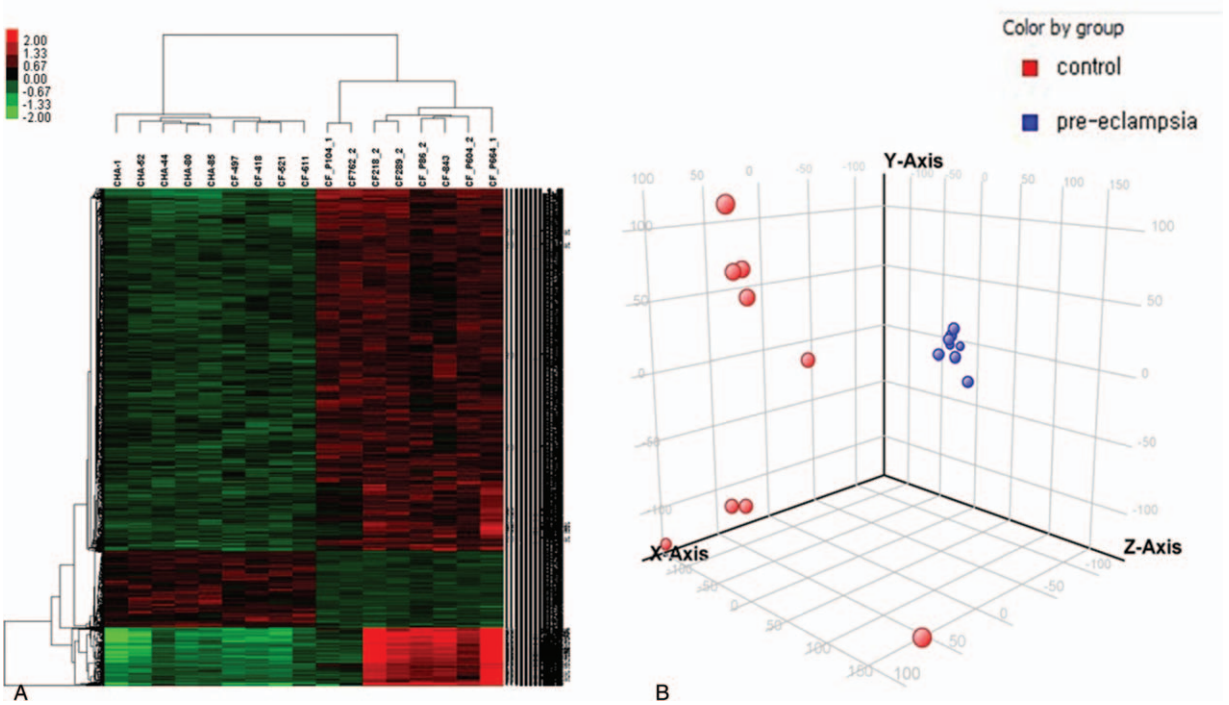

Figure 1. (A) Heatmap produced by hierarchical clustering of genes and samples, presenting differentially expressed genes in preeclampsia versus control. (B) Principal component analysis (PCA) of the top 3 identified components of transcriptome presenting the separation by disease. Control (red) and preeclampsia (blue).

implantation and placental development require immune escape mechanisms from maternal immune attack against semi-allogenic fetal tissue.[15] Enrichment in the several autoimmune-associated pathways, such as the graft-versus-host disease, rheumatoid arthritis, and allograft rejection pathways in the PE group support the importance of maternal-fetal immune recognition.

The seasonal variation of PE incidence has been observed. Magnus and Eskild [16]conducted a cross-sectional population-based study with 1,869,388 deliveries in Norway between 1967 and 1998. The authors reported mothers of children born in August had the lowest risk for PE, while risk was highest in the winter months. Phillips et al[17] also identified the seasonal variation in the incidence of PE, which was more strongly associated with the timing of conception than the timing of delivery. The highest incidence of PE was associated with conception during the summer. However, there was no known exact mechanism of pathogenesis to explain the seasonal variation of PE development. One of the potential causes of seasonality is the seasonal variation in infectious diseases.[18] Interestingly, KEGG pathway analysis revealed that functional pathways associated with the immune response to infections, including viral myocarditis, Leishmaniasis, *Staphylococcus aureus* infection, and intestinal immune network for IgA production pathways, were enriched in PE in addition to the autoimmune-related pathway. A significant enrichment in pathways involved in various infections may support the role of infection in the development of PE. This result may provide clues for the role of infection in the development of PE.

Table 2

Functional annotation of differentially regulated genes in pre-eclampsia.

Category	No.
Apoptotic process	148
Behavior	32
Cell activation	73
Cell adhesion	72
Cell cycle	111
Cell development	131
Cell differentiation	270
Cell growth	18
Cell proliferation	58
Cell migration	63
Homeostatic process	111
Immune response	114
Inflammatory response	46
Response to stress	343
Signal transduction	446
Translation	189
Transport	519
Total	1841

Our study revealed that the ribosome pathway was enriched in the AF cell-free transcriptome of PE. Ribosomes are complex molecular machineries that consist of ribosomal RNAs (rRNAs) and proteins and they are responsible for the synthesis of protein during mRNA translation in the cell cytoplasm. In addition, ribosomal proteins (RPs) have several extraribosomal functions in addition to constituting the ribosome complex. Extraribosomal functions of RPs include regulating cell cycle and apoptosis, defending against viral infection, and repairing DNA damage.[19] We propose several hypotheses as to why the ribosome pathways were enriched in the PE group. The release of NAs into the extracellular fluid is thought to be related to cellular apoptosis, necrosis, and active secretion of cells.[20] rRNAs, which are involved in the ribosome pathway, comprise 80% of total intracellular RNAs.[21] Therefore, increased placental apoptosis in PE increase the abundance of rRNA in cf-NA.[22] Another

Table 3

KEGG pathway analysis with enriched genes in preeclampsia.

Category term	No. of genes	*P*	Fold enrichment	Benjamini-Hochberg *P*
Ribosome	67	7.5E-39	6.5	2.1E-36
Huntington disease	27	2.4E-3	1.9	2.9E-1
Viral myocarditis	12	3.1E-3	2.8	2.5E-1
Lysosome	19	4.0E-3	2.1	2.5E-1
Oxidative phosphorylation	20	5.0E-3	2.0	2.5E-1
Rheumatoid arthritis	15	5.9E-3	2.3	2.4E-1
Alzheimer disease	23	7.6E-3	1.8	2.6E-1
Intestinal immune network for IgA production	10	7.6E-3	2.8	2.4E-1
Fructose and mannose metabolism	8	8.6E-3	3.3	2.4E-1
Graft-versus-host disease	8	1.0E-2	3.2	2.5E-1
Glycolysis/gluconeogenesis	12	1.1E-2	2.4	2.5E-1
Type I DM	9	1.2E-2	2.8	2.5E-1
Leishmaniasis	12	1.7E-2	2.2	3.0E-1
Allograft rejection	8	1.9E-2	2.9	3.2E-1
Parkinson's disease	19	2.0E-2	1.8	3.2E-1
Phagosome	20	2.1E-2	1.7	3.1E-1
Asthma	7	2.3E-2	3.1	3.1E-1
Antigen processing and presentation	12	2.7E-2	2.1	3.4E-1
Cell adhesion molecules	18	3.8E-2	1.7	4.4E-1
Other glycan degradation	5	4.2E-2	3.7	4.5E-1
Staphylococcus aureus infection	9	4.8E-2	2.2	4.8E-1
Pyrimidine metabolism	14	4.9E-2	1.8	4.7E-1

hypothesis is regarding its role in stress response. Various cellular stresses, such as oxidative stress, inflammatory cytokines, and autoimmunity, have been reported in PE. To adapt to those stresses, cells synthesize proteins, thus activating ribosome pathways in PE.

There are few studies elucidated the pathophysiology of PE by analyzing proteome and transcriptome changes in the placenta.[23,24] Our research group conducted genome-wide expression profiling for 10 samples of placenta from pregnant women with PE and found a common factor in the dysregulation of ribosome,

Table 4

Selected differentially regulated genes of interest as potential biomarkers of preeclampsia.

Gene symbol	Gene name	Fold change (log_2 ratio)	Gene function
RPS29	Ribosomal protein S29	3.554	This gene encodes ribosomal protein subunit. It binds to Zinc and enhances the tumor suppressor activity of Ras-related protein 1A (KREV1).
EEF1A1	Eukaryotic translation elongation factor 1 alpha	3.448	This encodes an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. This is expressed in brain, placenta, lung, liver, kidney, and pancreas.
IGF2	Insulin-like growth factor 2	3.229	This encodes a member of the insulin family of polypeptide growth factors, which are involved in development and growth.
UBC	Ubiquitin C	2.900	The encoded protein is a polyubiquitin precursor. Ubiquitination is associated with protein degradation, DNA repair, cell cycle regulation, endocytosis, and regulation of other cell signaling pathways.
FAU	Ubiquitin-like and ribosomal protein S30 fusion	2.428	It encodes a fusion protein consisting of the ubiquitin-like protein fubi at the N terminus and ribosomal protein S30 at the C terminus.
FTL	Ferritin, light polypeptide	2.318	This encodes the light subunit of the ferritin. A function of ferritin is the iron storage in a soluble state. Defects in this light chain ferritin gene are associated with several neurodegenerative diseases.
TMSB10	Thymosin beta 10	2.084	Actin monomer binding and protein binding
ARNT2	Aryl hydrocarbon receptor nuclear translocator 2	−1.738	The encoded protein acts as a partner for several sensor proteins of the bHLH-PAS family, forming heterodimers with the sensor proteins that bind regulatory DNA sequences in genes responsive to developmental and environmental stimuli. Under hypoxic conditions, the encoded protein complexes with hypoxia-inducible factor 1alpha in the nucleus and this complex binds to hypoxia-responsive elements in enhancers and promoters of oxygen-responsive genes.
HIST1H4A	Histone cluster 1, H4a	−1.406	Histones are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fiber in eukaryotes.
ZNF346	Zinc finger protein 346	−0.875	The protein encoded by this gene is a nucleolar, zinc finger protein that preferentially binds to double-stranded (ds) RNA or RNA/DNA hybrids, rather than DNA alone. Zinc finger domains are required for its nucleolar localization. The encoded protein is involved in cell growth and survival.

proteasome, and oxidative phosphorylation pathways.[24] van Uitert et al[23] performed meta-analysis with the placenta transcriptome data of 11 studies representing 116 PE pregnancies and 139 normotensive controls. The authors showed the involvement of the hypoxia/HIF1A pathway in gene expression profiling of PE and presented CREBBP/EP300, which was involved in the pathological processes in PE, as a novel regulator. However, those studies analyzed transcriptome expression in placenta obtained after delivery, and therefore, the results did not reflect the defect in early placental development. We collected AF during early second trimester before PE developed.

Our results demonstrated that RNA associated with the ribosome pathway, insulin-like growth factor B, and ubiquitin C were upregulated more than 10-fold in PE compared with the control. These genes may be predictive biomarkers for PE during early pregnancy. We isolated cf-RNA from AF obtained through amniocentesis, which is an invasive procedure. For clinical application, further validation studies using maternal blood should be performed.

There are several limitations in this study. First, we performed this study with transcriptome data obtained from 8 PE samples and 9 controls. This sample size is relatively small to draw comprehensive conclusions. Second, we did not conduct a functional study to support our hypothesis based on bioinformatic analysis. Third, there may have been annotation biases in our bioinformatic analysis, especially as inequality across gene annotation resources has been reported.[25] The researchers formed hypotheses based on the genes involved in the enriched processes and selected the genes that had previous experimental evidence, leading to annotation bias. Lastly, we did not use controls for subject characteristics in this study, which may have affected the gene expression patterns we were able to observe.

Despite these limitations, there are several strengths to this study. This study is the first to examine AF-cf-transcriptomes in PE. In addition, AF samples were obtained during early second trimester before the subjects developed PE. It is difficult to obtain fetal developmental information during early pregnancy due to the onset of hypertension and significant proteinuria after 20 weeks of pregnancy.

Our study demonstrated the comparable differences in early second trimester AF-cf-transcriptomes between the PE and control groups. In PE, the ribosome pathway and immune-associated pathways were enriched, which may reflect the importance of stress response and infection and immune-tolerance mechanisms in the pathogenesis of PE, respectively. Further studies are required to clarify and validate our results.

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