Comparative Proteomic Profile of the Human Umbilical Cord Blood Exosomes between Normal and Preeclampsia Pregnancies with High-Resolution Mass Spectrometry

Ruizhe Jia\textsuperscript{a} Jingyun Li\textsuperscript{b} Can Rui\textsuperscript{a} Hui Ji\textsuperscript{a} Hongjuan Ding\textsuperscript{a} Yuanqing Lu\textsuperscript{a} Wei De\textsuperscript{c} Lizhou Sun\textsuperscript{d}

\textsuperscript{a}Department of Obstetrics, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, \textsuperscript{b}State key Laboratory of Reproductive Medicine, Department of Plastic\&Cosmetic Surgery, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, \textsuperscript{c}Nanjing Medical University, Nanjing, \textsuperscript{d}Department of Obstetrics and Gynecology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China

\textbf{Key Words}
Exosomes • Proteomic profile • Umbilical cord blood • Preeclampsia • High-resolution mass spectrometry

\textbf{Abstract}

\textbf{Background/Aims:} Exosomes are extracellular vesicles that are involved in several biological processes. The roles of proteins from human umbilical cord blood exosomes in the pathogenesis of preeclampsia remains poorly understood. \textbf{Methods:} In this study, we used high-resolution LC-MS/MS technologies to construct a comparative proteomic profiling of human umbilical cord blood exosomes between normal and preeclamptic pregnancies. \textbf{Results:} A total of 221 proteins were detected in human umbilical cord blood exosomes, with 14 upregulated and 15 downregulated proteins were definitively identified between preeclamptic and control pregnancies. Further bioinformatics analysis (Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis) indicated that these differentially expressed proteins correlate with enzyme regulator activity, binding, extracellular region, cell part, biological regulation, cellular process and complement and coagulation cascades occurring during pathological changes of preeclampsia. \textbf{Conclusion:} Our results show significantly altered expression profiles of proteins in human umbilical cord blood exosomes between normal and preeclampsia pregnancies. These proteins may be involved in the etiology of preeclampsia.

R. Jia and J.Li contributed equally to this work.
Introduction

Preeclampsia is a hypertensive disorder of pregnancy, which affects 2-8% of all pregnancies and remains one of the leading causes of maternal and fetal morbidity and mortality worldwide [1]. Although the etiology of preeclampsia is largely unknown, recent studies suggest that placental-derived exosomes and their biological content (RNAs and protein) contributed to maternal-fetal communication, immune modulation and trophoblast physiology during pregnancy [2-4]. Syncytin proteins incorporated in placenta exosomes show variation from patients with preeclampsia and are important for cell uptake [5].

Exosomes are microvesicle with a size of 40-160 nm that are released from various cell types including tumor cells, red blood cells, platelets, lymphocytes, and dendritic cells [6]. They have been isolated from biological fluids, including blood plasma, urine and human breast milk [7-9]. Recent study has indicated that exosomes are composed of a lipid bilayer, and contain proteins, mRNA and miRNA [10]. Exosomes have been demonstrated in regulating immune modulation, and increased levels of maternal circulating exosomes is associated with progression of human pregnancy [4, 11].

Previous studies have demonstrated that decreased endothelial progenitor cells and ionized calcium levels were found in umbilical cord blood in preeclampsia [12, 13]. There were significant differences in nucleated red blood cell count and blood rheological properties in the umbilical cord blood between healthy women and women with preeclampsia [14, 15]. These observations could imply that it is possible to identify functional and/or structural differences in the umbilical cord blood with respect to the risk of developing preeclampsia. To date, little is known about umbilical cord blood exosomes during pregnancy. In this study, we compared the proteomic profiling of human umbilical cord blood exosomes between normal and preeclampsia pregnancies using high-resolution LC-MS/MS technologies. We aimed to find potential proteins that are involved in the etiology of preeclampsia.

Materials and Methods

Ethics statement

This study was performed with approval from the Medical Ethics Committee of Nanjing Maternal and Child Health Care Hospital (No. [2012]55). Written informed consent was obtained from all patients.

Sample preparation

All samples and clinical information were collected at the Nanjing Maternal and Child Health Care Hospital affiliated to Nanjing Medical University. Umbilical cord blood samples were collected from the umbilical vein immediately after delivery of fetus during cesarean section (10 cases for PE and 10 cases for control) according to the standard operating procedure. PE was diagnosed in patients with systolic blood pressure (BP) ≥ 150 mmHg or diastolic BP ≥ 90 mmHg and with proteinuria ≥ 0.3 g/d (in a 24 h harvest) for a period exceeding 4 h (Table 1). The detailed patient characteristics are presented in Table 1. All mothers had the same range of age and gestational age.

Exosome purification and analysis

Exosomes were prepared from the umbilical cord blood. Briefly, umbilical cord blood was centrifuged at 3,000 g for 15 min at 4 degree. Supernatants were then centrifuged at 12,000 g for 30 min at 4 degree. Then supernatants were filtered through 0.45 μm polyvinylidene fluoride (PVDF) membrane, and isolated in a final ultracentrifugation at 100,000 g for 180 min at 4 degree. The exosome pellet was resuspended in PBS or lysis buffer. The resulting exosomes were next analyzed with the Nanosight Nano ZS device (Malvern Instruments, Malvern, UK).

Protein digestion, peptide labeling and deprecation

Umbilical cord blood exosomes protein extracts (100 μg) from normal and PE subjects were digested with trypsin (1 μg/μL). Then the mixture was vacuum freeze-dried, and resuspended in tetraethylammonium
Jia et al.: Exosomes Proteomic Profile Between Normal and Preeclampsia Pregnancies

bromide (TEAB) containing 0.1% SDS (water: TEAB=1:1). MALDI TOF/TOF was used to check the digestive efficiency for 1μL of the lysate. 10 cases of PE or 10 cases of control were randomly divided into 3 groups respectively, indicating the peptide sample of each group was a mixture from 3 or 4 patients. Labeling reagent was then added to the peptides, and isotopic labels of different sizes were used for the different samples. The labeled samples were then dried in vacuo and separated by HPLC and C18 reversed phase chromatography and desalted. The peptides were dissolved by formic acid (0.1%).

Mass spectrometry data acquisition
The labeled peptides were analyzed using high-resolution LC-MS (Thermo-fisher Q-Exactive Orbitrap) the same as previously described [16]. Briefly, the MS/MS spectra acquired from precursor ions were submitted to Mascot (version 2.3.01) using the Swissprot Human Library for database search and methionine oxidation for variable modification. The peptide tolerance was set at 15 ppm, MS/MS tolerance was set at 20 ppm, and the maximum number of missed cleavages was 1. Meanwhile, qualitative analysis was performed using the median normalization method with the minimum peptides was 1, the p value was set at <0.05, and the fold change was 1.3.

Bioinformatics analysis
To further investigate the significance of the differentially expressed proteins, we used SBC Analysis system (Shanghai Biotechnology Corporation, Shanghai, China). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were applied. Interaction picture of those nine specific proteins was drawn according to Human Protein Reference Database (HPRD) and the Molecular INTeraction database (MINT) databases.

Statistical analysis
Data were analyzed using SPSS 20.0 software package (SPSS, Chicago, IL, USA) with independent-samples T test between two groups. All values were represented as mean±standard deviation (SD). Statistical significance was defined as P < 0.05.

Results

Nanoparticle tracking analysis
Nanoparticle tracking analysis (NTA) was used to visualize exosomes size and total concentration. By applying the Stokes Einstein equation (Fig. 1A), particle size in the PE group was 120±37 nm compared with the control group (112±40 nm). For the concentration, there was 32.56±5.68 E8 particles/ml in the PE group when compared to the control group (27.33±6.47 E8 particles/ml). A video was taken and the NTA software (Version 2.3, Nano Sight Ltd, Amesbury, UK) tracks the Brownian motion of individual vesicles. A sample video frame shows the static image of exosomes (Fig. 1B).

| Table 1. Characteristics of control and PE group. Data are presented as mean ± SD. ** P < 0.01 compared with control |
|--------------------------------------------------|
| Control (n=10) | Preeclampsia (n=10) |
|---|---|
| Age (years) | 26.9 ± 4.8 | 25.3 ± 5.3 |
| Gestational age (weeks) | 38.6 ± 5.5 | 34.3 ± 4.6 |
| Manner of delivery | Cesarean section |
| Systolic Blood pressure (mmHg) | 122.3 ± 7.2 | 173.2 ± 10.3** |
| Diastolic Blood pressure (mmHg) | 71.9 ± 7.4 | 99.5 ± 9.2** |
| Proteinuria (g/24h) | 0 | 4.2 ± 2.5 |
| Newborn birth weight (g) | 3074.9 ± 131.3 | 2705.8 ± 121.0** |
| Umbilical cord blood volume (ml) | 47.6 ± 13.5 | 41.9 ± 12.8 |
Identification of umbilical cord blood exosomes proteins related to pathological development of preeclampsia

To identify proteins that were differentially expressed in the umbilical cord blood exosomes of normal and PE patients, 221 identified proteins were analyzed on the Thermo-fisher Q-Exactive Orbitrap. Examination of the mass spectrometry data with Mascot (version 2.3.01) revealed that 29 proteins showed significant (fold change $\geq$1.3, $P < 0.05$) differential expression between the normal and PE patients (Table 2). Compared to the control, 14
proteins were upregulated and 15 proteins were downregulated in the preeclamptic pregnancies.

**Bioinformatics analysis of differentially expressed proteins using SBC Analysis system**

To examine the expression signatures of dysregulated proteins, we analyzed upregulated and downregulated proteins according to chromosome distribution. Differentially expressed proteins were located in different chromosomes with most proteins located in chromosome 4 (Fig. 2). GO analysis revealed that these 29 differentially expressed proteins were mainly involved in enzyme regulator activity and binding for the molecular functions (Fig. 3A). The most relevent cellular components for these differentially expressed proteins were extracellular region, cell part and cell (Fig. 3B) that was involved during the pathological changes of PE. For further identification of important biological processes, the results showed that these differentially expressed proteins were significantly involved mostly in biological regulation and cellular process (Fig. 3C). Indeed we found these biological processes are all present in PE development. Furthermore, KEGG pathway analysis indicated that complement and coagulation cascades are mostly associated with PE (Figure 3D). Further analysis identified 9 differentially expressed proteins were related with complement and coagulation cascades (C4BPA, C4BPB, F13B, FGA, FGB, FGG, MBL2, PROS1, VWF; detailed information of these proteins were listed at Table 2). We subsequently analyzed the interaction networks...
of these nine proteins according to Human Protein Reference Database (HPRD) and the Molecular INTeraction database (MINT) databases. The results indicated that VWF (von Willebrand factor), PROS1 (vitamin K-dependent protein S) and FGA (Fibrinogen alpha chain) were at the core of interaction networks (Fig. 4).

Discussion

Preeclampsia (PE) is a specific disorder characterized by the new onset of proteinuria, edema, hypertension and a series of other systematic disorders during pregnancy. A growing body of evidence suggests that placental proteome alterations coordinate the pathological development of PE [17-19]. However, the etiology of PE remains to be elucidated. In this paper, we show that 29 differentially expressed proteins were identified in human umbilical cord blood exosomes between normal and preeclampsia pregnancies with high-resolution mass spectrometry. Importantly, KEGG pathway analysis showed that complement and coagulation cascades are mostly associated with PE, suggesting a possibility that human umbilical cord blood exosomes proteins may be involved in the etiology of preeclampsia via the complement and coagulation cascades. Based on the above and our previous work [20, 21], we obtained a direction for future study on differentially expressed exosomal proteins in umbilical cord blood from PE.

Research on exosomes, most notably in the field of PE, has been increasing over recent years and has demonstrated that these vesicles are involved in cell uptake and placental functions [5, 22]. Recent findings suggest that exosome-associated proteins mediate different exosomal functions, such as miRNA-dependent modulation of gene expression, induced cell signaling and intercellular communication [23-25]. Our study indicated that exosomal proteins from the umbilical cord blood may play crucial roles in the pathogenesis of PE. Furthermore, GO analysis revealed similar information that these differentially expressed exosomal proteins were mainly involved in enzyme regulator activity, binding, extracellular region, cell part, biological regulation and cellular process (Fig. 3).
Many proteins in umbilical cord blood have been reported to be associated with PE. Higher soluble Fas ligand levels were identified in umbilical cord blood of PE patients [26]. Large amounts of MMP-9 were found in umbilical cord plasma of preeclamptic subjects [27]. Umbilical cord blood levels of soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) were higher in preeclamptic pregnant [28]. Methemoglobin levels were higher in umbilical cord blood of women PE [29]. Our study characterized 29 differentially expressed proteins in umbilical cord blood exosomes between PE and control samples. Among them, three proteins (VWF, PROS1 and FGA) involved in complement and coagulation cascades were found at the core of interaction network according to KEGG pathway and interaction network analysis (Figure 3 and Figure 4). Previous study reported that elevation in VWF and reduction in its proteolytic enzyme ADAMTS13 activity might have a role in the pathogenesis of PE [30]. FGA has been identified to be serological markers capable of diagnosing PE [31]. In addition, the anticoagulant PROS1 interacting with the complement regulator C4b-binding protein (C4BP) is a direct physical link between blood coagulation and complement pathways [32]. Our study found that PROS1 was upregulated in PE umbilical cord blood exosome, whereas VWF and FGA were downregulated in PE umbilical cord blood exosome compared with control subjects (Table 2). Those proteins might represent other new mechanisms for PE development during pregnancy. The relevance of those proteins in umbilical cord blood exosomes to PE needs to be further investigated.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (81200442), Scientific Research Foundation from Department of Health of Jiangsu Province of China (Z201309), Maternal and Child Health Research Foundation of Jiangsu Province of China (F201436), and Nanjing Medical Science and Technique Development Foundation (QRX11209).

Disclosure Statement

All authors have no conflicts of interest to declare.

References

1 English F-A, Kenny LC, McCarthy FP: Risk factors and effective management of preeclampsia. Integr Blood Press Control 2015;8:7-12.
2 Ouyang Y, Mouillet JF, Coyne CB, Sadovsky Y: Review: Placenta-specific microRNAs in exosomes - good things come in nano-packages. Placenta 2014;35:S69-73.
3 Redman C-W, Sargent IL: Microparticles and immunomodulation in pregnancy and pre-eclampsia. J Reprod Immunol 2007;76:61-67.
4 Taylor D-D, Akyol S, Gercel-Taylor C: Pregnancy-associated exosomes and their modulation of cell signaling. J Immunol 2006;176:1534-1542.
5 Vargas A, Zhou S, Ethier-Chisson M, Filip D, Lafond J, Gilbert C, Barbeau B: Syncytiotrophoblastic proteins incorporated in placenta exosomes are important for cell uptake and show variation in abundance in serum exosomes from patients with preeclampsia. FASEB J 2014;28:3703-3719.
6 Cocucci E, Racchetti G, Meldolesi J: Shedding microvesicles: Artefacts no more. Trends Cell Biol 2009;19:43-51.
7 Torregrossa P-P, Gutzeit C, Johansson S, Admyre C, Stenius E, Alm J, Scheynius A, Gabrielsson S: Differences in exosome populations in human breast milk in relation to allergic sensitization and lifestyle. Allergy 2014;69:463-471.
8 Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C: Exosomal-like vesicles are present in human blood plasma. Int Immunol 2005;17:879-887.
9 Pitkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A 2004;101:13368-13373.

10 Frydrychowicz M, Kolecka-Bednarczyk A, Madejczyk M, Yasar S, Dworacki G: Exosomes - structure, biogenesis and biological role in non-small-cell lung cancer. Scand J Immunol 2015;81:2-10.

11 Southcombe J, Tannetta D, Redman C, Sargent I: The immunomodulatory role of syncytiotrophoblast microvesicles. PLoS One 2011;6:e20245.

12 Kwon J-Y, Maeng YS, Kwon YG, Kim YH, Kang MH, Park YW: Decreased endothelial progenitor cells in umbilical cord blood in severe preeclampsia. Gynecol Obstet Invest 2007;64:103-108.

13 Yusuf K, Kamaludddeen M, Hasan SU, Al-Awad E, Finch RA, Akierman AR: Ionized calcium levels in umbilical cord blood of women with preeclampsia and normotensive pregnancies. J Matern Fetal Neonatal Med 2012;25:203-205.

14 Ćorba R, Yilmaz A, Tsikouras P,Wieg C,Teichmann A, von Tempelhoff GF: Rheological parameters in the umbilical cord blood in moderate and severe forms of preeclampsia. Clin Hemorheol Microcirc 2013;55:391-401.

15 Faraji D-R, Ghanbari A, Asgharnia M, Kian M: Comparison of nucleated red blood cells in the umbilical cord of term neonates in healthy women and women with preeclampsia. Iran J Reprod Med 2013;11:25-30.

16 Miao Z-J, Chen M, Wu H, Ding HJ, Shi ZH: Comparative proteomic profile of the human placenta in normal and fetal growth restriction subjects. Cell Physiol Biochem 2014;34:1701-1710.

17 Wang F-Q, Wang L, Shi ZH, Liang GL: Comparative n-glycoproteomic and phosphoproteomic profiling of human placental plasma membrane between normal and preeclampsia pregnancies with high-resolution mass spectrometry. PLoS One 2013;8:e80480.

18 Wang F-Q, Shi ZH, Wang P, You W, Liang GL: Comparative proteome profile of human placenta from normal and preeclamptic pregnancies. PLoS One 2013;8:e78025.

19 Shi Z-H, Long W, Zhao C, Guo XR, Shen R, Ding HJ: Comparative proteomics analysis suggests that placental mitochondria are involved in the development of pre-eclampsia. PLoS One 2013;8:e64351.

20 Jia R-Z, Ding GC, Gu CM, Huang T, Rui C, Wang YX, Lu Q: Cdx2 enhances htr-8/svneo trophoblast cell invasion by altering the expression of matrix metalloproteinases. Cell Physiol Biochem 2014;34:628-636.

21 Liu L, Zhang X, Rong C, Rui C, Ji H, Qian YJ, Jia RZ, Sun LZ: Distinct DNA methylomes of human placentas in normal and preeclampsia pregnancies. PLoS One 2013;8:e78025.

22 Lokesou A-G, Touduc C, Barbeau B: Implication of human endogenous retrovirus envelope proteins in placental functions. Viruses 2014;6:4609-4627.

23 Fox M-J, Gao H, Smith-Künzmann WR, Liu Y, Mosley AL: The exosome component rrp6 is required for rna polymerase ii termination at specific targets of the nrd1-nab3 pathway. PLoS Genet 2015;10:e1004999.

24 Yamaguchi T, Izumi Y, Nakamura Y, Yamazaki T, Shiota M, Sano S, Tanaka M, Osada-Oka M, Shimada K, Miura K, Yoshiyama M, Iwao H: Repeated remote ischemic conditioning attenuates left ventricular remodeling via exosome-mediated intercellular communication on chronic heart failure after myocardial infarction. Int J Cardiol 2015;178:239-246.

25 Zhang J, Li S, Li L, Li M, Guo CY, Yao J, Mi SL: Exosome and exosomal microRNA: Trafficking, sorting, and function. Genomics Proteomics Bioinformatics 2015;13:17-24.

26 Kuntz T-B, Christensen RD, Stegner J, Duff P, Koenig JM: Fas and fas ligand expression in maternal blood and in umbilical cord blood in preeclampsia. Pediatr Res 2001;50:743-749.

27 Galewska Z, Romanowicz L, Jaworski S, Bankowski E: Gelatinase matrix metalloproteinase (mmp)-2 and mmp-9 of the umbilical cord blood in preeclampsia. Pediatr Res 2001;50:743-749.

28 Tuten A, Erman H, Korkmaz GG, Oncul M, Gelisgen R, Sozer V, Acikgoz S, Simsek G, Uzun H: Comparison of maternal and umbilical cord blood soluble lectin-like oxidized low-density lipoprotein receptor 1 levels in early- and late-onset preeclampsia. Arch Gynecol Obstet 2014;290:1007-1013.

29 Yusuf K, Wilson RD, Kamaludddeen M, Franta J, Hasan SU, Akierman A: Methemoglobin levels in umbilical cord blood of women with intrauterine growth restriction and preeclampsia. J Matern Fetal Neonatal Med 2014;27:789-794.

30 Aref S, Goda H: Increased vwf antigen levels and decreased adams13 activity in preeclampsia. Hematology 2013;18:237-241.

31 Wen Q, Liu LY, Yang T, Alev C, Wu S, Stevenson DK, Sheng G, Butte AJ, Ling XB: Peptidomic identification of serum peptides diagnosing preeclampsia. PLoS One 2013;8:e65571.

32 Dahlback B: C4b-binding protein: A forgotten factor in thrombosis and hemostasis. Semin Thromb Hemost 2011;37:355-361.