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Original Paper

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

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Key Words
Exosomes • Proteomic profile • Umbilical cord blood • Preeclampsia • High-resolution mass spectrometry

Abstract
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. ***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. ***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. ***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.

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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 preeclamptic 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 depuration

Umbilical cord blood exosomes protein extracts (100 μg) from normal and PE subjects were digested with trypsin (1μg/μL). Then the mixture was vacuum freeze-dried, and resuspended in tetraethylammonium

Table 1. Characteristics of control and PE group. Data are presented as mean ± SD. ** $P < 0.01$ compared with control

	Controls (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	Caesarean 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

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).

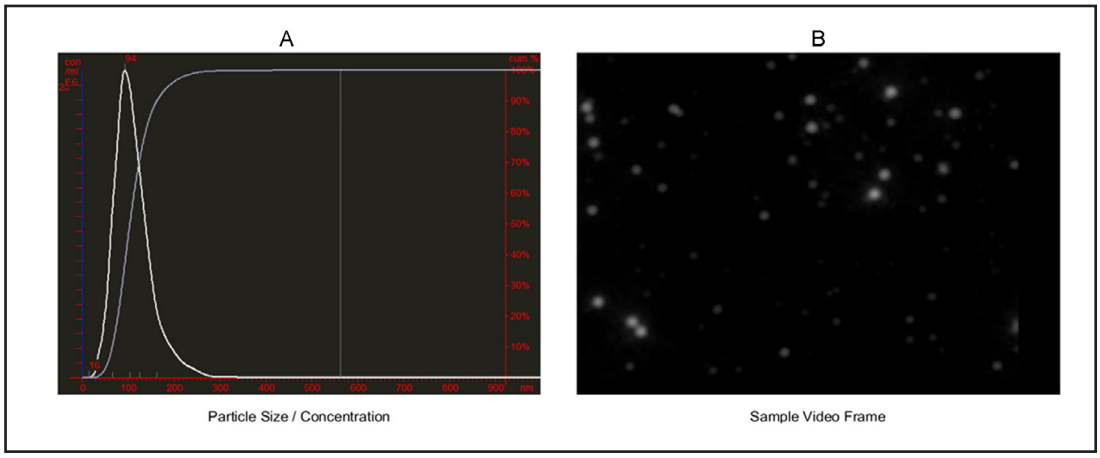

Fig. 1. Representative nanoparticle tracking analysis report. (A) Particle size and concentration analysis using Stokes Einstein equation. (B) A sample video frame shows the static image of exosomes. A white dot points to an exosome.

Table 2. The list of differentially expressed proteins in human umbilical cord blood exosomes from control and preeclampsia pregnancies

Protein ID	Protein names	*P* value	Fold Chang
P20851	C4b-binding protein beta chain(C4BPB)	3.43E-03	2.37
P04003	C4b-binding protein alpha chain(C4BPA)	2.02E-03	2.27
P07225	Vitamin K-dependent protein S(PROS1)	2.36E-03	2.18
P00450	Ceruloplasmin(CP)	6.77E-04	1.90
P01861	Ig gamma-4 chain C region(IGHG4)	1.77E-06	1.86
P04196	Histidine-rich glycoprotein(HRG)	1.15E-03	1.46
B9A064	Immunoglobulin lambda-like polypeptide 5(IGLL5)	2.28E-05	1.40
H3BM74	NEDD8 ultimate buster 1(NUB1)	4.17E-02	1.39
P01779	Ig heavy chain V-III region TUR	3.63E-05	1.38
P02776	Platelet factor 4(PF4)	2.76E-03	1.37
P01771	Ig heavy chain V-III region HIL	2.10E-03	1.36
O75382	Tripartite motif-containing protein 3(TRIM3)	8.66E-04	1.35
P01604	Ig kappa chain V-I region Kue	3.24E-04	1.35
P06317	Ig lambda chain V-VI region SUT	1.62E-04	1.31
P02790	Hemopexin(HPX)	8.65E-03	-1.33
P23083	Ig heavy chain V-I region V35	8.70E-03	-1.37
P04275	von Willebrand factor(VWF)	1.01E-02	-1.39
P05160	Coagulation factor XIII B chain(F13B)	4.14E-04	-1.43
P02766	Transthyretin(TTR)	3.86E-03	-1.52
P02771	Alpha-fetoprotein(AFP)	2.36E-05	-1.57
F5H4W9	Serum paraoxonase/arylesterase 1(PON1)	4.27E-02	-1.77
P02652	Apolipoprotein A-II(APOA2)	1.87E-02	-1.88
J3KNB4	Cathelicidin antimicrobial peptide(CAMP)	1.89E-02	-2.02
P43652	Afamin(AFM)	3.89E-02	-2.05
P02647	Apolipoprotein A-I(APOA1)	4.35E-02	-2.78
P11226	Mannose-binding protein C(MBL2)	7.34E-04	-2.96
P02671	Fibrinogen alpha chain(FGA)	4.18E-02	-3.41
C9JC84	Fibrinogen gamma chain(FGG)	4.27E-02	-3.93
P02675	Fibrinogen beta chain(FGB)	3.78E-02	-4.18

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 Thermofisher Q-Exactive Orbitrap. Examination of the mass spectrometry data with Mascot (version 2.3.01) revealed that 29 proteins showed significant (fold change≥1.3, $P < 0.05$) differential expression between the normal and PE patients (Table 2). Compared to the control, 14

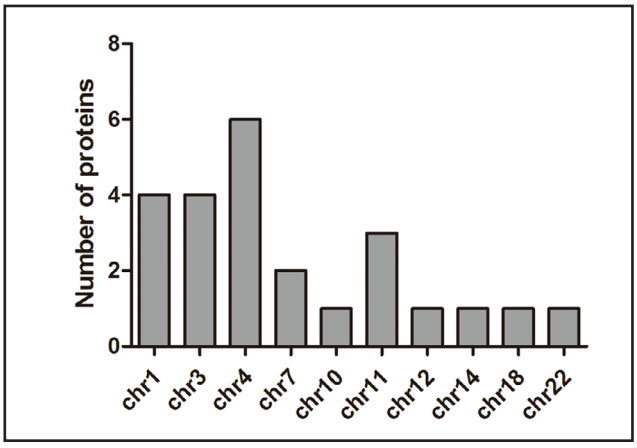

Fig. 2. Chromosome distributions of differentially expressed proteins. Chromosome distributions show the numbers of up and down regulated proteins in different chromosomes.

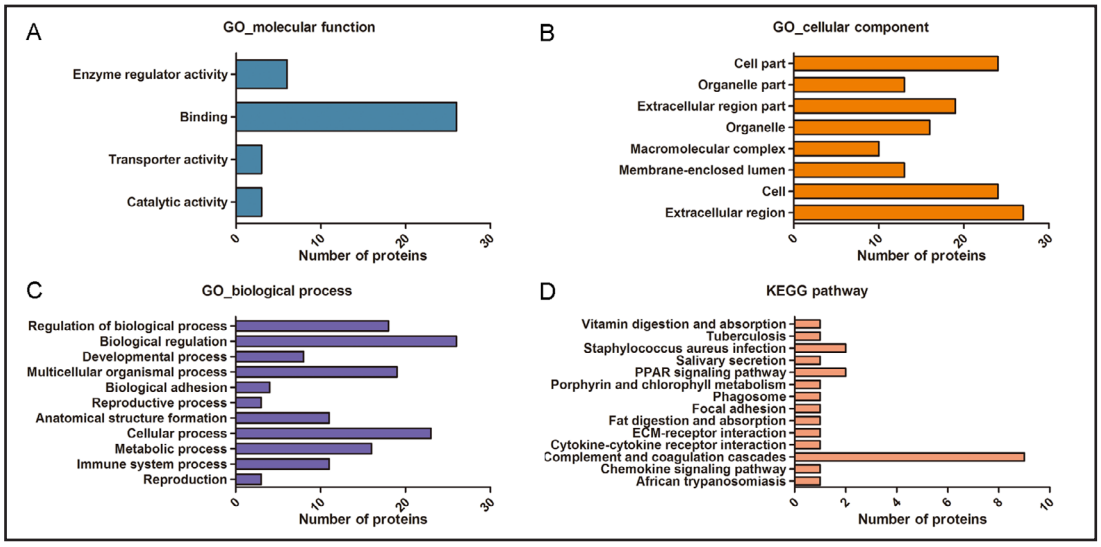

Fig. 3. GO and KEGG pathway analysis. The relative GO terms such as molecular functions (A), cellular component (B) and biological process (C) that associated with the differentially expressed proteins are listed. (D) The relative KEGG pathway that associated with the differentially expressed proteins are listed.

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

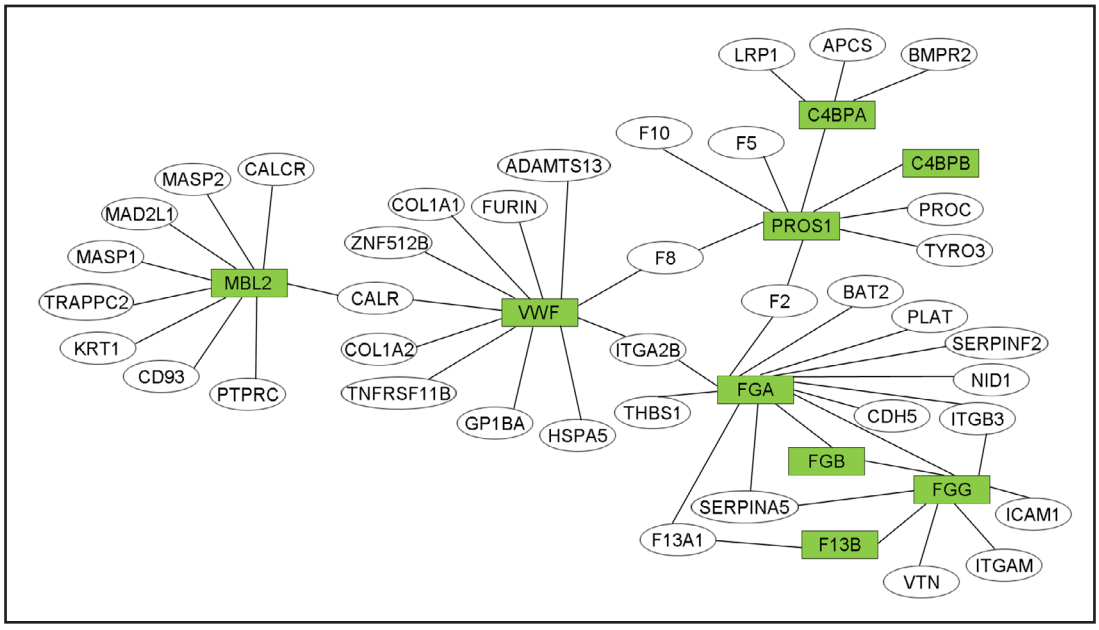

Fig. 4. Interaction networks of these nine proteins related with complement and coagulation cascades. The interaction network was drawn according to Human Protein Reference Database (HPRD) and the Molecular INTeraction database (MINT) databases. Green box indicated these nine proteins. Other proteins are denoted with ellipse.

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

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Disclosure Statement

All authors have no conflicts of interest to declare.

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