Original Article
Proteomic identification of candidate plasma biomarkers for preeclampsia in women with pregnancy-induced hypertension

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Received June 23, 2017; Accepted August 23, 2017; Epub October 1, 2017; Published October 15, 2017

Abstract: Preeclampsia (PE), a complication of pregnancy, is the leading cause of maternal and perinatal morbidity and mortality. This study aimed to characterize biomarkers for the early diagnosis of PE. We performed a comparative proteomic analysis on the plasma obtained from PE and healthy pregnant women. We analyzed the plasma samples using two-dimensional differential gel electrophoresis (2D-DIGE) coupled with Ultraflex III, a MALDI-TOF-TOF (matrix-assisted laser desorption/ionization-time-of-flight) mass spectrometer and identified differentially expressed proteins (DEPs). We analyzed the abundance levels of these DEPs by enzyme-linked immunosorbent assay (ELISA) to further confirm their role as putative PE biomarkers. We identified a total of 56 DEPs, of which 48 were down-regulated and 8 were up-regulated in women with PE. The identities of 8 of these DEPs were characterized by mass spectrum analysis, including LG3BP (lectin, galactoside-binding, soluble, 3 binding protein), APOA1 (apolipoprotein A-I), FETUA (alpha-2-HS-glycoprotein), CFAI (complement factor I), CD5L (CD5 antigen-like), K2C6A (keratin, type II cytoskeletal 6A), PON1 (paraoxonase/arylesterase 1) and HP1 (haptoglobin). Finally, the differential expression of these 8 proteins was verified by ELISA. In summary, we applied the 2D-DIGE and Ultraflex III-TOF/TOF platform to identify potential plasma biomarkers of PE. Of these, plasma LG3BP, APOA1, FETUA, CFAI, CD5L, K2C6A, PON1 and HP1 were promising candidates for predicting PE.

Keywords: Preeclampsia, pregnancy, plasma, biomarker, proteomic identification

Introduction

Preeclampsia (PE) is a complication of pregnancy affecting about 2%-8% of all pregnancies [1]. According to the guidelines for the diagnosis and treatment of hypertensive disorder complicating pregnancy, PE is characterized by the onset of hypertension and proteinuria after the 20th week of gestation [2]. It represents one of the leading causes of maternal and perinatal morbidity and mortality worldwide and seriously impacts maternal and child health [3, 4]. The incidence of PE has been increasing due to an increase in maternal age, obesity and women entering pregnancy with medical comorbidities. Several studies have shown that the etiology and pathogenesis of PE is closely associated with abnormal placentation and vasculogenesis [5], placental and vascular endothelial dysfunction [6], immune factors and the systemic inflammatory response [7, 8], endoplasmic reticulum stress [9] and inadequate trophoblast invasion [10]. However, the accurate etiology of preeclampsia remains largely unknown [11-13].

At present, diagnostic criteria of PE includes abnormally increased blood pressure and proteinuria. Pregnancy-induced hypertension (PIH) is a condition characterized by a blood pressure of ≥ 140 mmHg (systolic) or 90 mmHg (diastolic) during at least two blood pressure measurements, ideally separated by a period of rest [14]. A mercury sphygmomanometer is considered the gold standard for measurement [15], however, this instrument is being gradually withdrawn from clinical use for health and safety reasons. Proteinuria is a condition characterized by a total protein of 300 mg/day in a 24-hour urine collection. In practical terms, the
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New methods for early detection, prevention and management of PE have the potential to revolutionize clinical practice in the coming years. The purpose of this study was to identify plasma proteins associated with PE in pregnant women, using a proteomic approach. We successfully characterized 8 protein biomarkers that were significantly differentially expressed in plasma from women with PE compared with plasma from women with healthy pregnancies. These biomarkers included LG3BP (lectin, galactoside-binding, soluble, 3 binding protein), APOA1 (apolipoprotein A-I), FETUA (alpha-2-HS-glycoprotein), CFAI (complement factor I), CD5L (CD5 antigen-like), K2C6A (keratin, type II cytoskeletal 6A), PON1 (paraoxonase/arylesterase 1) and HP1 (Haptoglobin).

LG3BP is implicated in immune responses associated with natural killer (NK) and lymphokine-activated killer (LAK) cell cytotoxicity. In addition, LG3BP plays an anti-tumorigenic role in colorectal cancer (CRC) by suppressing Wnt signaling [16]. APOA1 has anti-inflammatory and anti-fibrotic effects in the bleomycin and silica-induced lung fibrosis models [17, 18]. These findings suggest that APOA1 could affect immune factors and the systemic inflammatory response. FETUA, a glycoprotein expressed in the serum, is involved in several processes such as endocytosis, brain development and the formation of bone tissues. In addition, plasma FETUA is a promising candidate in hypopharyngeal squamous cell carcinoma (HSCC) screening [19]. CFAI is essential in regulating the complement cascade, and is associated with a predisposition to atypical hemolytic uremic syndrome, a disease characterized by acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia [20]. CD5L plays critical roles as a pattern recognition receptor (PRR) of bacterial and fungal components and in the control of key mechanisms in inflammatory responses, such as infection, atherosclerosis and cancer [21]. HP1 functions to bind free plasma hemoglobin. This allows degradative enzymes to gain access to the hemoglobin, and at the same time prevent the loss of iron through the kidneys and protect the kidneys from damage by hemoglobin [22, 23].

Materials and methods

Study population

A total of 82 pregnant women were enrolled in this study, which were divided into two groups, the first included 38 women with pregnancy-induced hypertension (PIH); the second group, which served as the control group, consisted of 44 healthy pregnant women. All samples and clinical information were collected at The Third Affiliated Hospital, Sun Yat-sen University. Plasma samples were collected form the elbow vein of pregnant woman according to the standard operating procedure. 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) were diagnosed with PE [14]. The detailed patient characteristics are presented in Table 1. All pregnant women were within the same age and gestational age range. Written informed consents were collected from all participating subjects.

Sample preparation

The ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma-Aldrich, USA) was used to remove albumin and IgG from plasma samples. The procedure was strictly performed in accor-

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients in the control and PE groups</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Gestational age (weeks)</td>
</tr>
<tr>
<td>Systolic Blood pressure (mmHg)</td>
</tr>
<tr>
<td>Diastolic Blood pressure (mmHg)</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
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<tr>
<td>Newborn birth weight (g)</td>
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</tbody>
</table>

Data are presented as mean ± SD. **P < 0.01 compared with control.
dance with the manufacturer’s instructions. Briefly, the beads were washed with 400 μL equilibrium buffer, and then centrifuged (10,000 rpm, 10 min, 4°C), added to 80 μL plasma sample, incubated for 10 min and centrifuged again (10,000 rpm, 10 min, 4°C). The eluate was added to beads for another process of adsorption, and then collected and added with the appropriate volume of acetone and incubated at -20°C overnight. The precipitate was centrifuged (12,000 rpm, 20 min, 4°C), dried and stored at -80°C till further 2D Electrophoresis analysis. All chemicals and biochemicals used in this study were of analytical grade.

2D electrophoresis

2-DE was performed according to the instrument manufacturer’s instructions (ETTAN IPGPHOR 3 Manual, GE Healthcare). Immobilized pH gradient (IPG) strips, buffer and cover oil were purchased from GE Healthcare. DTT was purchased from Amresco. The ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma) was used to reduce high-abundant protein interference in plasma samples. About 1,200 μg of protein samples were diluted to 460 μl with lysis buffer (with 1% DTT, 1% IPG Buffer, 0.25% Bromophenol Blue), and loaded onto a 24 cm IPG strip with linear pH gradient 4-7 (GE Healthcare, Uppsala, Sweden). The strips were hydrated at room temperature for 12 h with cover oil. Isoelectric focusing (IEF) was performed on an Ettan IPGphor3 isoelectric focusing system as follows: 0.5 h at 300 V, 0.5 h at 700 V, 1.5 h at 1500 V, a gradient to 9000 V for 3 h, and 9000 V up to 52,000 Vh for strips. After IEF, the IPG strips were equilibrated in equilibration solution and kept at -20°C. For the following vertical electrophoresis, the strips were transferred to an Ettan Dalt system (GE Healthcare). The second dimension was carried out at 2 W/gel for 45 min and then 17 W/gel for 4.5 h at 16°C, and was terminated when the bromophenol dye front migrated to the lower end of the gels [24]. Gels were visualized by silver staining method.

Image analysis

After staining, gels were scanned with UMAX Powerlook 1100 and image analysis was performed with the Image Master 2D platinum 5.0 (GE Healthcare, Uppsala, Sweden). To quantify the differential proteins in the plasma of women with pregnancy-induced hypertension (PIH) and healthy pregnant women, the Student’s t-test was performed. In the statistical analysis only the spots presented in all three replicate gels that matched with its counterpart were considered. Spots with Student’s t P < 0.05 and at least 1.5-fold relative change in their quantities were further analyzed.

Ultraflex III-TOF/TOF MS/MS and database search

The protein spots of interest were manually excised from 2-DE gels and digested in-gel with trypsin (Promega, USA). Protein spots were first washed three times with MilliQ water for 30 min and destained three times with the destaining solution containing 25 mM NH₄HCO₃ and 50% methanol for 30 min each at 37°C. These were subsequently dehydrated with 100 μL of 100% ACN, vortexed for 2 min and ACN removed. Next, these spots were incubated in 50 ul of 100 mM NH₄HCO₃, then treated with 100 ul 50% CAN and air dried at room temperature for 1 h. Selected spots were digested with trypsin at a concentration of 20 ng/ul for 12 h at 37°C. The digested protein peptides were mixed with HCCA matrix (0.1% TFA, 50% ACN) for peptide map fingerprinting (PMF), then analyzed by an Ultraflex III-TOF/TOF mass spectrometer (Bruker Dalton, Germany) equipped with a neodymium with laser wavelength 355 nm. For protein identification, the acquired MS/MS data were uploaded on the flexAnalysis software (Bruker Dalton, Germany). Then, a BLAST search using NCBI (http://www.ncbi.nlm) was performed on the unnamed proteins to find the corresponding homologous proteins.

Enzyme-linked immunosorbent assay

The levels of differentially expressed proteins in plasma from women with pregnancy-induced hypertension and the control group were measured using enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO and CusAb Company, Wuhan, China), according to the manufacturer’s instructions. In general, samples were thawed at room temperature and then centrifuged at 3,000 rpm for 10 min. Samples were diluted to appropriate concentrations, then the ELISA assay was performed. The supplied standards were diluted and measured to establish standard curves. When the samples and stan-
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Standards were added to the wells, unbound protein was removed by washing, and the conjugate was added. After a color reaction with a substrate, the optical density was recorded using an automated ELISA reader (Thermo Fisher Scientific, USA) at a wavelength of 450 nm. Finally, the absorbance at 450 nm was converted to a concentration unit referring to the standard reference curves. Each sample was analyzed in triplicate. In this assay, each group (PE and control) had 35 individuals as described earlier.

Statistical analyses

The significance of protein expression differences between the PE and control groups were analyzed by independent samples. Student-t test was conducted using the SPSS 20.0 software package (SPSS, Chicago, IL, USA). All values were represented as mean ± standard deviation (SD). Statistical significance was defined by P < 0.05.

Results

Comparison of plasma protein profiles between PE and control groups

First, two-dimensional electrophoresis and image analysis were performed to compare the plasma protein profiles between the PE and control groups. For each group of protein extracts from the plasma of PE and control pregnant women, 2-DE gels were prepared in triplicate. More than one thousand protein spots were detected in each 2-DE image after silver staining with good reproducibility, and only the DEPs showing changes > 1.5-fold were analyzed in detail. These results revealed 56 DEPs (8 with higher and 48 with lower abundance) in plasma samples of the PE group, compared with that from the healthy group (Figure 1).

Protein identification via ultraflex III TOF/TOF MS

To further investigate these DEPs, 23 statistically significant candidates were selected for Ultraflex III TOF/TOF MS analysis after excision from 2-DE gels. The acquired MS/MS data were uploaded onto the flexAnalysis software (Bruker Dalton, Germany). Then, a BLAST search using NCBI (http://www.ncbi.nlm) was performed for the unnamed proteins to find the corresponding homologous proteins. Protein identification was based on homology to Homo sapiens proteins. We verified the identity of 8 proteins including LG3BP (lectin, galactoside-binding, soluble, 3 binding protein), APOA1 (apolipoprotein A-I), FETUA (alpha-2-HS-glycoprotein), CFAI (complement factor I), CD5L (CD5 antigen-like), K2C6A (keratin, type II cytoskeletal 6A), PON1 (paraoxonase/arylesterase 1) and HP1 (haptoglobin) (Table 2). To evaluate the quality of the identified proteins, the theoretical and experimental ratios of the molecular mass (Mr) and isoelectric point (pl) were determined and presented in a radial chart as the radial and annular radius labels, respectively (Table 2). These results showed that approximately 95% of the identified proteins exhibited a relative Mr ratio in the range of 1.0 ± 0.05 and 93% of the identified proteins exhibited a relative pl ratio in the range of 1.0 ± 0.05, suggesting that most of the identified proteins displayed experimental Mr and pl values similar to their theoretical values. In addition, the identity of these pro-
Table 2. Identification of the DEPs from plasma samples by Ultraflex III TOF/TOF MS

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Protein Accession No.</th>
<th>Theor. pl/Mr</th>
<th>Exper. pl/Mr</th>
<th>Matched peptide</th>
<th>Coverage (%)</th>
<th>Mascot score</th>
<th>t-test</th>
<th>Relative change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A05</td>
<td>Galectin-3-binding protein</td>
<td>LG3BP_HUMAN</td>
<td>4.90/65.32</td>
<td>5.13/66.20</td>
<td>8</td>
<td>24</td>
<td>259</td>
<td>2.60E-22</td>
<td>∞</td>
</tr>
<tr>
<td>A11</td>
<td>Apolipoprotein A-I</td>
<td>APOA1_HUMAN</td>
<td>5.50/30.77</td>
<td>5.56/30.76</td>
<td>5</td>
<td>26</td>
<td>167</td>
<td>4.00E-13</td>
<td>1.64</td>
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<tr>
<td>A15</td>
<td>Alpha-2-HS-glycoprotein</td>
<td>FETUA_HUMAN</td>
<td>5.53/39.32</td>
<td>5.43/40.10</td>
<td>5</td>
<td>16</td>
<td>123</td>
<td>1.00E-08</td>
<td>1.61</td>
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<tr>
<td>A16</td>
<td>Complement factor I</td>
<td>CFAI_HUMAN</td>
<td>7.53/65.71</td>
<td>7.72/68.07</td>
<td>6</td>
<td>23</td>
<td>197</td>
<td>4.00E-16</td>
<td>∞</td>
</tr>
<tr>
<td>A22</td>
<td>CD5 antigen-like</td>
<td>CD5L_HUMAN</td>
<td>5.15/38.08</td>
<td>5.28/39.60</td>
<td>3</td>
<td>28</td>
<td>265</td>
<td>6.40E-23</td>
<td>∞</td>
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<tr>
<td>A24</td>
<td>Keratin, type II cytoskeletal 6A</td>
<td>K2C6A_HUMAN</td>
<td>8.17/60.03</td>
<td>8.09/60.29</td>
<td>7</td>
<td>18</td>
<td>215</td>
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<td>2.47</td>
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<tr>
<td>A26</td>
<td>Serum paraoxonase/arylesterase 1</td>
<td>PON1_HUMAN</td>
<td>4.89/39.74</td>
<td>5.08/39.90</td>
<td>7</td>
<td>30</td>
<td>362</td>
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<td>1.52</td>
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<tr>
<td>A29</td>
<td>Haptoglobin</td>
<td>HPT_HUMAN</td>
<td>6.57/45.20</td>
<td>6.13/45.86</td>
<td>7</td>
<td>33</td>
<td>240</td>
<td>2.00E-20</td>
<td>∞</td>
</tr>
</tbody>
</table>

*aAssigned spot numbers as indicated in Figure 1. bDatabase accession numbers according to UNIProt. *cThe theoretical (c) and experimental (d) values of molecular weight (Mr, kDa) and pl for the identified proteins. *dPercentage values of coverage (%) of the matched peptides in the whole protein sequence. *eAverage abundance volume value of the target protein spots in the whole 2-DE gels.
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Figure 2. Comparison of the expression levels of the 8 identified proteins. The selected protein spots corresponding to DEPs in the 2-DE gels are shown. Abundance of the target protein spots in the 2-DE gels from PE and NT plasma samples. Each group include 3 cases of plasma samples. Analysis of the spots gray intensity corresponding to the identified proteins in PE and NT plasma samples. Data was normalized processed. Error bars represent the standard deviation (SD) of three replicates. *P < 0.05, compared with control.

Validation of proteins by ELISA

To verify the abundance levels of proteins identified by 2D-DIGE and Ultraflex III TOF/TOF MS, we evaluated LG3BP, APOA1, FETUA, CFAI, CD5L, K2C6A, PON1 and HP1 by ELISA. The differences in plasma protein levels between PE and normal pregnant women are shown in Figure 3. The results showed that the protein levels of LG3BP, APOA1, FETUA, CFAI, CD5L, K2C6A, PON1 and HP1 were significantly decreased in the PE group. These ELISA results are consistent with the proteomic data, and further suggest that LG3BP, APOA1, FETUA, CFAI, CD5L, K2C6A, PON1 and HP1 may be explored as potential biomarkers in the early diagnosis of PE in pregnant women.

Discussion

Although early diagnosis of PE is effective in its treatment, no accepted screening methods are currently available for this disease. Therefore, an urgent need exists for highly sensitive and specific biomarkers that can detect PE at an early stage. Differential proteomics is a widely used technique to identify all types of biomarkers in disease diagnosis research [25].

To the best of our knowledge, we are the first to report the use of 2D-DIGE/Ultraflex III TOF/TOF to identify potential plasma biomarkers of PE. Our proteomics analysis revealed 56 differentially expressed protein spots (8 up-regulated and 48 down-regulated). Eventually, we identified 8 plasma proteins unique to PE. These proteins have not been previously reported as biomarkers of PE in the literature. Moreover, the
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LG3BP is up-regulated in many cancers and is implicated in tumor metastatic processes, as well as in other cell adhesion and immune functions, and may be important in immune progression [26, 27]. In our study, LG3BP was significantly decreased in the plasma of pregnant women with PIH, suggesting that LG3BP may be a useful biomarker for PE diagnosis. APOA1 is a component of the high-density lipoprotein responsible for the transport of cholesterol into the liver. It exhibits an anti-inflammatory and an anti-fibrotic effect on lung fibrosis [17, 18]. Therefore, APOA1 potentially affects immune factors and the systemic inflammatory response. It is also a potential biomarker of liver fibrosis/cirrhosis [28]. In preeclampsia, APOA1 is typically accompanied with liver damage and transaminase elevation. Our 2D-DIGE analysis revealed that the APOA1 level is down-regulated in PE plasma. Therefore, the down-regulation of plasma APOA1 showed potential diagnostic value in the PE plasma. These results suggested that APOA1 may be a useful biomarker of PE. CFAI is essential in regulating the complement cascade, and is associated with a predisposition to atypical hemolytic uremic syndrome, a disease characterized by acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia [20]. Hemolysis, the condition of elevated liver enzymes and low platelets (HELLP), is a typical clinical symptom of PE. Moreover,

combination of these identified proteins may be further evaluated as PE-specific biomarkers.

CD5L has critical roles as a PRR of bacterial and fungal components functioning in the con-

Figure 3. Verification of LG3BP, APOA1, FETUA, CFAI, CD5L, K2C6A, PON1 and HP1 expression levels between PE and healthy pregnant women by ELISA analysis. The histograms represent mean protein expression and variation. The Student’s t-test was performed (*indicates P < 0.05). These ELISA results were consistent with the data from 2D-DIGE, which further suggested that LG3BP, APOA1, FETUA, CFAI, CD5L, K2C6A, PON1 and HP1 can be potential biomarkers for the early diagnosis of PE.
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trol of key mechanisms in inflammatory responses, such as infection, atherosclerosis and cancer [21]. HP1 functions to bind free plasma hemoglobin and allows degradative enzymes to gain access to the hemoglobin. This prevents the loss of iron through the kidneys and protects the kidneys from damage by hemoglobin [22, 23]. Interestingly, another study reported that HP1 treatment prevents HB-hemoglobin hypertension [29].

In conclusion, this study adopted a quantitative proteomic approach and identified 8 potential biomarkers of PE. These putative biomarkers may be useful tools in the early diagnosis and monitoring of PE. However, the potential of utilizing these markers for screening and treating PE still needs to be investigated further.

Acknowledgements

The authors wish to thank the students and other staff in the 3rd Affiliated Hospital, Sun Yat-sen University (Guangzhou, China) for technical assistance.

Disclosure of conflict of interest

None.

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