Miao et al.: Proteomics on Fetal Growth Restriction

Cellular Physiology and Biochemistry

Comparative Proteomic Profile of the Human Placenta in Normal and Fetal Growth Restriction Subjects

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Key Words
TMT • Fetal growth restriction • Placenta • Oxidative stress • Apoptosis

Abstract

**Background:** Fetal growth restriction (FGR) is the main cause of intrauterine fetal death and the second leading cause of death in the neonatal period. A large body of evidence suggests that FGR may be associated with the placenta, although its etiology and pathogenesis remain to be fully elucidated. **Methods and Results:** To better understand the molecular mechanisms underlying the pathological development of the placenta in FGR, we used tandem mass tags (TMTs) to construct a large-scale comparative proteomic profile of human placentas from normal and FGR pregnancies. A total of 1,198 kinds of proteins were identified in the control and FGR placentas, of which 95 were differentially expressed between two groups. Ingenuity Pathway Analysis (IPA) was used to organize these differentially expressed proteins into networks of interacting proteins and to identify the modules of functionally related proteins. Western blotting was used to verify the expression patterns of several randomly selected proteins. **Conclusion:** The placentas of women with FGR displayed significant proteome differences compared with normal pregnancy. The results indicate that a variety of mechanisms and proteins may contribute to the development of FGR. Further studies and validations are required to elucidate the exact roles of these proteins in FGR pathogenesis.

Z. Miao and M. Chen contributed equally to this work.
Introduction

Fetal growth restriction (FGR) is a major complication of pregnancy and affects up to 10% of all pregnancies [1]. It is the main cause of intrauterine fetal death and the second leading cause of death in the neonatal period [2]. Although the primary mechanism of FGR is still unknown, a considerable body of evidence suggests that FGR could be associated with many factors such as impaired placental function, inadequate trophoblast invasion, deficient spiral arterial remodeling, and increased apoptosis of trophoblastic cells [3-6]. Notably, recent studies have indicated that impaired placental function may hold the key to the etiology of FGR [3, 7].

The placenta is the interface between maternal and fetal circulation, and it plays several significant roles in pregnancy, such as transporting gases, nutrients, and waste products, preventing the rejection of fetal allografts and producing peptides and hormones [3-8]. In short, placental function holds a key to fetal development or its failure. Recent studies have made some progress in better understanding the molecular mechanisms underlying the pathological development of the placenta in patients with FGR. For example, abnormalities of trophoblast invasion and villous vascular development will lead to a failure in establishing adequate uteroplacental blood flow and may increases the risk of oxidative stress and acidosis [9, 10], indicating the imbalance between antioxidants and reactive oxygen species (ROS) production (oxidative stress) is a critical risk factor in FGR [11-13]. Besides, increased apoptosis was significantly observed in placenta trophoblasts, suggesting apoptosis may be one of the pathogenesis of FGR [14, 15].

Dysfunctions of several molecules, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, alpha-1-antitrypsin (SERPINA1), insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), might correlate with abnormal trophoblast invasion and vascular development, and oxidative stress in the placenta of FGR subjects [16-22]. The expression levels of apoptosis associated factors, such as ATP-dependent RNA helicase (DDX42), Bcl-2, p53, low-density lipoprotein (LDL), apo B and matrix metalloproteinases, changed during FGR development [6, 23-28]. However, thus far, no large-scale proteomic analyses have been used to investigate the regulatory factors involved in the placenta of pregnancies with FGR. Therefore, here, we aimed to establish a comparative proteomic profile of human placentas in normal and FGR pregnancies using TMTs.

Materials and Methods

Sample preparation

This study was conducted in accordance with the declaration of Helsinki and performed with approval from the Ethics Committee of the Nanjing Maternity and Child Health Care Hospital affiliated to Nanjing Medical University. Written informed consent was obtained from all participants.

Placental tissues were obtained from twenty pregnant women (10 cases for FGR and 10 cases for control) according to the standard operating procedure. All mothers had undergone cesarean section delivery at the Maternal and Child Health Hospital of Nanjing and had the same range of age and gestational age. FGR refers to a fetus that has failed to achieve its genetically determined growth potential and was defined as estimated fetal weight (EFW) less than the tenth percentile with respect to the reference value for current pregnancy age. The details of patient characteristics are presented in Table 1. For each placenta sample, 0.5 g of placental tissue was dissected from the maternal side of the placenta (in the central region excluding the calcified area), rinsed with 0.9% saline, and frozen in liquid nitrogen prior to use.

Protein extraction

Proteinminer (Aurum serum protein mini kit, Bio-Rad) before proteomic analysis was used to remove high abundance proteins to allow the visualization of low abundant proteins as previously described [29].
The protein was preserved at -20°C. The supernatant was reduced using 10 mM dithiothreitol (DTT) at 56°C for 1 h, and then alkylated by incubation with 55 mM of iodoacetetyl moiety biotin (IAM-biotin) in a dark room for 1 h. Precooled acetone was added to the sample liquid until the total volume was five times the original volume, and the mixture was precipitated at -20°C for at least 1 h. The mixture was then centrifuged at a rate of 1000 g for 20 min at 4°C. The precipitate was added to 300 µL of buffer with a final concentration of 50% Tetraethylammonium Bromide (TEAB) containing 0.1% SDS. Then, ultrasound was used to enhance homogenates and cell lysis. Finally, Bradford assay was used to quantify the protein.

**Protein digestion**

Placenta protein (100 µg) from normal and FGR subjects was diluted with TEAB containing 0.1% SDS. Then, 3.3 µg of 1 µg/µL trypsin was added, and the mixture was immersed in a 37°C water bath for 24 h. Then, 1 µg of trypsin was added, and the mixture was incubated in a 37°C water bath for 12 h. Thereafter, the mixture was vacuum freeze-dried, and resuspended in 30 µL of TEAB containing 0.1% SDS (water: TEAB = 1:1). Finally, MALDI TOF/TOF was used to check the digestive efficiency for 1µL of the lysate.

**Peptide labeling and depuration**

10 cases of FGR or 10 cases of control were randomly divided into 3 groups respectively, indicating the protein sample of each group was a mixture from 3 or 4 patients. The labeling reagent was equilibrated to room temperature. Then, 70 µL of isopropyl alcohol was added to the labeling reagent and vortexed for 1 min. The homogenized labeling reagent was then added to the peptides, and isotopic labels of different sizes were used for the different samples. The mixture was vortexed again and equilibrated at room temperature for 2 h. The labeled samples were then dried in vacuo and separated by HPLC and C18 reversed phase chromatography and desalted. Finally, the peptides were dissolved by adding 0.1% formic acid.

**Mass spectrometry data acquisition and identification**

The labeled peptides were analyzed using high-precision LC-MS (Thermo-fisher Q-Exactive Orbitrap). The MS/MS spectra acquired from precursor ions were submitted to Mascot (version 2.3.01) using the following search parameters for quantitative retrieval: the Swissprot Human Library was used for database search, the enzyme used was trypsin, urea methylation was used for fixed modification, methionine oxidation was used 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, the following search parameters were used for qualitative analysis: the protein ratio was determined using the median value, the minimum peptides was 1, the median normalization method was used, the p value was set at <0.05, and the fold change was 1.2.

**Western blotting**

Western blot analysis of all twenty samples was performed as described previously [30]. Lysates from the placentas of normal and FGR subjects were separated on 15% SDS-PAGE gels and the proteins were then transferred on to nitrocellulose membranes (Amersham Biosciences, RPR303D). The membranes were blocked in Tris-Buffered Saline and Tween 20 (TBST) containing 5% nonfat milk powder for 1 h, incubated overnight with primary antibodies against CSNK2B (1:3000 dilution; Abcam Ab133576, Cambridge, UK), GOSR2 (1:2000 dilution; Abcam Ab115642), HNRNPH2 (1:2000; Abcam Ab157498), SERPIN A3 (1:3000 dilution; Abcam Ab88527), SERPIN A1 (1:2000 dilution; Novus 2B12) and GAPDH (1:2000 dilution; Abcam 10044). The membranes were then washed three times in TBST and incubated with secondary antibodies conjugated to horseradish peroxidase. The blots were visualized using an ECL detection kit (Amersham Biosciences, RPR05006).

Table 1. Clinical characteristics of controls and patients with fetal growth restriction. Data are presented as the means ± SD. * P < 0.05, ** P < 0.01

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 10)</th>
<th>FGR (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>28.20 ± 3.71</td>
<td>26.30 ± 2.41</td>
<td>0.193</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1.30 ± 0.48</td>
<td>1.40 ± 0.70</td>
<td>0.714</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.06 ± 2.96</td>
<td>25.73 ± 2.62</td>
<td>0.597</td>
</tr>
<tr>
<td>Gestation weeks</td>
<td>38.64 ± 1.06</td>
<td>37.62 ± 2.20</td>
<td>0.207</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3305.00 ± 338.67</td>
<td>1949.00 ± 325.93</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
Ab9485), then washed three times (10 minutes each) with TBST. The membranes were then incubated for 1 h with alkaline phosphatase (AP)-conjugated anti-mouse or rabbit IgG (1:1000 dilution; Promega, S372B, WI, USA). The T protein levels were evaluated by detecting alkaline phosphatase activity using a Lumi-Phos kit (Pierce Biotechnology, KJ1243353).

Statistical analysis

For dimethyl labeling, the following criteria were used to consider a protein for further statistical analysis: two or more high-confidence unique peptides could be identified, the p value was <0.1, and the fold difference was >1.5 or <0.5. Then, the Student’s t test was used to find the significantly altered proteins with the SPSS software (version 18.0).

The visualized bands of the Western blot analysis were quantified with Quantity One software (Bio-Rad). The protein ratio was determined using the median value. The Student’s t test was used to generate p values. Differences were considered significant at a p value of <0.05.

Bioinformatics analysis

Gene ontology (GO) analysis was used to determine the biological significance of the identified proteins by exploring the relationship between biological terms and genes associated with three structured ontologies including the biological process, molecular function, and cellular components. The identified proteins were divided into different categories according to biological function. Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) was used to organize differentially expressed proteins into networks of interacting proteins and to identify modules of functionally related proteins involved during the pathological changes of placenta in FGR. Proteins whose expression was significantly differentially regulated (fold change ≥1.2, p value ≤ 0.05) were selected for the analysis. A detailed description is given in the online repository (http://www.geneontology.org and http://www.ingenuity.com).

Results

Identification of placental proteins related to pathological development

To identify proteins that were differentially expressed in the placentas of normal and FGR patients, the 1198 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 95 proteins showed significant (p < 0.05) differential expression between the normal and FGR placentas (Table 2).

Bioinformatics analysis of differentially expressed proteins

Ingenuity Pathway Analysis (IPA) was used to elucidate significant molecular networks and pathways associated with FGR. Comparison of the expression of 95 differentially expressed proteins revealed that 36 proteins were downregulated and 59 were upregulated in FGR, with a fold change of 1.2 (Table 2). The networks of interacting proteins and modules of functionally related proteins were show in Figure 1. These proteins were linked to two major molecular networks that are linked to erythropoiesis and oxidative stress (i.e., hemoglobin (A) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (B) networks).

Western blotting

We validated the TMT results of the identified proteins with Western blotting using GAPDH as an internal reference. For this, we randomly selected the following five proteins (CSNK2B, GOSR2, HNRNPH2, SERPINA1 and SERPINA3) from the list of differentially expressed proteins. As shown in Figure 2, the Western blotting results were essentially in agreement with the results of the MS/MS analyses.
Table 2. The list of differentially expressed proteins in human placenta from control and fetal growth restriction pregnancies. Fold change = Controls/fetal growth restriction (FGR). Fold Change < 0: The protein showed higher expression in the FGR placenta. Fold change > 0: The protein showed higher expression in the controls.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein names</th>
<th>Fold Change</th>
<th>Protein names</th>
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<td>E7Q5L4</td>
<td>Gpdp SNAP receptor complex member 2 (GSR2)</td>
<td>4.47</td>
<td>Gpdp SNAP receptor complex member 2 (GSR2)</td>
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<td>G3W7S3</td>
<td>Alpha-1-antitrypsin/alpha-1-proteinase inhibitor 5 (SERPINA5)</td>
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<td>High mobility group protein B3 (HMG3) High mobility group protein B2a (HMG2a) High mobility group protein B4 (HMG4)</td>
<td>1.57</td>
<td>High mobility group protein B3 (HMG3) High mobility group protein B2a (HMG2a) High mobility group protein B4 (HMG4)</td>
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Discussion

FGR is a major complication of pregnancy and affects up to 10% of all pregnancies [1]. It is the main cause of intrauterine fetal death and the second leading cause of death in the neonatal period [2]. Placenta is known as a critical determinant of fetal growth. Many previous studies have shown that structural and functional alterations in the placenta from FGR pregnancies might affect fetal growth and development. However, the fundamental mechanisms underlying these defects are poorly understood [3-6]. Therefore, we attempted to gain a comprehensive map of the proteins that are differentially expressed in FGR placentas.

Here, we carried out a comparative proteome study to determine the proteins that are differentially expressed in human placentas of normal and FGR pregnancies. A total of 1198 unique proteins expressed in the human placenta were identified with high confidence. Of these, 95 were found to be differentially expressed between these two groups. Ingenuity Pathway Analysis (IPA) was used to elucidate the significant molecular networks and pathways associated with FGR. Thirty five of these 95 total differentially expressed proteins...
are linked to two major molecular networks that are linked to erythropoiesis and oxidative stress.

Several proteins such as NADPH oxidase, low-density lipoprotein (LDL), and SERPINA1 play critical roles in the pathological development of FGR. NADPH oxidase is an enzyme complex that can generate large amounts of reactive oxygen species (ROS). They are generated at the maternal-fetal interface at the decidual, trophoblast, and mesenchymal levels. Physiological ROS levels play an important regulatory role through various signaling transduction pathways in uterine function, embryogenesis, embryonic implantation, and
feto-placental development [16, 31, 32]. However, the imbalance between antioxidants and ROS production (oxidative stress) is a causative agent in FGR [11-13]. Nutritional and environmental factors are the sources of oxidative stress [33, 34]. There is considerable evidence that FGR is associated with oxidative stress [35-37]. SERPINA1, also known as alpha-1 antitrypsin (AAT), is an acute phase protein and one member of the serpin superfamily of serine protease inhibitors. SERPINA1 has been shown to be a broad-spectrum anti-inflammatory, immunomodulatory, anti-infective and tissue-repair molecule. Moreover, SERPINA1 can increase LDL binding and uptake, and upregulate the levels of LDL receptors [38, 39]. However, decreased SERPINA1 activity, which is associated with oxidative stress, has been reported in FGR placentas [17-19]. SERPINA1 has been shown to be oxidized by peroxide lipids and various oxidants, and oxidized SERPINA1 has multiple effects on cytokine expression, generation of ROS, and intracellular lipid metabolism. Furthermore, oxidized SERPINA1 can release elastase, which may induce vascular abnormalities and stimulate NADPH oxidase, catalyzing the production of superoxides, which in turn causes direct oxidative damage to biomolecules, including SERPINA1 itself [40-42]. All in all, NADPH oxidase, LDL, and SERPINA1 are all associated with oxidative stress, which plays an important role in the pathological changes observed in FGR placentas. Several studies have suggest that using antioxidants (e.g., vitamins C and E) to treat pregnancies that are at high risk of FGR have shown positive effects of reducing the incidence of maternal disease and improving the prognosis of fetuses with FGR [43-45].

Studies have shown that hypoxia can enhance erythropoiesis to improve the oxygen carrying and buffering capacities by increasing the erythrocyte mass and hemoglobin concentration, and high hemoglobin concentrations have been associated with FGR [46-48]. In this work, HBA1, HBA2, HBG1, HBG2, and HBB were all found upregulated in the placenta of FGR. Therefore, abnormal erythropoiesis may be one cause of FGR.

Apoptosis is an essential feature of normal placental development but is notably exaggerated in association with some pregnancy complications [49], such as FGR [14]. DDX42 is a putative ATP-dependent RNA helicase implicated in cellular growth and apoptosis. It is a major apoptosis inducer known to enhance p53 transactivation of proapoptotic genes [23]. In this study, DDX42 was approximately 4.5-fold upregulated. At present, the pathological mechanism underlying increased cell apoptosis in FGR is still not understood. Crocker et al. found an increased susceptibility to apoptosis of primary trophoblasts isolated from patients with FGR [15]. In any case, the end result would be placental dysfunction and suboptimal pregnancy outcomes.

In summary, through comparative proteome analysis of the placentas of normal and FGR pregnancies, we constructed a protein expression profile; identified modules of functionally related proteins; and outlined important proteins that play key roles in the incidence and development of FGR, such as NADPH oxidase, SERPINA1, and DDX42. Further functional analyses of these differentially expressed proteins will help in understanding the critical biological processes and discovery of potential biomarkers and therapeutic targets, and eventually improve the prognosis of fetuses with FGR.

Acknowledgement

This work was supported by Grant 81100436 from the National Natural Science Foundation of China; Grant SKLRM-KF-201109 from the State Key Laboratory of Reproductive Medicine Fund; grant 2013NJMU142 from the Nanjing Medical University Foundation; grant YKK14 from Nanjing Medical Development Project.

Disclosure Statement

All authors have no conflicts of interest to declare.
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