의학박사 학위논문

Identification of differentially expressed proteins associated with the development of preeclampsia by proteomics using multiple reaction monitoring (MRM)-based quantification

다중반응탐색법을 이용한 프로테오믹스 분석에 의한 전자간증 발생 관련 단백의 발굴

2014년 2월

서울대학교 대학원

의학과 산부인과학 전공

김 선 민

Identification of differentially expressed proteins associated with the development of preeclampsia by proteomics using multiple reaction monitoring (MRM)-based quantification

지도교수 박 중 신

이 논문을 의학박사 학위논문으로 제출함
2013년 10월

서울대학교 대학원
의학과 산부인과학 전공
김 선 민

김선민의 의학박사 학위논문을 인준함
2013년 12월

위 원 장 _____ (인)
부위원장 _____ (인)
위 원 _____ (인)
위 원 _____ (인)
위 원 _____ (인)

Abstract

Identification of differentially expressed proteins associated with the development of preeclampsia by proteomics using multiple reaction monitoring (MRM)-based quantification

Sun Min Kim

Obstetrics & Gynecology

The Graduate School

Seoul National University

Introduction: Defective deep placentation is an abnormal transformation of the spiral arteries in the junctional zone of the myometrium. It results in significant obstetrical complications such as preeclampsia, fetal growth restriction, placental infarcts with fetal death, placental abruption, preterm labor, and premature rupture of membranes. Preeclampsia is a representative

disease of those with defective deep placentation. We performed proteomic analysis including multiple reaction monitoring (MRM)-based quantification with maternal plasma samples at the estimated time of deep placentation. The aim of this study was to determine the proteomic biomarkers associated with the development of preeclampsia.

Methods: A case-control study was conducted. Maternal plasma was obtained from women with singleton pregnancies between 16 and 21 weeks of gestation in which deep placentation appeared to have occurred during that period. Thirteen women subsequently diagnosed with preeclampsia were selected as cases and an equal number of matched women who delivered at term without complications served as controls. Differential proteome profiling was conducted with the previously stored plasma using an LTQ-Velos mass spectrometer. Proteins potentially associated with preeclampsia were further validated by multiple reaction monitoring (MRM)-targeted proteome analysis.

Results: We identified a large group of proteins differentially expressed in the cases compared to the controls by proteome profiling. Subsequently, MRM-targeted proteome analysis resulted in two significantly up-regulated proteins (complement C1s subcomponent and alpha-1 microglobulin / bikunin precursor (AMBP)).

Conclusions: We identified proteins that are differentially expressed during deep placentation in the maternal plasma of those who subsequently

developed preeclampsia. We propose that these proteins may be involved in the remodeling process of the spiral arteries even before the manifestation of clinical disease. These proteins could be served as potential biomarkers to predict obstetrical complications associated with defective deep placentation.

Keywords: proteomics, multiple reaction monitoring, defective deep placentation, physiologic transformation of spiral artery, preeclampsia, pregnancy

Student number: 2011-31114

Contents

List of Tables

List of Figures

Introduction

The placenta is essential for the maintenance of pregnancy; it supplies water and nutrients to the fetus, removes waste products, produces hormones that maintain pregnancy, and plays a role as a barrier to maternal immunologic activity. Physiologic transformation of the spiral arteries during pregnancy is necessary for normal placentation. During the transformation, vascular lumens become dilated and straightened, and trophoblasts replace the muscular and elastic arterial wall by a thick layer of fibrinoid material(1). That is vascular adaptation for maximal blood perfusion to the intervillous spaces through the low-resistant and high-capacity uteroplacental vessels. The transformation of spiral arteries occurs from the decidua even to the upper myometrium(1). The depth as well as the proportion of spiral artery transformation is also critical for blood flow through the placenta. Deep placentation is characterized by transformation of spiral arteries in the junctional zone of the myometrium, which is at a deeper level than the decidua. Failure of physiologic transformation of the spiral arteries in the myometrial junctional zone will result in defective deep placentation(2, 3). Additionally, obstructive arterial lesions such as acute atherosis contribute to the defective deep placentation.

Preeclampsia is the first-described and representative disease associated with defective deep placentation(2, 4). The placental bed biopsy results of patients with preeclampsia show a lack of transformed spiral arteries in the myometrial

junctional zone and obstructive arterial lesions(5-10). Defective deep placentation is now considered to underlie the pathogenesis of other pregnancy complications such as fetal growth restriction, intrauterine fetal death, placental abruption, preterm labor, and premature rupture of membranes(3, 10-15).

Physiologic transformation of the spiral arteries is a multistep process(16). Via decidualized endometrium and uterine natural killer (uNK) cells, decidua-associated remodeling initiates morphological changes of the spiral arteries with various angiogenic factors(17). Then, endovascular trophoblast-associated remodeling transforms the spiral arteries into low-resistant and high-capacity vessels. Endovascular trophoblast invasion into the junctional zone begins around 14 to 15 weeks of gestation. Trophoblasts conduct a type of destructive action. The musculoelastic structure is lost and re-endovascularization appears by apoptosis of endothelial and vascular smooth muscle cells; the penetration into arterial wall of trophoblast completes the transformation of the spiral arteries. Endovascular trophoblast-associated remodeling activity is maximized by 18 weeks of gestation and subsequently gradually declines. One third of the placenta bed spiral arteries contain endovascular trophoblasts in their myometrial segments in that period(18). The mechanism of failure in the transformation of spiral arteries is unclear. We hypothesize that there are differentially expressed proteins in the maternal blood between pregnant women who undergo defective deep placentation and subsequently developed preeclampsia and normal pregnant women at the time of the active phase of endovascular trophoblast-associated remodeling.

Several angiogenic or antiangiogenic proteins have been reported that they have a relationship with preeclampsia. Soluble fms-like tyrosine kinase 1 (sFlt-1) is an antiangiogenic protein that has an increased level in the placenta and maternal blood; conversely, angiogenic proteins such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) have been reported to have low serum concentrations in patients with preeclampsia(19-22). This change occurs even before the onset of clinical preeclampsia(23-25). Although some proteins have been shown to be altered prior to the onset of clinical disease, none of these have yet been regarded as a useful biomarker for screening.

Multiple reaction monitoring (MRM) is an advanced and high-tech method for validation and quantification of targeted proteins identified by tandem mass spectrometry (MS/MS). Comparing to the conventional quantitative methods, the remarkable strength of the MRM technique is the facility of generating a quantitative assay for the proteins that their specific antibodies are not available. The antibody-free method, MRM can quantify the proteins by the specific information for the target proteins, such as their unique peptides and the transitions for the peptides(26). MRM exploits the triple quadrupole (QqQ) LC-MS for quantitative analysis. Two levels of mass selection are able to effectively filter out background ions; thus, preserving a specific fragment ion of the target peptide. MRM restricts the acquisition mass range around the target peptide because the narrower the mass range, the

higher the specificity. The QqQ-based MRM analysis does not scan the entire mass spectra and it increases the sensitivity by one or two orders of magnitude compared to the conventional full scan technique(27). MRM-based quantification is superior to conventional immunoassay in reproducibility and assay development(26), and it performs reliable quantification in highly complex mixtures across multiple samples(27-30). Moreover, the multiplexed MRM technique can quantify the hundreds of target proteins simultaneously in a single experiment(27). It is a favorable procedure for highly multiplexed biomarker verification. Linear ion trap mass spectrometry (LTQ) alone is not enough to produce accurate quantifications and cannot reliably detect low levels of proteins.

Proteomic analysis using MRM technique has not been explored in the area of obstetrics. We performed this study to identify differentially expressed proteins between patients who subsequently developed preeclampsia and controls with maternal plasma at the time of active transformation of the spiral arteries in the junctional zone of the myometrium by proteomic analysis using MRM, which is an advanced quantitative method. Our eventual goal was to identify the proteins in maternal blood, which have the potential to be biomarkers for the prediction of preeclampsia and investigate the pathogenesis of defective deep placentation.

Materials and Methods

Study population

A case-control study was performed with stored maternal plasma obtained at the early second trimester (16-21 weeks of gestation). The study population consisted of women with a singleton pregnancy without chronic disease or pregnancy-associated complication at the time when maternal blood was collected. Women subsequently diagnosed with preeclampsia were selected as cases (n = 13) and an equal number of normotensive women who delivered normal weight neonate at term without significant medical or obstetric complications were selected as controls. Age (within two years), parity (nullipara vs. multipara), gestational age at sampling (within one week), and sampling time (within six months) were considered for selection of controls. Preeclampsia was defined as new-onset hypertension (systolic blood pressure ≥ 140 mm Hg and/or diastolic pressure ≥ 90 mm Hg on two occasions at least four hours but not more than seven days apart) and significant proteinuria (≥ 300 mg/24 h or ≥ 1+ on dipstick) after 20 weeks of gestation in a previously normotensive woman(31). A diagnosis of severe preeclampsia was based on the presence of preeclampsia plus one or more of the following criteria: persistent systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 110 mm Hg, proteinuria ≥ 5 g in a 24-hour urine collection or ≥ 3+ on dipstick in random urine samples, oliguria (< 500 mL/d), cerebral or visual

disturbances, pulmonary edema, epigastric or right upper-quadrant pain, impaired liver function, thrombocytopenia (< 100,000 platelets/mL), or fetal growth restriction(31). The Institutional Review Board of Seoul National University Hospital approved the collection of these samples and clinical information and use for research purposes.

Sample preparation

Maternal blood was obtained by venipuncture and collected into tubes containing ethylenediamine tetraacetic acid (EDTA). Samples were centrifuged at 700 x *g* for 10 minutes and the supernatant was then stored in polypropylene tubes at -70˚ C until assay. Samples with significant hemolysis were excluded. Before the in-gel digestion, six highly abundant proteins (human albumin, IgG, α1-antitrypsin, IgA, transferrin and haptoglobin) were removed from the plasma sample. Then, purified hydrophobic chromatography fractions containing protein extracted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were divided into 10 fractions. Each protein gel band was cut and subjected to in-gel tryptic digestion following the general protocol. Briefly, protein bands were excised, destained, and washed. Proteins were reduced with 20 mM dithiothreitol and alkylated with 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were digested with 12.5 ng/μL sequencing-grade modified porcine trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate overnight

at 37° C. Peptides were extracted from the gel slices with 50% (v/v) acetonitrile in 5% (v/v) formic acid. The eluates were dried under vacuum and stored -20° C until assay.

Mass spectrometry analysis and database search

Protein profiling with the digested peptides was done in duplicate on a LTQ-Velos mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts). Peptides were separated on a microcapillary column with a linear gradient of 2–38% solvent B (98% ACN and 0.1% FA in water) for 90 min at a flow rate of 300 nL/min. The spray voltage was set at 1.9 kV and the heated capillary temperature was set at 325° C. The LTQ-Velos was run in data-dependent mode with one survey MS scan in the mass range 400–1400 m/z, followed by five MS/MS scans using a normalized collision energy of 35%. Collected MS/MS raw files were converted to mzXML files using the TPP (Trans-Proteomic Pipeline, version 4.4). Peptides were assigned using the SEQUEST algorithm (Thermo Fisher Scientific, version 27, rev.11) against the SWISS-PROT human database (version 3.14). All searches were semi-tryptic, and allowed two missed cleavages. Precursor ion and fragment ion mass tolerances were set at 1.5 and 0.5 Da, respectively. Carbamidomethylation of cysteine was utilized for fixed modification, and oxidation of methionine for variable modification. Identification of peptides was approved if they were

defected at > 95% probability as specified by the PeptideProphet algorithm(32).

We used the previously described statistical method, Power Law Global Error Model (PLGEM), which provides signal-to-noise (STN) and the *P* value required for the calculation of variance(33). Candidate proteins were selected by means of PLGEM and Ingenuity Pathway Analysis (IPA) analysis and literature search.

For MRM analysis, maternal plasma proteins were reduced with 6M urea and dithiothreitol (DTT) at a final concentration of 10 mM and alkylated with iodoacetamide at 30 mM. For digestion, protein extracts were diluted in 50 mM ammonium bicarbonate to a final concentration of 1M urea; then trypsin was added 1/50 (trypsin/protein) ratio. They were digested overnight at 37° C.

Selection of MRM peptide transitions

We optimized the condition for MRM analysis. MRM peptide transition, a selection of a peptide precursor ion (Q1) and its fragment ions (Q3) for the targeted protein, is the essential process for successful analysis. Peptides were analyzed on an Agilent 6490 triple quadrupole mass spectrometer (Agilent Technologies). Separation of peptides were performed on the Agilent SB-C 18 set at 1.8 μm, 2.1 x 50 mm with a linear gradient of 0–40% solvent B (0.1% FA in ACN) for 60 min at a flow rate of 400 uL/min. The spray voltage was

set at 1.9 kV, and the temperature of the nitrogen drying gas was set at 200° C. MRM peptide transitions were established using two scan modes. The product ion scan analysis was performed in the following steps. Precursor ion mass was input to MassHunter Acquisition Analysis software (version B. 6.0, Agilent Technologies). Fragmentor voltage was fixed at 380 V and collision energy was modulated according to each target peptide's precursor mass (i.e., 500−600 m/z, 12−17 V; 600−700 m/z, 18−22 V; 700−800 m/z, 23−30 V; and 800 ≤ m/z, 31−35 V). MS/MS scan range was set from 400 to 2,000 m/z, in accordance with the precursor ion mass and charge states, and the scan time was set to 500 ms. Both MS and MS/MS ions were monitored with a unit resolution mass window (0.7 FWHM, Full Width at Half Maximum) in Q1 and Q3. The unbiased Q3-ion monitoring assay was conducted in MRM mode following the steps provided by the manufacturer. The four most intense fragment y and/or b ions were selected from LTQ MS/MS spectra of target peptides. Both fragmentation voltage and collision energy were adapted at the same values used for the full MS/MS scan method. Dwell time of each transition was set to 20 ms, and MRM Q1/Q3 transitions were monitored with 0.7 FWHM. Chromatographic elution profiles of each transition ion and estimation of chromatographic peak area were accomplished using MassHunter Quantitative Analysis (MHQA) software (version B. 6.0, Agilent Technologies).

MRM analysis

The multiplexing MRM assay was performed in triplicate with 93 transitions for 46 MRM target peptides, which had been determined from both full MS/MS scan and unbiased Q3-ion monitoring modes. The integration of peak areas for MRM transitions was calculated using MHQA software (version B. 6.0, Agilent Technologies). The coefficient of variation (CV) of the integrated peak area for external standard peptide was determined by the ratio of the standard deviation to the mean of triplicate measurements. Each integrated peak area of MRM transition was calibrated to the external standard peptide.

Results

Study population

The clinical characteristics of the cases and controls are shown in Table 1. There were no statistically significant differences between the two groups in regard to maternal age, parity, gestational age at blood sampling, and body mass index. The characteristics of preeclampsia after diagnosis are presented in Table 2. Of the patients with preeclampsia, 61.5% were diagnosed with severe preeclampsia. Almost half of the patients with preeclampsia had a growth restricted fetus. However, no patients had impaired liver function or thrombocytopenia.

Table 1. Characteristics of study population and pregnancy outcome

	Case	Control	*P* value
Age[a] (y)	35(31-40)	35(31-40)	0.636
Nulliparity	53.8%	46.2%	>1.00
BMI[a,b]	22.2(18.5-25.2)	22.6(19.2-29.1)	0.664
GA at sampling[a] (wk)	17.5(16.1-21.0)	17.1(16.3-20.5)	0.315
GA at delivery[a] (wk)	37(29.3-39.3)	39.3(37.6-40.7)	<0.001
Birthweight[a] (g)	2600(840-3410)	3310(3000-3830)	<0.001

BMI, body mass index*; GA*, gestational age

[a] Values are given as median (range)

[b] BMI at sampling time

Table 2. Characteristics of patients with preeclampsia as to the severity

Severe preeclampsia [a]	61.5 (8/13)
Early onset preeclampsia (diagnosis before 34 weeks of gestation) [a]	38.5 (5/13)
24hr urine protein (mg/day) [b]	1110 (304-7640)
Persistent systolic blood pressure ≥160 mm Hg or diastolic blood pressure ≥110 mm Hg [a, c]	61.5 (8/13)
Fetal growth restriction [a]	46.2 (6/13)
Serum creatinine (mg/dL) [b]	0.79 (0.7-1.1)
Abnormal liver enzyme (> twice normal range)	0
Low platelet count (<100,000 /μL)	0

[a] Values are given as % (number)

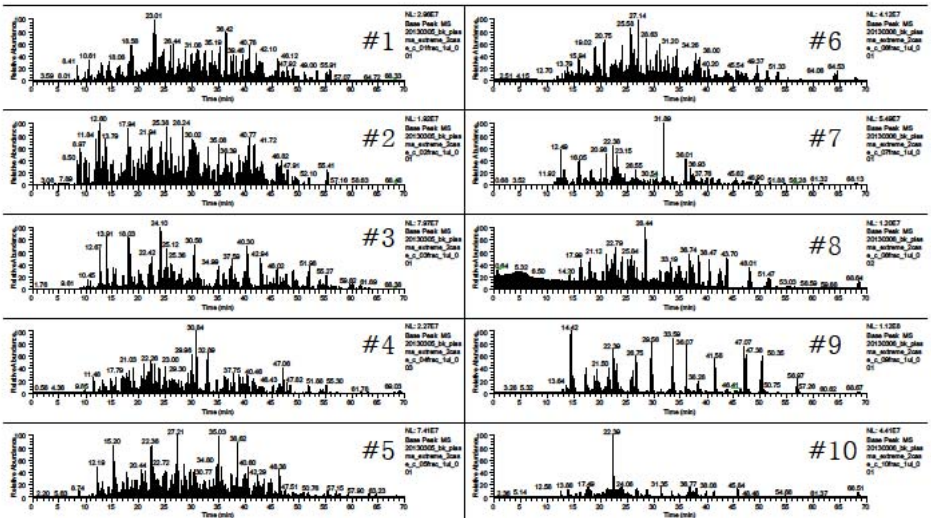
[b] Values are given as median (range)

[c] On 2 occasions at least 4 hours but no more than 7 days apart

Profiling by LTQ-Velos mass spectrometer

Depleted plasma samples were subjected to a LTQ-Velos mass spectrometer for duplicate analysis as described above. To maximize the difference in distinctive proteins between cases and controls, we selected the three most extreme cases and their controls for profiling. The three most extreme cases were the patients with a severer degree of preeclampsia. Figure 1 shows the LTQ chromatogram of cases (A) and controls (B). By a database search and PLGEM analysis, we discriminated between the 120 protein candidates showing significantly distinctive and consistent patterns between cases and controls for at least two pairs of the identified proteins ($P < 0.1$)(33). Among 120 candidates, 86 proteins of interest were selected by means of ingenuity pathway analysis (IPA) software and a literature search. Some were selected due to a large difference in up or down ratios between cases and controls.

(A)



(B)

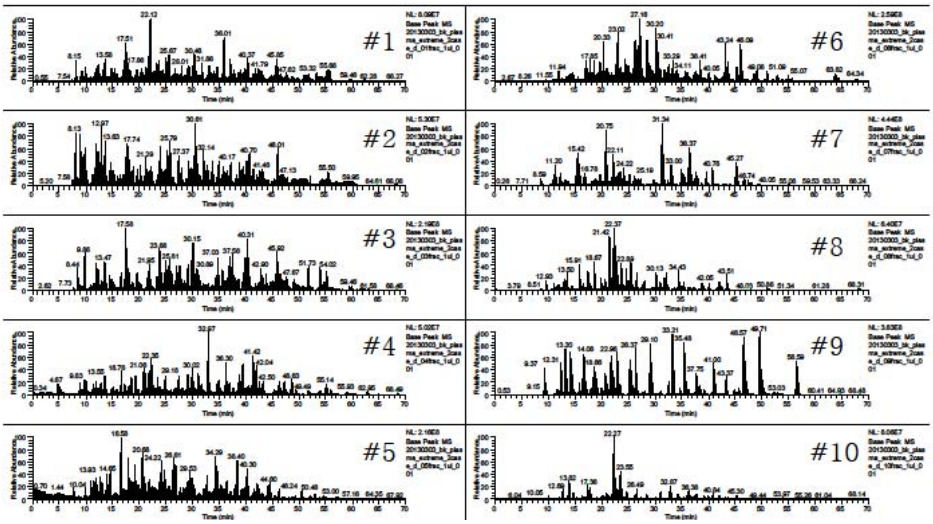


Figure 1. LTQ chromatogram; (A) control, (B) disease

MRM results

Determination of SRM peptide transitions

We determined a total of 38 target proteins appropriate for MRM analysis, 17 up-regulated and 21 down-regulated proteins, in cases compared to controls (Table 3). Selection of MRM peptide transitions representing target proteins of interest were performed using previously described criteria(34, 35). The unique peptide was selected for each target protein; it was determined by its representativeness, length of unmodified peptides (10−20 amino acids), and detectable charge states within the m/z scan window.

Table 3. Optimized MRM target proteins

Up-regulated targets	Down-regulated targets
Ceruloplasmin	Alpha-2-macroglobulin
Complement component C8 gamma chain	Angiotensinogen
Coagulation factor XIII A chain	Apolipoprotein E
cDNA FLJ58413, highly similar to Complement component C7	Isoform 1 of Clusterin
Haptoglobin	Isoform 1 of Complement factor H
Hemoglobin subunit beta	Complement C3 (Fragment)
Vitamin D-binding protein isoform 1 precursor	Protein AMBP
Hemoglobin subunit alpha	Hemopexin
Transthyretin	Complement C5
Galectin-3-binding protein	Complement factor I
Complement C1s subcomponent	Complement component C8 beta chain
Complement component C9	Serum paraoxonase/arylesterase 1
Alpha-1-acid glycoprotein 1	Vitronectin
Isoform 1 of C-reactive protein	Histidine-rich glycoprotein
Isoform 1 of Alpha-1-antitrypsin	Antithrombin-III

Isoform 2 of Ig mu chain C region	Apolipoprotein M
Apolipoprotein C-I	Apolipoprotein C-II
	Apolipoprotein C-III
	Plasma protease C1 inhibitor
	Isoform 1 of Serum albumin
	Isoform 1 of N-acetylmuramoyl-L-alanine amidase

AMBP, alpha-1-microglobulin/bikunin precursor

Detection of target proteins

A multiplexing MRM assay was performed in triplicate with 93 predetermined peptide transitions for 46 target peptides using the doubly-charged precursor ions. Figure 2 presents a chromatographic tracing of the peptide transitions. We estimated the MRM responses by integrating the peak areas of MRM peptide transitions, followed by normalization with transitions of the external peptide, beta-galactosidase extracted from *E. coli* to compensate for possible systematic bias. The external standard peptide was used for assurance about the reliability of the selected MRM peptide transitions for the target peptides mixed in complex biological samples.

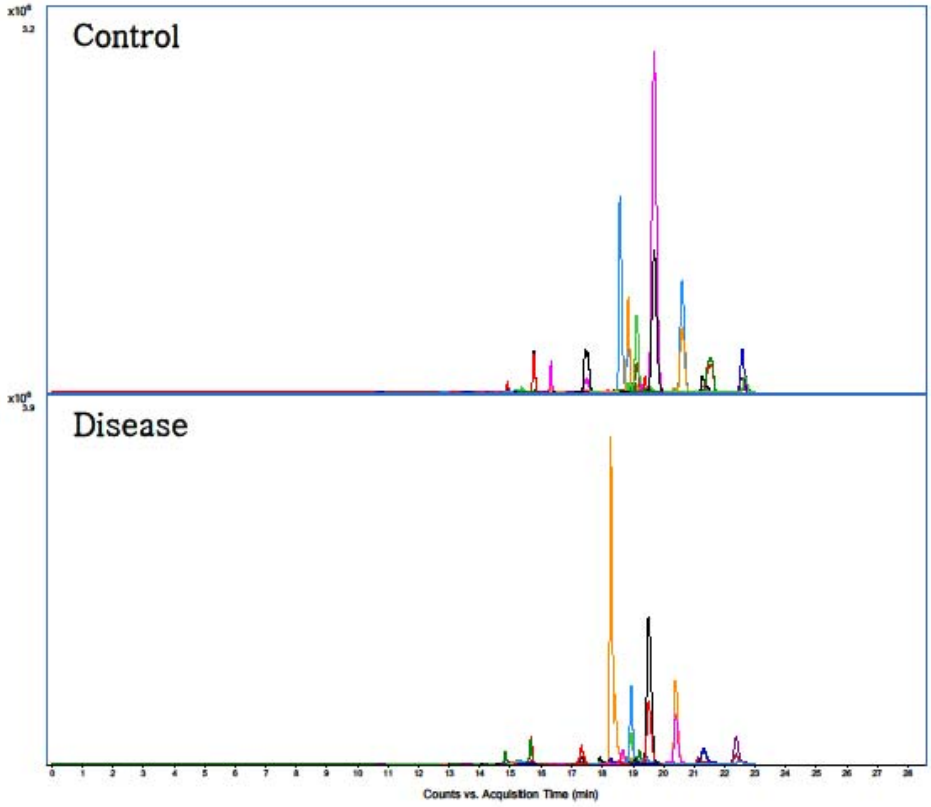


Figure 2. MRM chromatogram

Chromatographic elution profiles of selected fragment ions generated by MRM mode.

Figure 3 and 4 showed the average value and standard deviation of signal intensity quantified by MRM assay in each protein. Two target proteins were successfully quantified with statistical significance displaying the consistent up-regulated patterns expected from profiling. Complement C1s subcomponent and alpha-1-microglobulin/bikunin precursor (AMBP) were significantly up-regulated in the patients who subsequently developed preeclampsia. Haptoglobin showed tendency to be elevated in the plasma of cases compared with that of controls, but we failed to obtain the statistical significance (Figure 3). On the other hand, there were two proteins showed down-regulated patterns in the cases compared with controls (apolipoprotein M (Apo M), complement C5) but, statistically significant difference could not be obtained in these proteins (Figure 4). They may obtain statistical significance if the large number of subjects were included. The coefficient of variance for the target peptide transitions was reliable (≤ 20%) with triplicate analysis.

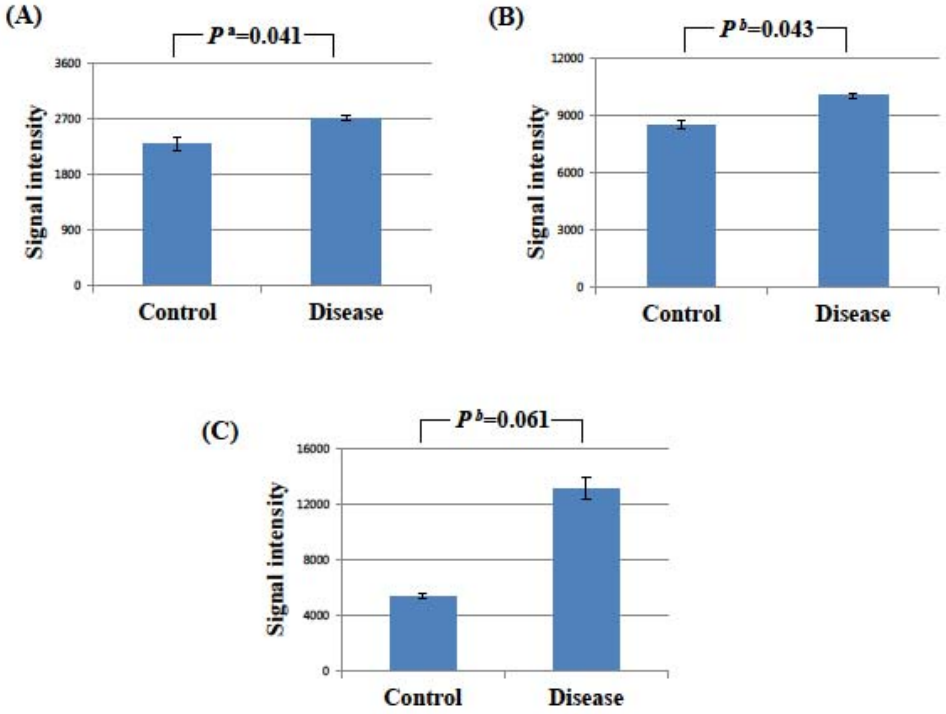


Figure 3. The comparison average value and standard deviation of signal intensity of the up-regulated proteins quantified by MRM assay; (A) complement C1s subcomponent, (B) alpha-1-microglobulin/bikunin precursor (AMBP), (C) haptoglobin

[a] Student's t-test on normal distributions

[b] Mann-Whitney *U*-test in on non-normal distributions

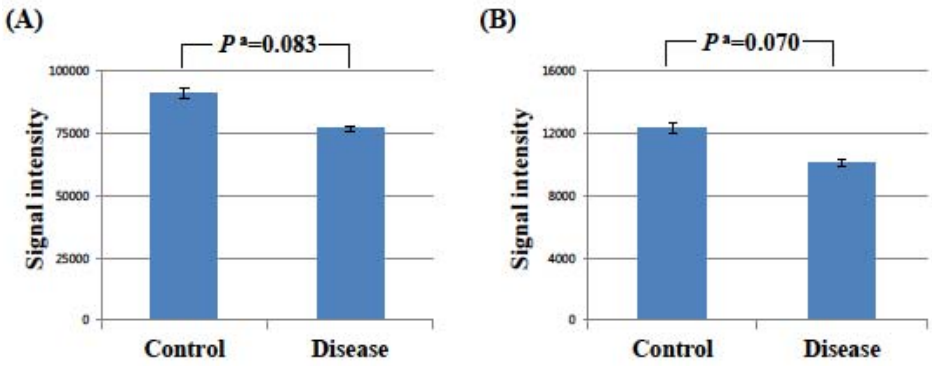


Figure 4. The comparison average value and standard deviation of signal intensity of the down-regulated proteins quantified by MRM assay; (A) apolipoprotein M, (B) complement C5

[a] Student's t-test on normal distributions

[b] Mann-Whitney *U*-test in on non-normal distributions

Discussion

The principal findings of this study

This study demonstrates that there are distinctive proteins in the maternal plasma, differentially expressed at the time of deep placentation. We applied MRM analysis, an advanced quantitative proteomic technique, to an obstetrical disease for the first time. When we selected the subjects with relatively severer disease among the patients with preeclampsia, both the number and degree of differentially expressed proteins between cases and controls were increased. This phenomenon can be explained by the premise that the physiologic transformation of spiral arteries is not an ‘all or none’ concept. The range of defective deep placentation is wide, and the severity of associated diseases is diverse(36).

Advantage of MRM analysis as a technique for clinical biomarker identification

MRM is an advanced technique of proteomics that can quantify multiple proteins detected by mass spectrometry simultaneously in a single assay. We can quantify by MRM the proteins of which antibodies are not obtainable. MRM is a highly reproducible quantification method when the adequate

conditions are established. It is preferable for high throughput validation of a number of putative biomarkers in complex biologic samples(37, 38). MRM analysis has been applied to identify biomarkers of human diseases with various biological samples, such as the cerebrospinal fluid from patients with Alzheimer's disease(39) and Parkinson's disease(40), pathologic tissue from breast cancer patients(41), and plasma samples from various cancers(42, 43). In the field of obstetrics, the MRM technique is also expected to be utilized to identify biomarkers for prediction of disease development or outcome.

Immunologic factors in defective placentation

Preeclampsia affects more nulliparous women than multiparous, and the risk of preeclampsia decreases with subsequent pregnancies. Several studies have demonstrated that a different partner with a subsequent pregnancy may be associated with an increased risk of preeclampsia(44, 45). These epidemiologic features suggest that immune maladaptation, or loss of maternal immune tolerance to paternally derived placental and fetal antigen is involved in the development of preeclampsia. Some proteins identified in this study (complement components) are associated with the immune system. A previous study reported that there was an association between complement system to angiogenic factor imbalance and placental dysfunction(46).
NK cells in the decidua are known to release cytokines and growth factors necessary for arterial transformation of the placenta(47). In early pregnancy,

30% of the cells in the decidua are leukocytes; 70% are uNK cells(48). Defective deep placentation may be influenced by aberrant function of uNK cells because the spiral artery is remodeled by extravillous trophoblasts with the aid of activated uNK cells. In preeclampsia, the extent and depth of remodeling of spiral arteries is far less than that of a normal pregnancy. Complement components can modulate the activation of NK cells. Changes of complement components in this study may be a result associated with activity of uNK cells. The exact mechanism whereby uNK cells influence trophoblast invasion remains unclear; however, it is assumed that uNK cells allow the trophoblasts to reach the inner myometrium. Various cytokines and angiogenic factors including interferon-ϒ, interleukin-8, VEGF, all of which can affect either vascular function or trophoblast migration, are released by uNK cells(49). They may regulate implantation by monitoring trophoblast invasion and ensure a balance between adequate fetal nutrition and maternal survival.

Inflammation and oxidative stress in defective placentation

Inflammatory response is developed by tissue damage and subsequent immunologic activation including the complement system. Compared to a normal pregnancy, endothelial cell dysfunction and systemic activation of inflammatory response is exaggerated in preeclampsia(50). Defective

placentation associated with inflammation can be considered to be the cause of preeclampsia(4). Alpha-1-microglobulin is a type of lipocalin transport protein and is known to be involved in processes of inflammatory response. A report by Olsson et al. described elevated alpha-1-microglobulin in the plasma, urine, and placentas of patients with preeclampsia(51). Alpha-1-microglobulin/bikunin precursor (AMBP) was also up-regulated in the patients who subsequently developed preeclampsia in this study. AMBP was overexpressed in the amniotic fluid of patients with preterm labor(52). Preterm labor, an obstetric complication associated with intra-uterine infection and/or inflammation is one of the phenotypes of defective deep placentation, which is milder abnormality of spiral artery remodeling than preeclampsia.

Lipid metabolism and defective deep placentation

Obstructive arterial lesions such as acute atherosis are found in the severe form of defective deep placentation. Preeclampsia is one of the diseases characterized by the absence or insufficiency of physiological remodeling of the spiral arteries in the junctional zone of myometrium; in addition, the vascular lesion is also found in patients with preeclampsia and hypertension during pregnancy(53, 54). Apo M, which showed down-regulated pattern, is bound to high density lipoproteins (HDL); it is known to modulate the bioactivity of sphingosine-1-phosphate (S1P) bioactivity, which is a lipid that influences angiogenesis and endothelial function(55). Overall, apolipoproteins

can play an essential role in atherosclerosis by controlling inflammatory responses and lipid transports(56, 57). Thus, Apo M identified in this study may get involved in defective deep placentation.

Unanswered questions

Several angiogenic and antiangiogenic proteins are known to be associated with the pathogenesis of preeclampsia. Among them, sFlt-1 is a noted protein which is a variant of the receptor for PlGF and VEGF. The serum levels of sFlt-1 increase in women destined to develop preeclampsia before disease onset as well as women with preeclampsia(22). However, sFlt-1 was not identified in this study. We suppose that this inconsistency may be resulted from the methodological aspect for protein detection and the low abundance of sFlt-1 in blood. At first, sFlt-1 had been identified as mRNA through the gene expression profiling with the placental tissue of patients with preeclampsia and controls(19). The serum levels of sFlt-1 were usually less than 10 nanograms per milliliter in pregnant women with or without preeclampsia(22). It may be too small amount to be detected by the protein profiling with mass spectrometry.

Weakness of this study and suggestions for further study

The individual variations of human biological samples contributed to the final MRM results. Relatively a small number of cases were included in this study.

Therefore, if only a few values are extremely high or low against the overall tendency, they can influence the average value of the entire cases, and even countervail the overall tendency. The more subjects enrolled in the analysis, the lower effect of individual variation.

We failed to establish a MRM transition for some of the candidate proteins because of very low amounts of those unique peptides in biological samples or different ionization mechanism of the peptides between profiling and MRM assay. In some cases, but not in this study, MRM transition cannot be installed when there is no unique peptide for a specific protein in database. Standardized proteotypic peptides (PTPs), which are unique peptides identifying the targeted protein, are important factor in MRM transitions.

We compared relative abundance of the proteins between cases and controls in this study. This relative quantification meets the aim of this study which was to find the candidate proteins differentially expressed between cases and controls. However, absolute concentrations for specific proteins can be also determined by MRM technique. Isotopically labeled synthetic peptide standards for the targeted proteins are required for the absolute quantification(58).

In conclusion, we identified distinguishing proteins differentially expressed between patients who subsequently developed preeclampsia and normal pregnant women at the time of deep placentation by proteomics with MRM technique. MRM is an advanced technique with high selectivity and

reproducibility for quantification of proteins in the targeted proteomic approach with complex biological samples. We can quantify specific proteins by MRM even if their antibodies are not available. Besides, once the transitions for a target protein are established, repeated quantifications are possible in new cases subsequently. The findings of this study can be applied to other obstetrical complications associated with defective deep placentation in addition to preeclampsia. If there are common proteins among these diseases, they may be useful for understanding of the mechanism of defective deep placentation. We expect that these identified proteins may have potential to be biomarkers for obstetrical complications associated with defective deep placentation.

References

1. Brosens I, Robertson WB, Dixon HG. The physiological response of the vessels of the placental bed to normal pregnancy. The Journal of pathology and bacteriology. 1967;93(2):569-79.

2. Brosens JJ, Pijnenborg R, Brosens IA. The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature. American journal of obstetrics and gynecology. 2002;187(5):1416-23.

3. Khong Y, Brosens I. Defective deep placentation. Best practice & research Clinical obstetrics & gynaecology. 2011;25(3):301-11.

4. Brosens IA, Robertson WB, Dixon HG. The role of the spiral arteries in the pathogenesis of preeclampsia. Obstetrics and gynecology annual. 1972;1:177-91.

5. Brosens IA. Morphological changes in the utero-placental bed in pregnancy hypertension. Clinics in obstetrics and gynaecology. 1977;4(3):573-93.

6. Gerretsen G, Huisjes HJ, Elema JD. Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. British journal of obstetrics and gynaecology. 1981;88(9):876-81.

7. Frusca T, Morassi L, Pecorelli S, Grigolato P, Gastaldi A. Histological features of uteroplacental vessels in normal and hypertensive patients in relation to birthweight. British journal of obstetrics and

gynaecology. 1989;96(7):835-9.

8. Meekins JW, Pijnenborg R, Hanssens M, van Assche A, McFadyen IR. Immunohistochemical detection of lipoprotein(a) in the wall of placental bed spiral arteries in normal and severe preeclamptic pregnancies. Placenta. 1994;15(5):511-24.

9. Guzin K, Tomruk S, Tuncay YA, Naki M, Sezginsoy S, Zemheri E, et al. The relation of increased uterine artery blood flow resistance and impaired trophoblast invasion in pre-eclamptic pregnancies. Archives of gynecology and obstetrics. 2005;272(4):283-8.

10. Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. British journal of obstetrics and gynaecology. 1986;93(10):1049-59.

11. De Wolf F, Brosens I, Renaer M. Fetal growth retardation and the maternal arterial supply of the human placenta in the absence of sustained hypertension. British journal of obstetrics and gynaecology. 1980;87(8):678-85.

12. Khong TY, Liddell HS, Robertson WB. Defective haemochorial placentation as a cause of miscarriage: a preliminary study. British journal of obstetrics and gynaecology. 1987;94(7):649-55.

13. Sheppard BL, Bonnar J. An ultrastructural study of utero-placental spiral arteries in hypertensive and normotensive pregnancy and fetal growth retardation. British journal of obstetrics and gynaecology. 1981;88(7):695-705.

14. Kim YM, Chaiworapongsa T, Gomez R, Bujold E, Yoon BH, Rotmensch S, et al. Failure of physiologic transformation of the spiral arteries in the placental bed in preterm premature rupture of membranes. American journal of obstetrics and gynecology. 2002;187(5):1137-42.

15. Kim YM, Bujold E, Chaiworapongsa T, Gomez R, Yoon BH, Thaler HT, et al. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. American journal of obstetrics and gynecology. 2003;189(4):1063-9.

16. Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. Placenta. 2006;27(9-10):939-58.

17. Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K, et al. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. The Journal of clinical endocrinology and metabolism. 2001;86(4):1823-34.

18. Pijnenborg R, Bland JM, Robertson WB, Dixon G, Brosens I. The pattern of interstitial trophoblastic invasion of the myometrium in early human pregnancy. Placenta. 1981;2(4):303-16.

19. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. The Journal of clinical investigation. 2003;111(5):649-58.

20. Koga K, Osuga Y, Yoshino O, Hirota Y, Ruimeng X, Hirata T, et al. Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. The Journal of clinical

endocrinology and metabolism. 2003;88(5):2348-51.

21. Ahmad S, Ahmed A. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. Circulation research. 2004;95(9):884-91.

22. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. The New England journal of medicine. 2004;350(7):672-83.

23. Polliotti BM, Fry AG, Saller DN, Mooney RA, Cox C, Miller RK. Second-trimester maternal serum placental growth factor and vascular endothelial growth factor for predicting severe, early-onset preeclampsia. Obstetrics and gynecology. 2003;101(6):1266-74.

24. Park CW, Park JS, Shim SS, Jun JK, Yoon BH, Romero R. An elevated maternal plasma, but not amniotic fluid, soluble fms-like tyrosine kinase-1 (sFlt-1) at the time of mid-trimester genetic amniocentesis is a risk factor for preeclampsia. American journal of obstetrics and gynecology. 2005;193(3 Pt 2):984-9.

25. Vatten LJ, Eskild A, Nilsen TI, Jeansson S, Jenum PA, Staff AC. Changes in circulating level of angiogenic factors from the first to second trimester as predictors of preeclampsia. American journal of obstetrics and gynecology. 2007;196(3):239 e1-6.

26. Picotti P, Aebersold R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. Nature methods. 2012;9(6):555-66.

27. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction

monitoring for quantitative proteomics: a tutorial. Molecular systems biology. 2008;4:222.

28. Hoofnagle AN, Wener MH. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. Journal of immunological methods. 2009;347(1-2):3-11.

29. Picotti P, Rinner O, Stallmach R, Dautel F, Farrah T, Domon B, et al. High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. Nature methods. 2010;7(1):43-6.

30. Whiteaker JR, Lin C, Kennedy J, Hou L, Trute M, Sokal I, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. Nature biotechnology. 2011;29(7):625-34.

31. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2002;77(1):67-75.

32. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. Analytical chemistry. 2003;75(17):4646-58.

33. Lee YY, McKinney KQ, Ghosh S, Iannitti DA, Martinie JB, Caballes FR, et al. Subcellular tissue proteomics of hepatocellular carcinoma for molecular signature discovery. Journal of proteome research. 2011;10(11):5070-83.

34. Cham Mead JA, Bianco L, Bessant C. Free computational resources

for designing selected reaction monitoring transitions. Proteomics. 2010;10(6):1106-26.

35. Sherman J, McKay MJ, Ashman K, Molloy MP. Unique ion signature mass spectrometry, a deterministic method to assign peptide identity. Molecular & cellular proteomics : MCP. 2009;8(9):2051-62.

36. Brosens I, Pijnenborg R, Vercruysse L, Romero R. The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. American journal of obstetrics and gynecology. 2011;204(3):193-201.

37. Carr SA, Anderson L. Protein quantitation through targeted mass spectrometry: the way out of biomarker purgatory? Clinical chemistry. 2008;54(11):1749-52.

38. Paulovich AG, Whiteaker JR, Hoofnagle AN, Wang P. The interface between biomarker discovery and clinical validation: The tar pit of the protein biomarker pipeline. Proteomics Clinical applications. 2008;2(10-11):1386-402.

39. Choi YS, Hou S, Choe LH, Lee KH. Targeted human cerebrospinal fluid proteomics for the validation of multiple Alzheimer's disease biomarker candidates. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2013;930:129-35.

40. Lehnert S, Jesse S, Rist W, Steinacker P, Soininen H, Herukka SK, et al. iTRAQ and multiple reaction monitoring as proteomic tools for biomarker search in cerebrospinal fluid of patients with Parkinson's disease dementia. Experimental neurology. 2012;234(2):499-505.

41. Muraoka S, Kume H, Watanabe S, Adachi J, Kuwano M, Sato M, et

al. Strategy for SRM-based verification of biomarker candidates discovered by iTRAQ method in limited breast cancer tissue samples. Journal of proteome research. 2012;11(8):4201-10.

42. Percy AJ, Chambers AG, Yang J, Borchers CH. Multiplexed MRM-based quantitation of candidate cancer biomarker proteins in undepleted and non-enriched human plasma. Proteomics. 2013.

43. Yoneyama T, Ohtsuki S, Ono M, Ohmine K, Uchida Y, Yamada T, et al. Quantitative targeted absolute proteomics-based large-scale quantification of proline-hydroxylated alpha-fibrinogen in plasma for pancreatic cancer diagnosis. Journal of proteome research. 2013;12(2):753-62.

44. Robillard PY, Hulsey TC, Alexander GR, Keenan A, de Caunes F, Papiernik E. Paternity patterns and risk of preeclampsia in the last pregnancy in multiparae. Journal of reproductive immunology. 1993;24(1):1-12.

45. Trupin LS, Simon LP, Eskenazi B. Change in paternity: a risk factor for preeclampsia in multiparas. Epidemiology. 1996;7(3):240-4.

46. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. The Journal of experimental medicine. 2006;203(9):2165-75.

47. King A. Uterine leukocytes and decidualization. Human reproduction update. 2000;6(1):28-36.

48. King A, Burrows T, Verma S, Hiby S, Loke YW. Human uterine lymphocytes. Human reproduction update. 1998;4(5):480-5.

49. Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, et

al. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. Journal of leukocyte biology. 2006;80(3):572-80.

50. Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. American journal of obstetrics and gynecology. 1999;180(2 Pt 1):499-506.

51. Olsson MG, Centlow M, Rutardottir S, Stenfors I, Larsson J, Hosseini-Maaf B, et al. Increased levels of cell-free hemoglobin, oxidation markers, and the antioxidative heme scavenger alpha(1)-microglobulin in preeclampsia. Free radical biology & medicine. 2010;48(2):284-91.

52. Bujold E, Romero R, Kusanovic JP, Erez O, Gotsch F, Chaiworapongsa T, et al. Proteomic profiling of amniotic fluid in preterm labor using two-dimensional liquid separation and mass spectrometry. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet. 2008;21(10):697-713.

53. Brosens I. A Study of the Spiral Arteries of the Decidua Basalis in Normotensive and Hypertensive Pregnancies. The Journal of obstetrics and gynaecology of the British Commonwealth. 1964;71:222-30.

54. De Wolf F, Robertson WB, Brosens I. The ultrastructure of acute atherosis in hypertensive pregnancy. American journal of obstetrics and gynecology. 1975;123(2):164-74.

55. Christoffersen C, Nielsen LB. Apolipoprotein M: bridging HDL and endothelial function. Current opinion in lipidology. 2013;24(4):295-300.

56. Hansen JB, Fernandez JA, Noto AT, Deguchi H, Bjorkegren J, Mathiesen EB. The apolipoprotein C-I content of very-low-density lipoproteins is associated with fasting triglycerides, postprandial lipemia, and carotid atherosclerosis. Journal of lipids. 2011;2011:271062.

57. van der Ham RL, Dehnavi RA, van den Berg GA, Putter H, de Roos A, Berbee JF, et al. Apolipoprotein CI levels are associated with atherosclerosis in men with the metabolic syndrome and systemic inflammation. Atherosclerosis. 2009;203(2):355-7.

58. Kuzyk MA, Smith D, Yang J, Cross TJ, Jackson AM, Hardie DB, et al. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. Molecular & cellular proteomics : MCP. 2009;8(8):1860-77.

국문초록

다중반응탐색법을 이용한 프로테오믹스 분석에 의한 전자간증 발생 관련 단백의 발굴

서론: 임신 초기 태반형성 과정에서 탈락막보다 더 깊은 층인 자궁근층에서 나선동맥의 생리적 변환이 정상적으로 이루어지지 못하는 것을 깊은 태반형성 부전이라고 한다. 이는 다양한 산과적 합병증의 발생과 관련이 있다고 알려져 있으며 전자간증은 그 대표적인 질환이다. 본 연구는 나선동맥의 생리적 변환이 활발하게 이루어지는 것으로 알려진 시기의 모체 혈장을 이용하여 전자간증과 대조군에서 다르게 발현되는 물질을 프로테오믹스 기법을 이용하여 탐색하고, 발굴된 물질이 깊은 태반형성 부전 과정에서 어떤 의미를 갖는지 알아보고자 한다.

방법: 나선동맥의 생리적 변환이 이루어지는 것으로 추정되는 시기

인 임신 16-21 주 사이에 채취한 모체 혈장이 보관되어 있는 단태 임산부를 대상으로 하였다. 실험군은 이후에 깊은 태반형성 부전의 대표적인 질환인 전자간증이 발생한 경우로 하고, 대조군은 주산기 합병증 없이 만삭에 정상 체중아를 분만한 임산부로 선정하였다. 보관되어 있는 모체 혈장을 이용하여 프로테오믹스 분석을 시행하였으며, 선정된 후보 단백들에 대해 다중반응탐색법을 이용한 정량을 시행하여 실험군과 대조군 사이에서 유의한 차이를 보이는지 알아보았다.

결과: LTQ-Velos mass spectrometer로 찾은 수백 개의 단백 중에 실험군과 대조군 간에 유의한 차이를 보이면서 태반형성 과정과 관련 가능성이 있어 보이는 후보 단백들을 선정하였다. 최종적으로 38개의 단백에서 각 단백을 대표할 수 있는 고유의 펩타이드를 이용하여 다중반응탐색법을 이용한 정량 분석을 위한 조건을 확립하고, 각각의 단백에 대하여 정량을 시행하였다. 실험군에서 대조군에 비하여 유의하게 농도가 높은 단백 (complement C1s subcomponent and alpha-1-microglobulin/bikunin precursor (AMBP))들의 존재를 확인하였다.

결론: 다중반응탐색법을 이용한 정량화라는 새로운 프로테오믹스 기법을 적용하여 모체 혈장에서 후에 전자간증이 발생하는 군과 대조

군 사이에 차이를 보이는 단백을 탐색해 보았다. 이러한 물질들은 임상적인 질환이 나타나기 이전에 변화를 보이는 것으로 생각되며, 나선동맥의 생리적 변환 과정에도 영향을 미칠 수 있을 것으로 추정된다. 전자간증 이외의 깊은 태반형성 부전과 관련된 다른 질환들에도 적용시켜 볼 수 있을 것이다. 다중반응탐색법은 복잡한 생물학적 시료를 이용한 실험에서 특정 단백에 대한 항체가 없이도 높은 처리 효율과 특이성을 가지고 단백을 반복적으로 정량할 수 있는 방법이다. 본 연구 결과는 향후 깊은 태반형성 부전 질환의 발생을 예측하는 생체표지자 개발에 활용될 수 있을 것이다.

주요어: 프로테오믹스, 다중반응탐색법, 깊은 태반형성 부전, 나선동맥의 생리적 변환, 전자간증, 임신

학번: 2011-31114