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Original Article

Proteomic profiling reveals α1-antitrypsin, α1-microglobulin, and clusterin as preeclampsia-related serum proteins in pregnant women

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ABSTRACT

Objective: Preeclampsia is a major cause of mortality in pregnant women but the underlying mechanism remains unclear to date. In this study, we attempted to identify candidate proteins that might be associated with preeclampsia in pregnant women by means of proteomics tools.
Materials and methods: Differentially expressed proteins in serum samples obtained from pregnant women with severe preeclampsia ($n = 8$) and control participants ($n = 8$) were identified using two-dimensional gel electrophoresis (2-DE) followed by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). Additional serum samples from 50 normal and 41 pregnant women with severe preeclampsia were analyzed by immunoassay for validation.
Results: Ten protein spots were found to be upregulated significantly in women with severe preeclampsia. These protein spots had the peptide mass fingerprints matched to α1-antitrypsin, α1-microglobulin, clusterin, and haptoglobin. Immunoassays in an independent series of serum samples showed that serum α1-antitrypsin, α1-microglobulin, and clusterin levels of severe preeclampsia patients ($n = 41$) were significantly higher than those in the normal participants ($n = 50$; α1-antitrypsin 295.95 ± 50.94 mg/dL vs. 259.31 ± 33.90 mg/dL, $p = 0.02$; α1-microglobulin 0.029 ± 0.004 mg/mL vs. 0.020 ± 0.004 mg/mL, $p < 0.0001$; clusterin 77.6 ± 16.15 μg/dL vs. 67.6 ± 15.87 μg/dL, $p < 0.05$).
Conclusion: Identification of these proteins by proteomics analysis enables further understanding of the pathophysiology of preeclampsia. Further studies are warranted to investigate the role of these biomarkers in prediction of this disease.

Introduction

With an incidence of approximately 5% around the world, preeclampsia is a major cause of mortality in pregnant women [1]. The incidence of preeclampsia in Taiwan is reported to be 2.03%; nevertheless, it contributes to 23.7% of perinatal maternal death [2]. Unfortunately, the cause of preeclampsia remains unclear to date. Preeclampsia is associated with abnormal placentation, reduced placental perfusion, and systemic vasospasm. Hormone factors as well as vascular and hemostatic hyper-reactivities involving the renin–angiotensin system, eicosanoids, and platelets have all been implicated [3].

The pathogenic mechanisms behind preeclampsia are totally different from other hypertensive disorders. Biochemical markers related to angiogenesis are generally chosen because the

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development of new blood vessels from existing endothelium is essential for normal placental development [4]. A number of potentially useful biochemical markers of angiogenesis such as vascular endothelial growth factor, placental growth factor, and endoglin have been implicated in preeclampsia [5,6]. Inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin, etc.] are known to be potent activators of the vascular endothelium and have been shown to be produced by trophoblasts and Hofbauer cells in the placenta [7]. Rinehart et al [8] reported that expression of the placental cytokines TNF-α, interleukin 1β, and interleukin 10 were increased in pregnant women with preeclampsia. To identify the patients at risk of developing severe preeclampsia at an early stage would be of great value to prevent the condition, to observe it closely in advance, and to offer the patients appropriate treatment. However, there are only a few tests available to screen for preeclampsia at the present time.

Comparative proteome analysis is a new technology to identify specific proteins or peptides which would be candidates of interest involving some of the diseases [9]. Novel proteins associated with preeclampsia could provide clues to the pathophysiology of the disorder and might also be used as specific markers for early detection and treatment. The goal of the present study was to identify candidate proteins that might be associated with preeclampsia in pregnant women by means of proteomics tools.

Materials and methods

Sample collection and preparation

The study was designed as a case-control study. After the Institute Review Board approved the study, 58 pregnant women with severe preeclampsia were identified at our medical center between August 2005 and July 2006. As case controls, we enrolled another 58 healthy gestational age-matched control individuals in a manner of one healthy pregnant woman being selected to the study when a severe preeclampsia case was collected. The criteria used for the diagnosis of preeclampsia were established in accordance with the guidelines of the American College of Obstetricians and Gynecologists (ACOG). Severe preeclampsia was defined as blood pressure $\geq$160/110 mmHg after the 20th week of gestation and proteinuria 2.0 g/24 h or $\geq$3 by dipstick, serum creatinine >1.2 mg/dL, platelet count <100,000/mm^3, elevated alanine transaminase (ALT) or aspartate aminotransferase (AST) and persistent headache, epigastric pain, or other cerebral or visual disturbance. None of the women had uterine contraction, rupture of membrane, vaginal bleeding or were in labor. We initially identified 58 women with severe preeclampsia, but nine were excluded—four because of pre-existing hypertension or pre-existing proteinuria and five for preterm labor or rupture of membrane at initial presentation. Samples of the corresponding nine healthy pregnancies were already analyzed and maintained in the control group. As a result, a total of 107 women were included and 6-mL peripheral blood samples were obtained from every patient. In the first part of our study, blood samples of eight women with severe preeclampsia and eight healthy pregnant women were collected as a screening group for proteomic analysis. In the second part of our study, blood samples from 41 women with severe preeclampsia and 50 healthy pregnant women were collected as a validation group for serum immunoassay. Blood samples were centrifuged at 3000 rpm for 15 minutes and the serum samples were stored in microtubes at −80°C until assayed.

Two-dimensional gel electrophoresis and image analysis

For two-dimensional gel electrophoresis (2-DE), 200 μg of serum sample was added to 250 mL of rehydration solution to become a test solution. Rehydration solution consisted of 8M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% immobilized pH gradient (IPG) buffer (Amersham Biosciences, Piscataway, NJ, USA), 40mM dithiothreitol (DTT), and 0.002% bromophenol blue as tracking dye. First-dimensional isoelectric focusing (IEF) was carried out using an IPGphor system (Amersham Biosciences). Test solution (250 μL including the sample) was applied to each gel strip (Immobiline DryStrip gels 13 cm, pH 4.0–7.0; Amersham Biosciences). IEF conditions were 16 hours at 30 V, then 1 hour at 500 V, 1 hour at 1000 V, and 4 hours at 8000 V. Prior to the second-dimension separation, gels were equilibrated. The sodium dodecyl sulfate (SDS) equilibration buffer contained 50mM Tris-HCl, 6M urea, 30.45% glycerol, 2% w/v SDS, and 0.002% bromophenol blue as tracking dye. First equilibration was performed by adding 100 mg DTT to 10 mL of SDS equilibration buffer for 15 minutes. Then, 250 mg iodoacetamide was added to 10 mL of SDS equilibration buffer for 15 minutes to achieve second equilibration. Protein separation by size in the 2-DE was performed in 1.5-mm-thick linear 10% polyacrylamide gels (18 cm × 16 cm) at 10 mA/gel for 20 minutes, and then at 25 mA/gel until the tracking dye reached the anode. In all experiments, the 2-DE was performed immediately after IEF. After SDS polyacrylamide gel electrophoresis, the proteins were detected with silver staining. The gel was scanned using a GS710 imaging densitometer (Amersham Biosciences) and analyzed using Image Master 5.0 software (Amersham Biosciences). Image Master 5.0 software was used to locate and measure protein spots and compare spots between gels. Spot measurements were normalized by expressing the optical density of a spot as a percentage of the sum of the optical densities for all spots detected on the gel.

In-gel proteolytic digestion

The spots were excised from gels using a pipet tip of large orifice, sliced into 0.6-mL micro centrifuge tubes and then washed three times with MilliQ water. The gel pieces were then destained with 30mM potassium ferricyanide and 100mM sodium thiosulfate and washed three times with 200 μL of a solution containing 50mM ammonium bicarbonate/100% acetonitrile (3:2). The gel pieces were then shrunk in 100% acetonitrile followed by drying under vacuum in an Eppendorf Concentrator 5301 (Hambury, Germany) at 30°C. The desiccated gel pieces were rehydrated with 3 μL of freshly prepared enzyme solution (20 ng/μL) containing sequencing grade modified trypsin in 25mM ammonium bicarbonate and incubated at 4°C for 1 hour. To keep the gel wet overnight at 37°C, 3 μL of 25mM ammonium bicarbonate was added. After 16 hours, 3 μL of solution in the tube was added to 2 μL of 100% acetonitrile and 1% trifluoracetic acid (TFA) and then sonicated for 10 minutes. Then, the supernatant was recovered. The acetonitrile/acid step was repeated, and the supernatants pooled. The peptide mixture was prepared for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis.

MALDI-TOF/MS analysis

Samples of freshly prepared matrix solution containing 1.5 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA), 0.1% TFA, 80% ceric ammonium nitrate (CAN), and 20% MilliQ water were prepared for MALDI-TOF/MS analysis. The peptide calibration standard II mixture allows calibrations (5 × 250 calibration points) and testing of MALDI-TOF mass spectrometers in a mass range between approximately 700 Da and 4000 Da. The standard calibration mixture contains nine standard peptides: bradykinin 1–7, angiotensin II, angiotensin I, substance P, bombesin, renin substrate, adrenocorticotropic hormone (ACTH) clip 1–17, ACTH clip 18–39,

and somatostatin 28. A 10-fold dilution of the standard calibration mixture and peptide mixture were spotted onto a freshly cleaned anchor chip 600/384 sample plate and allowed to crystallize, then the matrix solution was added to the sample plate and left to dry at room temperature. Peptide mixtures were analyzed using a matrix-assisted laser desorption tandem TOF mass spectrometer equipped with a 50-Hz 332-nm N_2 laser (Ultraflex II MALDI-TOF-TOF MS/MS, Bruker Daltonics, Bremen, Germany). Peptide matching and protein searches were performed automatically using MASCOT software (Matrix Sciences, London, UK). Proteins with a MASCOT score $\geq$65 and a peptide sequence coverage $\geq$20% matched peptides were considered as candidates for identification. For peptide identification, monoisotopic masses and a mass tolerance of 150 ppm were used. Only one missed trypsin cleavage was allowed. All extraneous peaks, such as trypsin autodigests, matrix, and keratin peaks were not considered for protein searches. The peptide masses were compared with the theoretical peptide masses of all available proteins from *Homo sapiens* using the SwissProt and National Center for Biotechnology Information (NCBInr) databases. A p value < 0.05 identified by the software was used as the criterion for affirmative protein identification.

Validation of elevated serum proteins in proteomic display by immunoassays

The serum levels of α1-antitrypsin, α1-microglobulin, clusterin, and haptoglobin (Hp) of the control group (additional 41 preeclamptic and 50 healthy pregnant women) were measured using enzyme-linked immunoassay (ELISA) for validation. The serum levels of α1-antitrypsin and Hp were measured by the BN system (Dade Behring, Siemens Healthcare, Erlangen, Germany). The serum levels of α1-microglobulin and clusterin were measured by an ELISA kit (BioVendor Laboratory Medicine, Inc., Modrice, Czech Republic).

Statistical analysis

Expressed as mean ± standard deviation, hematological parameters were compared between patient and control groups using the paired Student t test. A p value of < 0.05 was considered significant. Statistical analyses for the study were performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Demographic data between preeclampsia and normal pregnant women

The preliminary results of analysis of demographic data are shown in Table 1. The mean maternal age and gestational age (GA) of the study participants in both groups were 33.2 ± 3.2 years versus 33.1 ± 6.1years and 30.6 ± 1.3 weeks versus 32.7 ± 2.8 weeks, respectively, showing no significant difference. In the validation group, the mean maternal age and GA were 31.6 ± 4.3 years versus 32.8 ± 5.0 years and 34.9 ± 4.3 weeks versus 34.3 ± 4.2 weeks, respectively. However, pregnant women with preeclampsia had significantly higher mean systolic blood pressure (180.7 ± 11.6 mmHg vs. 125 ± 9.4 mmHg in screened participants and 172.2 ± 16.7 mmHg vs. 119.5 ± 9.9 mmHg in validated participants) and diastolic blood pressure (112.3 ± 5.7 mmHg vs. 72.2 ± 9.7 mmHg in screened participants and 107.5 ± 7.8 mmHg vs. 68.9 ± 10.42 mmHg in validated participants; $p < 0.001$), and lower mean fetal birth weight (1620 ± 675.5 g vs. 3041.4 ± 99.5 g in screened participants and 2012.4 ± 1035.6 g vs. 3187.4 ± 375.3 g in validated participants; $p < 0.005$).

Protein profiles of sera from screening group with preeclamptic and healthy pregnant women

A representative serum protein profile of a preeclampsia patient was displayed by 2-DE and silver stain as shown in Figure 1; the protein spots identified from silver stained gels were compared using the Image Master 5.0 software. There were several protein spots differentially showing upregulation and downregulation. However, only 10 protein spots were found to be more than twofold upregulated in the sera of preeclampsia patients (Figure 1).

Identification of differentially upregulated proteins

These 10 upregulated protein spots were trypsin-digested and then submitted to MALDI-TOF-MS for protein identification. With the use of MASCOT search software for protein identification, the results showed that all 10 upregulated protein spots had the peptide mass fingerprints (PMF) matching up to four corresponding proteins in the SwissProt (SWISS 2DPAGE) and NCBInr databases, which were α1-antitrypsin, α1-microglobulin, clusterin, and Hp. The details of the analytical results of these proteins are shown in Table 2.

Immunoassay of an independent series of serum samples

Additional immunoassay was performed to compare the serum levels of α1-antitrypsin, α1-microglobulin, clusterin, and Hp between the study groups of preeclampsia patients and normal pregnant women. The concentrations of α1-antitrypsin, α1-microglobulin, and clusterin increased linearly in standard serum diluted between 1/2400 and 1/300. Samples were measured after 1000-fold dilution. Sera of 41 preeclampsia patients and 50 normal pregnant women were measured and the levels of α1-antitrypsin, α1-microglobulin, clusterin, and Hp in both groups are shown in Figure 2. Mean serum levels of α1-antitrypsin, α1-microglobulin, and clusterin in the preeclamptic group were significantly higher than those in the control group (α1-antitrypsin 295.95 ± 50.94 mg/dL vs. 259.31 ± 33.90 mg/dL, $p = 0.02$; α1-microglobulin 0.029 ± 0.004 mg/mL vs. 0.020 ± 0.004 mg/mL, $p < 0.0001$; clusterin 77.6 ± 16.15 μg/dL vs. 67.6 ± 15.87 μg/dL, $p < 0.05$). Mean serum Hp levels were not significantly different between groups (83.32 ± 64.20 mg/dL vs. 89.11 ± 38.83 mg/dL, $p = 0.68$).

Discussion

In the present study, proteomic analyses were performed to find that α1-antitrypsin, α1-microglobulin, and clusterin were candidate proteins associated with preeclampsia in pregnant women. Although the underlying cause of preeclampsia remains unknown, a commonly accepted theory is that the disease progresses in two stages. First, an impaired cytotrophoblastic invasion of myometrial segments leads to the spiral arteries narrowing and therefore the blood supply to the fetus is restricted. Inadequate perfusion of the placenta results in local ischemia and oxidative stress with formation of free radicals and reactive oxygen species (ROS). All these compounds are toxic because they can cause oxidative damage to DNA, matrix molecules, cell membranes, and other tissue components. The second stage is the maternal response to oxidative stress, causing the wide spread of endothelial dysfunction which characterizes preeclampsia [10].

Alpha 1-antitrypsin is an “acute-phase” protein with the primary function to protect tissues against the proteolytic enzymes released after an inflammatory process [11]. As an “acute-phase” protein, the plasma α1-antitrypsin concentration can increase in response to stimuli such as infection or tissue trauma. Together

Table 1
Participant characteristics in the present study.

	Screened participants		p[a]	Validated participants		p[a]
	Normal pregnant women (n = 8)	Severe preeclamptic women (n = 8)		Normal pregnant women (n = 50)	Severe preeclamptic women (n = 41)	
Age (y)	33.2 ± 3.2	33.1 ± 6.1	NS	31.6 ± 4.3	32.8 ± 5.0	NS
GA (wk)	30.6 ± 1.3	32.7 ± 2.8	NS	34.9 ± 4.3	34.3 ± 4.2	NS
BW (g)	3041.4 ± 399.5	1620 ± 675.5	0.002	3187.4 ± 375.3	2012.4 ± 1035.6	0.004
SBP[b] (mmHg)	125 ± 9.4	180.7 ± 11.6	<0.001	119.5 ± 9.9	172.2 ± 16.7	<0.001
DBP[b] (mmHg)	72.2 ± 9.7	112.3 ± 5.7	<0.001	68.9 ± 10.42	107.5 ± 7.8	<0.001

Data are expressed as mean ± standard deviation.
BW = birth weight; DBP = diastolic blood pressure; GA = gestational age; NS = not significant; SBP = systolic blood pressure.
[a] For the difference between the preeclamptic and normal individuals, calculations were carried out by two-way analysis of variance.
[b] Data for SBP/DBP were collected upon the diagnosis of severe preeclampsia in the study group while in the control group the BP was measured soon after informed consent.

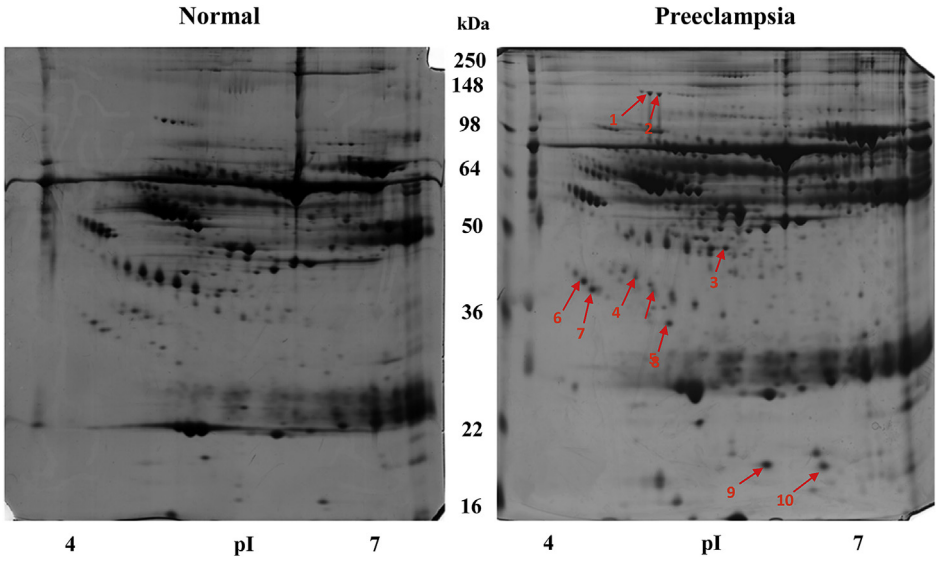

Figure 1. Representative serum protein profile patterns of normal and preeclamptic woman produced by two-dimensional gel electrophoresis and silver stain. The arrows indicate spots with significant differential expression, which were later identified to be α1-antitrypsin, α1-microglobulin, clusterin, and haptoglobin.

Table 2
Significant differentially expressed proteins identified in the serum of preeclamptic women.

Spot no.	NCBI entry	Protein name	pI/mass, kDa	Sequence coverage, MS/MSMS,%	MASCOT Score	No. of sequenced peptides	Functions[a]
1	gi1942629	α1-antitrypsin	5.4/44.3	42/7	76	2	a, e, f, o
2	gi1942629	α1-antitrypsin	5.4/44.3	37/8	70	2	a, e, f, o
3	gi4826762	Haptoglobin	8.5/45.2	35/5	132	2	a
4	gi32891795	Clusterin	6.7/48.7	32/6	74	1	b, c, e
5	gi32891795	Clusterin	6.7/48.7	30/4	81	1	b, c, e
6	gi1942629	α1-antitrypsin	5.4/44.3	45/7	73	2	a, e, f, o
7	gi1942629	α1-antitrypsin	5.4/44.3	45/7	75	2	a, e, f, o
8	gi 55957384	α1-microglobulin	5.9/39.9	39/16	128	3	b, c, e
9	gi4826762	Haptoglobin	8.5/45.2	46/10	122	2	a
10	gi4826762	Haptoglobin	8.5/45.2	40/8	133	2	a

MS = mass spectrometry; NCBI = National Center for Biotechnology Information.
[a] a = acute phase protein; b = transport scavenger proteins; c = immune modulation; e = protease inhibitor; f = coagulation; o = antiviral activity (based on the description on http://harvester.embl.de).

with Hp, fibrinogen, orosomucoid (1-acid glucoprotein), and ceruloplasmin, α1-antitrypsin is classified as one of the inflammation-sensitive plasma proteins (ISPs) that are used in clinical practice to measure the degree of inflammation and to differentiate between inflammatory diseases. Elevation of ISP levels was also associated with an increase in incidence of cardiovascular diseases [12,13]. Legge et al [14] showed that the serum levels of α1-antitrypsin in the severe hypertension patients were significantly higher when compared with the normotensive patients. However, the role of α1-antitrypsin in the pathogenesis of preeclampsia is

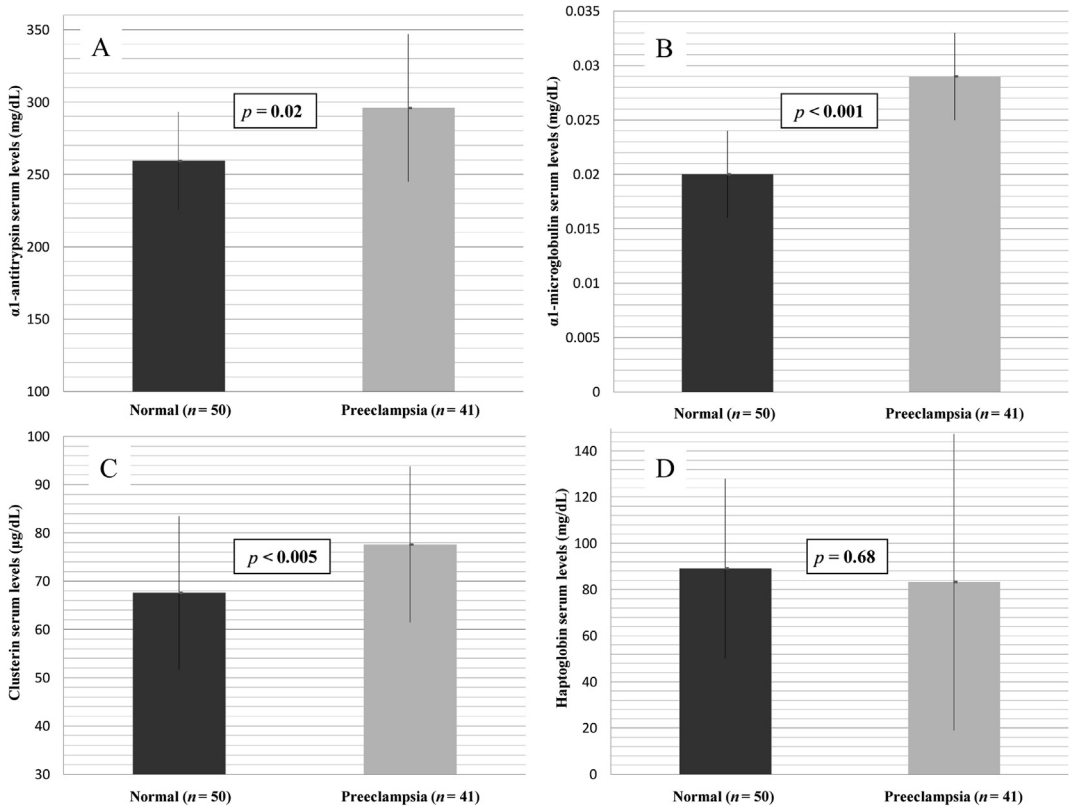

Figure 2. Immunoassays of four candidate proteins: α1-antitrypsin, α1-microglobulin, clusterin, and haptoglobin. The serum levels of (A) α1-antitrypsin, (B) α1-microglobulin, and (C) clusterin were increased significantly in preeclamptic women, whereas the serum levels of (D) haptoglobin were similar in both groups.

still unclear. Only one previous report showed that serum α1-antitrypsin levels increased as gestation progressed, however, the degree of increment was more significant in women with severe preeclampsia [15]. Serum α1-antitrypsin levels were demonstrated to be higher in women with severe preeclampsia similar to the results in the current study, however, further investigation is needed to evaluate whether this biomarker is feasible for the prediction of preeclampsia for women in their first trimester.

Human α1-microglobulin, a low-molecular-weight (26 kDa) glycosylated protein in plasma and tissue, is synthesized by liver cells and rapidly distributed via blood to the extravascular compartment [16]. In normal physiologic conditions it is freely filtered in the kidneys and completely reabsorbed in the proximal tubules. However, in the pathologic conditions such as preeclampsia, the load of high-molecular-weight protein increases which competes with reuptake of low-molecular-weight proteins such as α1-microglobulin, resulting in the significant leakage of this protein to the urine [17]. These data support the idea that the increased excretion of urinal α1-microglobulin in women with preeclampsia is a sign of endothelial dysfunction. Recent reports suggest that α1-microglobulin is involved in the defense against oxidative tissue damage (oxidative stress) and therefore its expression has been shown to be upregulated in response to ROS [18,19]. Increased serum α1-microglobulin levels of women in their first trimesters are believed to be a sign of subsequent development of preeclampsia [20]. Our results also demonstrated that the serum α1-microglobulin level was significantly higher in preeclampsia patients, reflecting its biological role against oxidative stress.

Clusterin (apolipoprotein J) is a 75–80-kDa disulfide-linked heterodimeric glycoprotein associated with apoptosis, oxidative stress, hypoxia, cancers, and neurological disorders [21]. Because of its biological characteristics, upregulation of clusterin expression might be associated with preeclampsia. Watanabe et al [22] were the first to reveal elevated serum levels of clusterin in patients with preeclampsia. Later, an immunohistochemical study also concluded that overexpression of clusterin in placental tissues might play an important role in the pathogenesis of preeclampsia [23]. We confirmed the significance of clusterin in preeclampsia by means of a proteomics technique and serum level analysis. However, the predictive role of serum clusterin levels for the women in their first trimester would actually be the most interesting part for future studies.

Hp is one of the five ISPs mentioned above; any inflammatory process may increase the plasma levels of Hp. In blood plasma, Hp binds to free hemoglobin (Hb) which is released from erythrocytes owing to hemolysis to counteract its oxidative activity and thus protects against cell damage [24,25]. The Hp–Hb complex is then removed by the reticuloendothelial system. This causes a decline in serum Hp levels. Owing to a genetic polymorphism, there are three different Hp genotypes in humans [25]. Previous studies showed that Hp with different genotypes would exhibit quite different biological activities due to their different binding affinities to Hb, reflecting the inconsistent results regarding the association between serum Hp levels and preeclampsia [26–28]. For example, Arinola et al [29] found that the serum Hp level was significantly raised in preeclampsia patients when compared with women having normal pregnancy. By contrast, Olsson et al [30] reported that the serum Hp concentrations of preeclampsia patients were significantly depressed. However, no significant difference of serum Hp levels was found between groups in our study.

In conclusion, we used a proteomic tool to identify three proteins: α1-antitrypsin, α1-microglobulin, and clusterin, which were

significantly associated with preeclampsia. These proteins are related to oxidative stress and inflammation, suggesting that oxidative stress, hypoxia, and inflammation are important events involved in the natural course of preeclampsia. Further studies are warranted to investigate these biomarkers in maternal serum alone or in combination as first-trimester predictors for this disease.

Conflicts of interest

The authors state that there are no conflicts of interest with regard to this article.

Acknowledgments

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