Vitamin D Binding Protein as screening biomarker candidate for late-onset preeclampsia without intrauterine growth restriction during 16 week of gestation.

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ABSTRACT

The aim of this investigation was conducted to proteomic analysis of plasma obtained from pregnant women who destined to develop late-onset preeclampsia without intrauterine growth restriction (IUGR) during 16th week of gestation. Plasma was obtained from primiparous women during 16th week of gestation. 2-DE proteomic analysis was done for plasma from 11 healthy pregnant women and 11 women who developed preeclampsia later. Using bioinformatic analysis with Progenesis SameSpots ver4.0 software and ANOVA test, expression of 2 spots were statistically different between two groups. In preeclamptic state, expression of both were decreased, one of these spots was vitamin D binding protein (p-value: 0.047), the other one will be discussed in another paper. According to results, we concluded that during 16th week of gestation, occurance of late-onset preeclampsia without IUGR is predictable. During this week, pathology of disease is present and may be the process of placental degeneration and impaired placentation are include in disease pathology.

Keywords: Preeclampsia; Proteomics; Biomarker Screening; Vitamin D Binding Protein**.**

INTRODUCTION

Preeclampsia (PE) is a complex multi-systemic syndrome characterized by hypertension and pregnancy-induced proteinuria. In all cases, the only effective treatment is delivery. Delivery leads in recovery that reflects the key role of placental factors in pathogenesis of disease. This disease is a leading cause of mothers mortality and morbidity [1-4] and throughout the world, affects 3-8% of pregnant women [4-6] and annually, affects more than 8million mothers and causes the death of more than 70000 mothers [7], generally, 10-15% of mothers mortality is directly related to preeclampsia and eclampsia, this disease affects 3-7% of nulliparous and 1-3% of multiparous pregnant women [4]. In Iran, the prevalence of severe preeclampsia is 3% and this disease causes 18.2% of mothers mortality and 12% of preterm deliveries [8]. The complications of this disease for mothers include: disseminated coagulopathy, acute renal failure, liver damage, pulmonary edema and convulsion (in eclampsia). May the infants of preeclamptic mothers be affected: one third has preterm birth, 20% of them have intrauterine growth restriction (IUGR) and prenatal death increases 3-10 times [9], oligohydramnious and placenta previa also occur [1].

Preeclampsia is associated with diseases that occur in future, including hypertension, stroke ischemic heart disease [4, 10, 11]. Risk factors of PE includes nulliparity [4, 9, 12], multiple pregnancies [1,4], previous PE [4, 9], mother's age> 35 years [4], to be born in Africa continent [4], mole pregnancies [4] and fetal congenital abnormalities [4], living in high altitudes [4], obesity [4, 13, 14], mother's co-existed disease related to pathophysilogy of PE, including

chronic hypertension, renal disease, diabetes (type 1 and 2), systemic lupus erythematous and thrombophilia [4, 15-17], immunologic interactions between parents [4], genetic predisposition [18].

Based on phenotype, PE can be classified into two subgroups of early-onset and late-onset or with and without IUGR [19-21]. In Iran, the prevalent phenotype is late-onset without IUGR [8].

According to above mentioned materials, it is beneficial to find screening biomarker(s) for PE, when the disease has not been established well or there is not any signs or symptoms of PE. The first step in biomarker determination is biomarker discovery and proteomics study is a way for this purpose. In this study, differences in the plasma proteome of healthy pregnant women and pregnant women destined to late-onset PE without IUGR was analyzed using 2 dimensional electrophoresis proteomic technique. This study was done during 16th week of gestation when there is no signs and symptoms of PE.

METHODS

Patients and phlebotomy

This case/control study was done in 16th week of gestation for primiparous women. In this study, blood samples of healthy pregnant women and pregnant women destined to late-onset PE without IUGR, was collected in the 16th week of pregnancy. Written consent was obtained before phlebotomy. The case group was pregnant women destined to late-onset PE without IUGR. The disease was diagnosed by an obstetrics and gynecologic specialist using the criteria of preeclampsia, the criteria of preeclampsia was diastolic blood pressure > 90 mmHg and systolic blood pressure > 140 mmHg in two times with 4 hours interval and one of these symptoms: proteinuria ≥ 0.3 g/24h or ≥ 2+ using dipstick or symptoms indicative of PE, including edema, epigaster pain and neurologic signs.

Neonates were declared IUGR whom birth weight was lower than 10th percentile for that gestational age.

The control group was healthy pregnant women BMI matched with case group, these women were normotensive and their children were not IUGR. Phlebotomy was done in 16th week of gestation. During venipuncture sampling, 4.5 ml EDTA blood was drawn [22-25]. Phlebotomy was done in Vali-asr Hospital which belongs to Tehran University of Medical Sciences.

Exclusion criteria was hypertension and proteinuria before 20th week of gestation, immunologic diseases, diabetes mellitus, multiple pregnancies, women with Body Mass Index (BMI) >30 on admission and women with IUGR pregnancy (is used for neonates born with birth weight lower than 10th percentile).

Plasma samples preparation

Blood collection was done by venipuncture rout and using needle with gauge 2°C (25-29), samples were collected in EDTA tubes and in less than 2 hours were centrifuged in 4ºC with 3000 rpm and 8 minute durance. The separated plasma was again centrifuged with above conditions. Five X 150 µl aliquots were prepared from each sample and the remainder was divided in 500 µl aliquots. Microtubes were immediately placed in -80ºC freezer. The entire process, from phlebotomy to placing plasma samples in freezer, was done in 4 hours [22-24, 30-34].

Using ProteoPrepImmunoDepletion20 Kit and according to its direction, aboundant proteins were depleted from plasma samples. These proteins were: Albumin, IgGs, Transferrin, Fibrinogen, Immunoglobulin A (IgAs), Alpha-2-Macroglobulin, IgMs(Immunoglobulin M), Alpha-1-Antitrypsin, Complement C3, C4 & C1q, Haptoglobin, Apolipoprotein A1, Apolipoprotein A2, Apolipoprotein B, Acid-1-Glycoprotein, Ceruloplasmin, IgDs (Immunoglobulin D), Prealbumin, Plasminogen.

Then using 2D-CleanUp Kit and according to its direction, remaining proteins were purified and precipitated and this precipitate was solved in rehydration solution. Then using 2D Quant Kit and according to its direction, protein concentration of rehydration solution was determined. Then 2-dimensional electrophoresis (2-DE) was done.

2-DE technique

For all samples, 2-DE analysis was done with triple repeat. First dimension was done using BIO RAD PROTEAN IEF Cell, 7 cm IPG with pH range 3-10. IsoElectric Focusing (IEF) was done in-gel and 30 µg protein was

loaded for each gel. For second dimension, Bio Rad Electrophoresis Tank was used. For this step, 12% SDS-PAGE gel was prepared. After electrophoresis completed, gels were stained using silver nitrate and scanned using GS-800 Calibrated Densitometer and documented.

Densitometric analysis of images and statistic evaluation of spots

For bioinformatic analysis, Progenesis SameSpots ver.4 software was used. For representing the proteome pattern, a reference gel (figure 1) was selected.

Then using this software, changes in expression of proteins in healthy and preeclamptic states were analyzed. The entire data was presented as ± 2 standard deviation (SD). Statistic significance was analyzed using t-test and p-values below 0.05 were considered statistically significant.

Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF MS) analysis

Using spot picker, protein spots were picked up and transferred to York University in England. Protein bands were destained using acetonitrile , and using dithiotreitol (DTT) and iodoacetamide were reduced and alkylated respectively and were trypsinized in 37ºC for 24 hours. Finally, the extracted peptides were sequenced and identified using MALDI-TOF MS.

RESULTS

The general information of studied population is mentioned in table 1. Statistical analysis for 33 gels of control group and 33 gels of case group was done. After selection of reference gel (figure 1) and statistical analysis, 303 spots were seen.

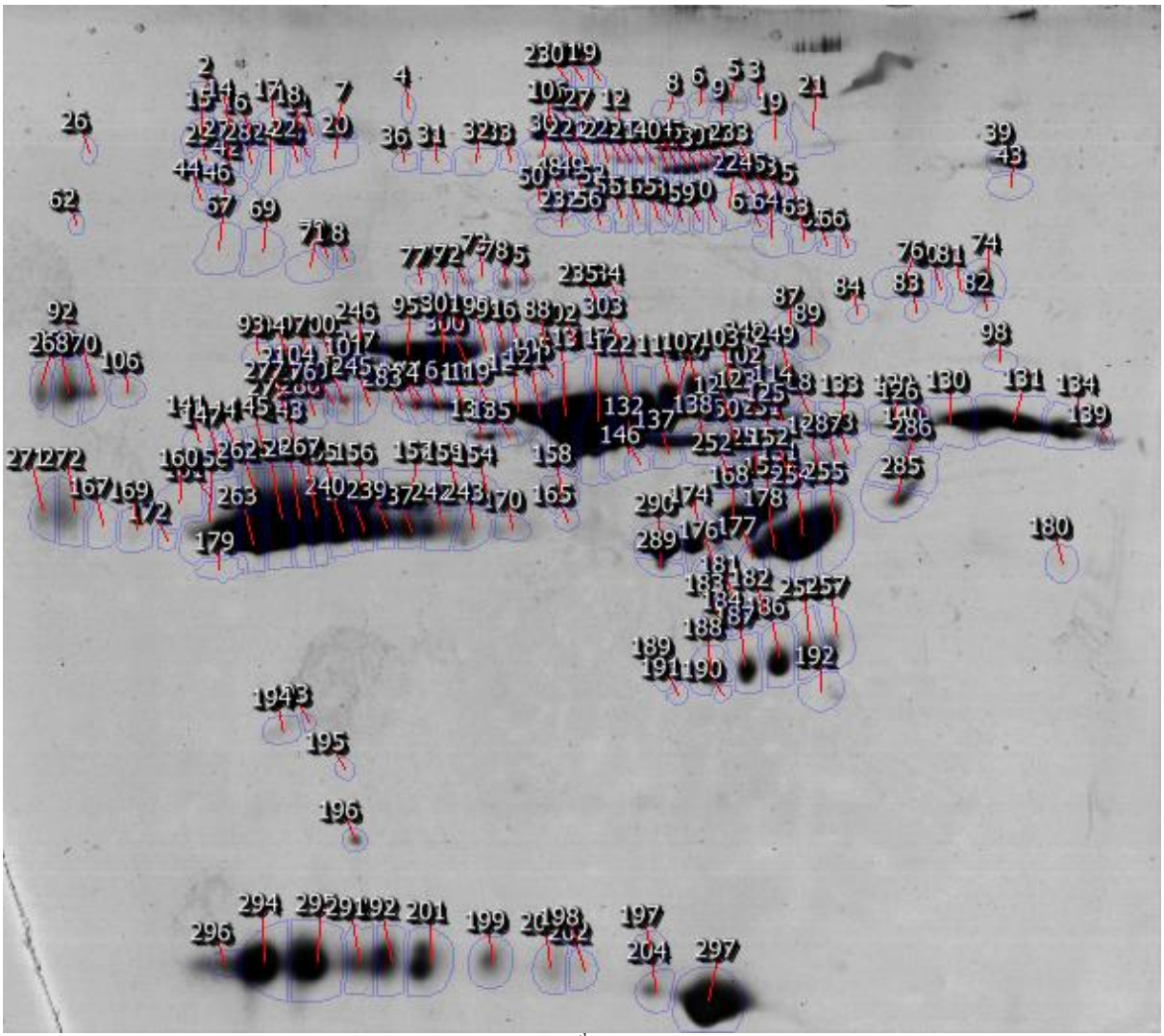
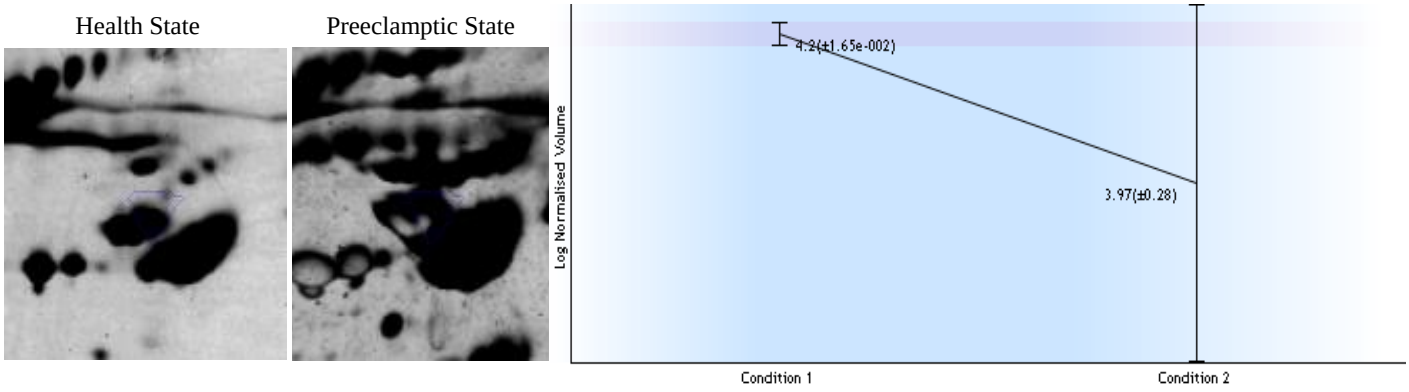


Figure 1. Reference 2-DE gel ofhealthy pregnant women 16th week plasma.303 spots were detected.



A B

Figure 2. Expression decrease plot of spot No. 153 and it's location. (A) location of spot No. 153 on gels of healthy and preeclamptic state. (B) Expression difference of VDBP in healthy (purple, 4.2±1.65) and preeclamptic (blue, 3.97±0.28) states. Results are represented as expression difference ± 2SD.

Table 1. Studied population general information using Mean (SD).

Maternal Results	Healthy	Preeclamptic without IUGR	p-value
Maternal Age (years)	28 (1.6)	29.56 (3.7)	0.357
Systolic Blood Pressure During 16 week of gestation (mm Hg)	103.33 (10)	110 (17)	0.399
Diastolic Blood Pressure During 16 week of gestation (mm Hg)	76.67 (5)	76.67 (5)	1
Maximum Systolic Blood Pressure During Pregnancy (mm Hg)	125 (5)	158.89 (8)	0.000
Maximum Diastolic Blood Pressure During Pregnancy (mm Hg)	70 (0)	90 (0)	
BMI ($kg.m^{-2}$)	24.050 (2)	25.144 (2.4)	0.379
Fetal Results			
Delivery Gestational Age (week)	39 (0)	39.11 (0.3)	0.435
Birth Weight (kg)	3.45 (0.12)	3.18 (0.3)	0.63

Table 2. Identified spot characteristics.

Spot No.	Healthy to Preeclamptic state	Protein	Theoretical molecular weight (kDa)	Theoretical pI	p-value
153	1.6	Vitamin D binding protein	51	5.33	0.047

Among these 303 spots, density of 2 spots was statistically different (significant) between the case and control groups. These 2 spots were No. 153 and 197. Density of both spots or expressions of these proteins were decreased in case group or PE condition. These 2 spots, 153 and 197, were determined using MALDI-TOF MS. The spot No. 153 (p-value: 0.047) was vitamin D-binding protein (table 2) and the other one will be revealed in another paper.

The location of this spot and change in its expression level is shown in figures 2. In part A of figure 2, a section of final gel for case and control groups is shown. In part B of figure 2, numbers on the plot represent amount of spot density or protein expression. For normalization and deleting highly varied data, the vertical axis is based on log. For normalization, spot density is divided by total density of the gel spots, so it has no unit.

DISCUSSION

This is the first investigation exclusively done for late-onset PE without IUGR during 16^{th} week of gestation. Using MALDI-TOF MS, 2 spots were identified. Because of the gestational week and phenotype studied, studied population, i.e., Iranian pregnant women, whom according to primipaternity theory, pregnancy pattern is similar to pregnancy pattern of most of the world, and also, the technique used, is done for the first time in the world.

Spot No. 153 was vitamin D binding protein (DBP or VDBP, Gc-globulin, Group-Specific Component) and serum amyloid P component. Vitamin D binding protein, belongs to serum

albumin family, this family includes serum albumin, afamin, α-fetoprotein and vitamin D binding protein. This protein also migrates as an α_1-globulin. This multifunctional protein binds and transports vitamin D and its metabolites, endotoxin and fatty acids in plasma and by binding to actin monomers, prevents its polymerization, this complex is rapidly cleared from bloodstream and therefore, the thrombotic effects of actin will be blocked [35-37]. 85-90% of plasma vitamin D and its metabolites is bound to VDBP [38, 39] and therefore slows its metabolism [40]. Its molecular weight and length are 51243 Da and 458 amino acids, respectively. Its plasma concentration is 20-55 mg/dl and its plasma half-life is approximately 2.5 days [41,42]. This protein is mainly synthesized in liver and is the main reservoir of vitamin D sterols. This plasma protein also presents in amniotic fluid [43, 44]. Also, expression of this protein on the surface of placental trophoblasts has been reported [45]. Three electrophoretic variants of this protein include: Gc2, Gc1s and Gc1f. Increased levels of this protein occurs in the third trimester of pregnancy and in patients consuming estrogen oral contraceptives. Estrogens increase VDBP plasma levels, therefore plasma concentration of this protein increases during pregnancy [46, 47]. Also, during normal pregnancy, cell-free actin levels and therefore, actin-VDBP complex levels are increased [48]. Decreased amount of this protein occurs in severe liver disease, protein-losing syndromes and trauma patients developed organ dysfunction and sepsis. Gc perhaps is important in bone formation and for immune system and presumably acts as a co-chemotactic factor in facilitating chemotaxis of neutrophils and monocytes in inflammation. This protein is also an acute phase reactant, although it is controversial [49, 50].

In this study, there is a 1.6 fold decrease of VDBP expression in case group or preeclamptic state. In a nested case-control study, blood levels of VDBP were measured. In this study, sampling was done during first trimester and was not done for a specific phenotype of PE. The levels of VDBP were similar in case and control group [51]. In another study, levels of actin-free VDBP (AFVDBP) were measured for early and late-onset PE, and these levels were not increased during 2nd and 3rd trimester of PE pregnancies but increased during the first trimester of PE pregnancies, presumably due to inflammatory process normally occurs during pregnancy [52]. In another study, sera of pregnant women with severe preeclampsia and healthy pregnant women were analyzed using 1D Gel-LC-MS/MS, phlebotomy was done during disease presentation. 51 proteins were differentially expressed, among them; VDBP was up-regulated [53]. Results of these three studies were controversial. However, in another study, auto-antibodies against two placental proteins has been reported: annexin A1 and VDBP. Subjects were Iranian pregnant women and study was done for late-onset PE [54]. Decreased VDBP in plasma of case group or preeclamptic patients may indicate increased placental tissue damage, because the protein is present in placental tissue. This damage may be a result of auto-immune response. Presence of VDBP polymorphisms may support this idea. According to primipaternity theory of preeclampsia, mother produces antibodies against father's antigens expressed in placental tissue. This phenomenon occurs in developing countries in which sexual intercourse between couples before marriage is rare, therefore, mother's tolerance against father's antigens is low [55, 56]. Our study and Gharesi-Fard study, both was performed on Iranian pregnant women with above mentioned pattern for marriage. There is a convergence between our study and Gharesi-Fard et al. study in two aspects: the protein involved in disease, i.e., VDBP, and the population studied, i.e., Iranian pregnant women. This may be indicative of culture effect on pregnancy patterns [55, 56] and it should be considered in study design. However, may be autoantibodies attach to VDBP and promote its clearance from bloodstream. According to above mentioned material, VDBP is a placental tissue protein, and there is a defect in placentation in PE pathogenesis, therefore, there is a consistence between decrease in VDBP and PE. Another point is that vitamin D deficiency is a risk factor for PE and hypertensive conditions [57, 58] and theoretically, decrease in VDBP is associated with decrease in vitamin D, i.e., the reservoir of vitamin D decreases [57, 59].

According to this point, decrease in VDBP expression is consistent with PE pathology.

CONCLUSION

In this study, first parity average age of case and control group reflects the limitation of pregnancy opportunity for this population that represents the importance of pregnancy care, of which prediction and diagnosis of diseases are its important aspects. According to results, PE pathogenesis is present in 16^{th} week of gestation; therefore PE can be predicted or may be prevented. In this period, placental tissue damage may be present; therefore tissue damage markers may be helpful for prediction of PE. Also immunity against father's antigens may be a cause of disease that implies the effect of culture on pregnancy pattern. Altered vitamin D metabolism may be a result of PE pathogenesis rather than a cause. Generally, it seems that a complex of above mentioned causes is involved in disease physiopathology and this marker is specific enough for PE prediction. Further investigation should be done for screening biomarker determination for PE.

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