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Screening Preeclamptic Cord Plasma for Proteins Associated with Decreased Breast Cancer Susceptibility

Hoi Pang Low, Ashutosh Tiwari, Jagadeesh Janjanam, Li Qiu, Chien-I Chang, William C. Strohsnitter, Errol R. Norwitz, Sun W. Tam, James E. Evans, Karin M. Green, Joao A. Paulo, Mats Lambe, Chung-Cheng Hsieh

Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA
Department of Chemistry, Michigan Technological University, Houghton, MI 49931, USA
Department of Obstetrics and Gynecology, Tufts Medical Center, Boston, MA 02111, USA
Proteomics and Mass Spectrometry Facility, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01545, USA
Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
Department of Medical Epidemiology and Biostatistics, Karolinska Institute, SE171 77 Stockholm, Sweden

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Abstract
Preeclampsia, a complication of pregnancy characterized by hypertension and proteinuria, has been found to reduce the subsequent risk for breast cancer in female offspring. As this protective effect could be due to exposure to preeclampsia-specific proteins during intrauterine life, the proteomic profiles of umbilical cord blood plasma between preeclamptic and normotensive pregnancies were compared. Umbilical cord plasma samples, depleted of 14 abundant proteins, were subjected to proteomic analysis using the quantitative method of nanoACQUITY ultra performance liquid chromatography–mass spectrometry with elevated energy mode of acquisition (NanoUPLC-MSE). Sixty-nine differentially expressed proteins were identified, of which 15 and 6 proteins were only detected in preeclamptic and normotensive pregnancies, respectively.

* Corresponding author.
E-mail: chung-cheng.hsieh@umassmed.edu (Hsieh CC).

1 Department of Chemistry and Biochemistry, Clark University, Worcester, MA 01610, USA.
2 Current address: Roskamp Institute Inc., Sarasota, FL 34243, USA.

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Introduction

Breast cancer is the most common cancer that affects women in the United States. However, there are few effective interventions to lower breast cancer risk. Epidemiological and experimental studies suggest that the intrauterine environment influences breast cancer risk in the offspring [1]. Among the maternal, gestational and newborn characteristics, strong inverse associations with breast cancer risk have been found for prenatal exposure to preeclampsia [1–3]. In a large population-based study that examined prenatal factors and adult risk of breast cancer in a cohort of Swedish women, women born from a preeclamptic (PE) pregnancy had a 59% reduction in breast cancer risk (relative risk 0.41, 95% confidence interval 0.22–0.79) [2]. Additionally, a meta-analysis has shown that among all women, PE-born offspring had a 52% lowered risk of breast cancer (relative risk 0.48, 95% confidence interval 0.30–0.78) [3]. This potential protective effect is as strong as that associated with tamoxifen/raloxifene intervention [4] or physical exercise [5].

Preeclampsia is a complication of 5–8% of all pregnancies in the United States that brings significant morbidity and mortality for both mother and baby [6,7]. The hallmark characteristics of preeclampsia are a new-onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive (N) woman. Although the underlying causes of the syndrome remain obscure, abnormal placenta development has been implicated [8]. There is also no compelling explanation for the inverse association between preeclampsia and subsequent breast cancer risk in the offspring, but a possible influence of the intrauterine environment has been implicated. Although preeclampsia has been associated with higher levels of umbilical cord alpha-fetoprotein (AFP) [9], insulin-like growth factor binding protein-1 (IGFBP-1) [10], leptin [11], triglycerides [12] and homocysteine [13], none of these efforts may play a role in reducing breast cancer risk. Of the 15 proteins with up-regulated expression in PE plasma (Table S1), of which 15 (Table 1) and 6 (Table 2) proteins were exclusive to PE and N pregnancies, respectively. Additionally, compared to N pregnancy, expression of 8 proteins was up-regulated more than twofold and expression of 7 proteins was down-regulated more than 1.5 fold in umbilical cord blood plasma from PE pregnancy.

Results

Identification of differentially expressed proteins between PE and N umbilical cord plasma

Screening of umbilical cord blood plasma samples by Nano-UPLC-MSE (as outlined in Figure 1) identified a total of 69 proteins (Table S1), of which 15 (Table 1) and 6 (Table 2) proteins were exclusive to PE and N pregnancies, respectively (Figure 2). An additional 48 proteins (Table S2) were detected in umbilical cord blood plasma from both PE and N pregnancies (Figure 2).

Identification of proteins with up-regulated expression in PE plasma

As daughters of PE births have a reduced risk for breast cancer [2,3], we hypothesize that proteins exclusive to umbilical cord plasma from PE pregnancies or up-regulated in PE plasma may play a role in reducing breast cancer risk. Of the 15 proteins that were exclusive to PE plasma (i.e., absent in N plasma), hemoglobin subunit alpha (P69905) was present in the largest quantity of approximately 190 fmol and with a mean spectral count of 4.7 (Table 1). Additionally, serum amyloid

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**Figure 1 Experimental workflow**
P-component (SAP; P02743), fibronectin (P02751), keratin type II cytoskeletal 6A or keratin 6A (P02538), fibrinogen alpha chain (P02671) and inter-alpha-trypsin inhibitor heavy chain H3 (Q06033) were present in amounts >20 fmol (Table 1).

To identify proteins that were up-regulated in PE relative to N plasma, we compared the mean quantities of each of the 48 proteins that were detected in both groups and identified 13 proteins that were up-regulated in PE plasma with a fold change \( \geq 2.0 \) when compared to that of N pregnancies (Table S1). An additional filtering criterion using a Bayes factor value >1 was applied and further reduced the number of proteins from 13 to 8 (Table 3). These proteins include complement C5 (C5; P01031), keratin type I cytoskeletal 10 or keratin 10 (P13645) and pigment epithelium-derived factor or serpin peptidase inhibitor clade F member 1 (PEDF or SERPINF1; P36955) that have a fold change >3 and a Bayes factor >5. Of note, this proteomic screen also detected the previously reported AFP (P02771) [9] (Table 3).

Identification of proteins with up-regulated expression in PE plasma

Conversely, proteins with expression that was only detected or up-regulated in umbilical cord blood plasma from N pregnancies (Table 2) when compared to PE pregnancies (Table 4) are candidate proteins for increased breast cancer risk. These include proteins that were absent in PE plasma such as immunoglobulin (Ig) kappa chain V-III region SIE (P01620), Ig heavy chain V-III region BRO (P01766), prothrombin (P00734), apolipoprotein A-I (P02647), complement component C6 (P03952) and POTE ankyrin domain family member E (Q6S8J3) (Table 2). Of the 48 proteins that were detected in both PE and N plasma, expression of 13 proteins was up-regulated in N plasma with a fold change \( \geq 1.5 \) when compared to PE plasma (Table S1). Applying an additional filter criterion of identifying proteins with a Bayes factor >1
This observation was supported by KEGG inhibitor or serpin peptidase inhibitor clade G member 1 C7, complement factor B (Factor B) and plasma protease C1 (Table 3) PE plasma (n = 23), and similarly proteins with expression absent (Table 2) or down-regulated (Table 4) in PE plasma, which display structural functions. These proteins include collagen alpha-1(I) chain, fibronectin, keratin 14, keratin 6A and keratin 10), which, except for fibronectin, played a role in ectoderm/epidermis development (Tables S3 and S4). Additionally, molecular functions that are unique to proteins with exclusive or up-regulated expression in PE plasma include serine-type endopeptidase activity (3 proteins: C1r, plasma kallikrein B and Factor B) and oxygen transporter activity (2 proteins: hemoglobin subunit alpha and hemoglobin subunit gamma 2) (Table S3).

The set of proteins with exclusive or up-regulated expression in PE plasma include serine-type endopeptidase activity (3 proteins: C1r, plasma kallikrein B and Factor B) and oxygen transporter activity (2 proteins: hemoglobin subunit alpha and hemoglobin subunit gamma 2) (Table S3).
complement activation (Tables S4 and S6). Consistent with the presence of these proteins in plasma, the majority of these proteins were clustered to the extracellular region or extracellular space in the cellular component analysis (Tables S7 and S8).

Validation of protein expression by Western blot analysis

To validate our findings, several proteins were selected for western blot analysis (Figure 3). For representative proteins with up-regulated expression in PE plasma, we chose 2 proteins with high fold change and Bayes factor values, i.e., C5 and PEDF. We also examined the expression of AFP, which has been previously reported in PE plasma [9] and therefore would serve as a positive control in our analysis. Factor H was selected as the representative protein with up-regulated expression in N plasma. The loading of equal amounts (10 μg) of total protein for each of the plasma samples analyzed was confirmed by Coomassie blue-stained SDS–PAGE gel visualization (Figure 3A) before the transfer to polyvinylidene difluoride (PVDF) membranes. First, the quantification feature of the NanoUPLC-MSE method was validated as the densitometric intensities for the bands of each sample immuno-positive for the AFP antibody corresponded significantly with the normalized fmol quantities determined from the bioinformatic analysis (Figure 3B; $R^2 = 0.91, P = 0.003$ significant at **P < 0.01, linear regression analysis). Second, although there were substantial variations within each group, which is not uncommon in human plasma samples, the mean densitometric intensities of the bands for AFP and C5 were higher, but not significantly ($P = 0.6$ and 0.3, respectively, t-test), for PE samples when compared to N samples (Figure 3B and C). However, the mean band intensity for PEDF was significantly ($P = 0.006$ significant at **P < 0.01, t-test) higher in PE than N samples (Figure 3C). Conversely, the mean densitometric band intensity for Factor H was higher, but not significantly ($P = 0.2$, t-test), in N plasma when compared to that of PE plasma (Figure 3C). These initial observations of protein expression are in accordance with the findings of the proteomic screen although confirmation of more protein expression using more samples is needed.

Discussion

To explore the mechanisms by which a PE intrauterine environment might confer protection against the subsequent development of breast cancer in female offspring [2,3,16], we
employed the quantitative proteomic technology of NanoUPLC-MS², a unique method to compare the relative abundance of protein between samples based on their absolute quantity from LC-MS data of tryptic peptides. In this method, the average MS signal response for the three most intense tryptic peptides per mole of protein has been found to be constant within a coefficient of variation of less than ± 10% [15]. Hence, given an internal standard, the absolute quantity of each protein could be determined in a complex mixture without the cumbersome generation of a calibration-response curve for specific polypeptides using numerous external reference peptides or in the use of radiolabeled amino acids.

Notably, the screen detected the previously reported AFP in the umbilical cord blood plasma of PE pregnancies [9]. In epidemiological studies, elevated levels of AFP, a glycoprotein produced by the fetal liver and yolk sac with known anti-estrogenic properties, have been independently associated with a reduced breast cancer risk [17] as well as indirectly associated with a reduction in breast cancer based on its association with low birth weight [18], ethnicity [19], multiple births [20] and hypertensive disorders of pregnancy [9,21]. In addition to AFP, the set of proteins identified to be up-regulated in PE samples can also be linked to anti-cancer effects. For example, PEDF or SERPINF1, a 50 kDa glycoprotein that was first discovered as a factor secreted by retinal pigment epithelial cells with neuronal differentiation and neurotrophic capabilities [22], has been reported to have additional roles in neuroprotection, anti-angiogenesis and anti-tumorigenesis [23]. Indeed, both AFP [14] and PEDF [24] have been explored for their roles as anti-cancer agents. To our knowledge, this is the first report of a significant up-regulation of PEDF in PE plasma.

Besides playing a role in the pathological events of preeclampsia [25,26], the identification of components of the complement system from PE umbilical cord plasma also points to a potential role of an anti-tumor immune response. It is conceivable that complement activation might be able to eliminate cancer cells and thus contribute to a lower cancer risk. For example, SAP, a member of the pentraxin family that is found exclusively in our PE plasma samples, may function to modulate host-immunodefence [37] and insulinomas [38]. It has been suggested that alpha-1-antitrypsin may function to modulate host-immunodefence mechanisms in favor of tumor cells and promote blood circulation within tumor tissues for tumor development [36,37]. Hence, the down-regulation of alpha-1-antitrypsin expression in PE plasma is again consistent with a model of decreased cancer risk by preeclampsia.

Our screen identified 13 potential high-risk breast cancer proteins whose expression is decreased in PE or up-regulated in N pregnancies (Tables 2 and 4). Previously reported high-risk proteins include insulin-like growth factor-1 (IGF-1), 1,25-dihydroxyvitamin D, insulin-like growth factor binding protein-3 (IGFBP-3), ghrelin, high-density lipoprotein (HDL)-cholesterol and Apo A-1 [10–12]. Hence, other than Apo A-1, which was also picked up by our screen, we report here 12 potential novel proteins that are postulated to be associated with a higher prenatal risk of breast cancer due to fetal programming. As an example, the expression of lumican, an abundant small leucine-rich proteoglycan in breast stroma, was significantly correlated with mammographic density, an important risk factor for breast cancer [39]. Future research is needed to determine the relevance of these proteins in relation to breast cancer risk and novel therapies can be designed to silence such potential high-risk molecules.

Current cancer research has focused mainly on the analysis of pathological samples from patients whereby proteins identified would be limited to those produced after disease formation. Consistent with a fetal-origin-of-disease hypothesis, we look for prenatal factors in the intrauterine environment using umbilical cord blood plasma samples. Although our findings support research for the analysis of the prenatal environment using umbilical cord blood to identify factors that mediate a protective effect toward disease outcomes, such as that of cancer, it must be pointed out that not all biomarkers associated with preeclampsia play a role in reducing breast cancer risk and it is unclear how these molecules, if they do play such a role, mediate their effects from the in utero environment into adult life. Speculatively, such molecules could have brought about genetic and/or epigenetic changes to the stem cells of the developing embryo and these ‘altered’ cells become more resistant or susceptible to disease onset in later life [40].

In summary, this pilot study identified candidate proteins in umbilical cord blood plasma that might play a role in the prevention of breast cancer later in life. The validity of our screen for prenatal factors is supported by the identification of several
PE proteins that have anti-estrogenic, anti-angiogenic and anti-tumorigenic activities, properties that are consistent with a lowered risk of a hormone-responsive cancer such as breast cancer. However, given the small sample size, conclusions are preliminary. Upon validation with a larger sample size and testing in animal models, such preeclampsia-associated proteins can serve as biomarkers for identifying individuals with different susceptibilities to breast cancer, give us insight into the potential prenatal mechanisms by which preeclampsia mediates its protective effect against breast cancer in female offspring, and lead to potential applications in cancer surveillance and prevention.

Materials and methods

Experimental workflow

The experimental workflow is summarized in Figure 1. Briefly, subjects with PE and N pregnancies were recruited and, at the time of birth, their umbilical cord blood was collected, processed and the plasma stored at −80 °C prior to analysis. The plasma was immuno-depleted of 14 abundant proteins with the Sepro IgY14 column and processed for analysis by the proteomic technology of NanoUPLC-MS<sup>E</sup>. Selected differentially expressed proteins were validated by western blot analysis.

Subject recruitment

The study protocol was approved by the institutional review boards of the University of Massachusetts Medical School and Tufts Medical Center, and informed consent was obtained from all participating subjects. Study subjects were recruited among pregnant women who delivered at Tufts Medical Center, Boston, MA. All subjects were 18 years or older with a singleton pregnancy, HIV- and hepatitis B-negative, and the fetus was free of anomalies by ultrasound examination. Preeclampsia and its severity were diagnosed using standard clinical criteria [7]. Briefly, preeclampsia was diagnosed by the presence of a persistently elevated systolic blood pressure (BP) ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg after 20 weeks of gestation along with a 24-h urinary protein output of ≥ 300 mg or ≥ 1+ proteinuria on dipstick.

Umbilical cord blood collection and processing

Infants were delivered according to standard obstetrical practices. Umbilical cord blood was collected from the umbilical vein using a blood collection bag containing 35 ml of citrate-phosphate-dextrose anticoagulant (Fenwal, Lake Zurich, IL). Samples were centrifuged at 20 °C for 30 min at 400g within 24 h of collection. The top plasma layer was harvested into 2-ml cryovials and stored at −80 °C until less than 200 µl of concentrated solution remained. Protein concentration was determined by the Bradford method. The concentrated samples were prepared in 50 mM ammonium bicarbonate with 0.1% RapiGest. The proteins were then reduced in 5 mM dithiotheroethiol at 60 °C for 30 min and alkylated in 15 mM iodoacetamide by incubation in the dark at room temperature for 30 min. A tryptic digestion was performed at 30 °C overnight with 1.5 µg of trypsin (Promega, Madison, WI). The RapiGest was acid cleaved by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.5% at 37 °C for 45 min. The particulates were centrifuged down and the peptides in the supernatant were passed through a 0.22 µm Amicon Ultrafree-MC spin-filter (Millipore) before analysis by NanoUPLC-MS<sup>E</sup>.

NanoUPLC-MS<sup>E</sup> analysis

Samples were individually analyzed by the non-data dependent absolute protein quantification method described by Silva and colleagues [15]. Before each injection, the protein concentration of the sample was adjusted to 500 ng/µl with 0.1% TFA. A tryptic digest of yeast alcohol dehydrogenase (ADH; 200 fmol/µl; Waters, Milford, MA) was added to the digests as an internal standard for quantification. Nano-flow separations of the tryptic peptides were performed on a NanoUPLC system (Waters). The column temperature was maintained at 35 °C. Partial loop sample injections (2 µl) were performed in a random order with three analyses of each sample. The UPLC system was equilibrated with 5% acetonitrile (ACN)/0.1% formic acid (FA) (mobile phase A). Separation of injected tryptic peptides was achieved by application of a linear 60-min gradient from 3% to 90% ACN in 0.1% FA which was passed through both the trapping (SymmetryC18, 180 µm × 20 mm; Waters) and analytical columns. A lock mass solution (200 fmol/µl of Glu-fibrinopeptide; Sigma) in 30% ACN/0.1% FA in water was delivered via the auxiliary solvent pump at 300 nL/min into the reference sprayer of the NanoLockSpray source. Alternating high and low collision energy mass spectra of NanoUPLC eluates were acquired using a Q-TOF Premier mass spectrometer (Waters). Nanospray ionization was performed using uncoated, pulled fused silica emitters (New Objective, Woburn, MA) at a potential of 3.5 kV. The time-of-flight (TOF) analyzer was operated in the V mode with a typical mass resolution of 10,000. Alternating low and high collision energy scans were used to obtain low collision energy (4 eV) MS spectra and programmed high collision energy (ramped from 15 to 40 eV) MS<sup>E</sup> mixed product ion spectra, both acquired at 0.6 s/scan. The instrument was calibrated using 13 fragment ions from a high energy scan of the [M + 2H]<sup>2+</sup> ion from Glu-fibrinopeptide. To correct any shifts in masses that occurred during analyses, a single-point lock mass correction was performed at 30 s intervals using the [M + 2H]<sup>2+</sup> ion from Glu-fibrinopeptide (m/z 785.8426). The raw files acquired were processed and searched against the database of human proteins generated from the UniProtKB/Swiss-Prot protein sequence database downloaded on September 9, 2009 (www.expasy.org), using the ProteinLynx Global Server (PLGS) Identity<sup>E</sup> version 2.4 software (Waters).
Bioinformatic and data analysis

Scaffold

Scaffold (version 3.00.03, Proteome Software, Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95% probability as specified by the Peptide Prophet algorithm [41]. Protein identifications were accepted if they could be established at >99% probability as assigned by the Protein Prophet algorithm [42] and contained at least 3 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Fold change determination

Relative abundance of each protein was assessed by first converting intensity values to fmol amounts by normalization to 200 fmol ADH internal control added in each run. As the average MS signal of the three most intense peptides from each protein is proportional to the abundance of that protein [15], the total amount in fmol of all the proteins identified for each run was determined and the fmol amounts of each protein was then normalized so that the sum of each run was the same for all the runs. Mean values for all the runs of each sample and the mean value for each of the two groups of samples were calculated. Fold changes were determined by comparing the means of the two groups. A value > 2.0 or > 1.5 (based on the upper bound of the 95% confidence interval) was used as our significance threshold for proteins whose expression was up-regulated in PE and N pregnancies, respectively.

Spectral counting

Since spectral counts correlate with protein abundance [43], relative abundance of each protein was also assessed by normalization of spectral counts based on the total spectral counts of each run, as reported previously [44]. Normalized spectral counts were subjected to QSPEC analysis [45] to obtain Bayes factor and false discovery rate (FDR) values as additional filtering criteria.

Ontology and pathway analysis

Gene ontology analysis [46] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [47] were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Database (http://david.abcc.ncifcrf.gov/) interface [48] for the proteins that were unique or significantly up-regulated in the two groups. For gene ontology analysis, only molecular functions, biological processes and cellular components with $P < 0.05$ (as specified by DAVID) were considered.

Western blot analysis

Plasma samples (10 μg total protein/well) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A non-reducing SDS–PAGE was run for C5 detection [49]. Proteins were transferred to PVDF membranes and blocked with 5% skim milk powder (for C5, Factor H and PEDF) or 5% bovine serum albumin (for AFP) in tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T) for 2 h at room temperature. After washing with TBS-T, the appropriate blot was incubated overnight at 4 °C with one of the following antibodies: anti-human Factor H (goat, 1:2000; AF4779, R&D Systems, Minneapolis, MN), anti-human AFP antibodies (chicken, 1:2000; AF1369, R&D Systems), mouse anti-human C5 (mouse, 1:500; sc-70476, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-human PEDF (mouse, 1:500; sc-53921, Santa Cruz Biotechnology). After washing at room temperature, the blots were incubated for 2 h at room temperature with 1:5000 to 1:10,000 of an appropriate secondary antibody conjugated to horseradish peroxidase. The protein of interest was detected using the ECL Western Blotting Detection System (GE Healthcare Bio-Sciences/Amersham Biosciences, Piscataway, NJ) in a LAS-4000 Luminescent Image Analyzer (Fujifilm Life Sciences, Valhalla, NY). Densitometry was performed using the image analysis software Multi Gauge V3.0 (Fujifilm Life Sciences). Statistical significance was determined by $t$-test and linear regression analyses at a two-sided $P < 0.05$.

Authors’ contributions

HPL, ML and CCH participated in conception and design of the study, data analysis and interpretation, and manuscript writing. AT and JJ participated in the western blotting analysis and manuscript writing. LQ and CIC participated in sample processing and collection of data. WCS and ERN participated in subject recruitment, data analysis and manuscript writing. SWT, JEE, KMG and JAP participated in proteomic analysis, assembly of data, and manuscript writing. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gpb.2013.09.009.

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