Alteration in the Expression of Proteins in Unexplained Recurrent Pregnancy Loss Compared with in the Normal Placenta

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Abstract. The placenta is a unique pregnancy-related tissue and plays a key role in occurrence of unexplained recurrent pregnancy loss (URPL). Abnormal placentation might play a key role in occurrence of URPL. Therefore, the purpose of this study was to compare the human placental proteome between URPL placentas and normal placental matched for gestational week. Total placental proteins were extracted, and the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique was used for separation of the placental proteomes. Protein spots differentially expressed between URPL and normal placentas were selected and identified by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF) technique after being digested in the gel. Moreover, quantitative real-time PCR and Western blot techniques were used to confirm the differential expression mass results for some differentially expressed proteins. The results indicated that at least 19 protein spots were differentially expressed between URPL and normal placentas (P < 0.05), and twelve of them were successfully identified. While only two proteins were downregulated (calumenin and enolase 1), the remaining ten spots (actin gamma 1 propeptide, cathepsin D propeptide, heat shock protein gp96, tubulin beta, tubulin alpha 1, glutathione S-transferase, vitamin D binding protein, prohibitin, actin beta, apolipoprotein A-I) showed increased expression in URPL cases in comparison with normal placentas. Real-time PCR also confirmed the downregulation of calumenin and upregulation of prohibitin and apolipoprotein A-I at the mRNA levels. In conclusion, the results of the present study showed that alteration in the expression of proteins involved in proliferation and migration of endothelial cells as well as control of coagulation by these cells might play an important role in the pathogenesis of URPL.

Key words: Abortion, Placenta, Pregnancy, Proteomics, Recurrent pregnancy loss (RPL)

Recent pregnancy loss (RPL) is defined as at least two or three sequential abortions before the 20th week of gestation [1]. RPL occurs in 1% to 5% of all pregnancies. Despite the fact that several factors such as environmental and stress factors, chromosomal abnormalities, coagulation protein defects and, endocrine, anatomical and autoimmune disorders are involved in approximately 60% of RPLs, in the remaining 40% of the cases, the etiology of abortion is unknown and is classified as unexplained RPL (URPL) [2]. Since any abnormalities in implantation and placental development can lead to miscarriage [3], abnormal placentation could be considered a plausible hypothesis for URPL development. In fact during normal placentation, cytotrophoblast cells invade the uterus in a tumor-like manner and engraft to the maternal blood vessels. These cells mediate the modification within the maternal arteries and convert them to the uteroplacental arteries of pregnancy. Therefore, an endothelial dysfunction may represent the cause of many pregnancy-related disorders such as preeclampsia and RPL [4]. In this respect, endothelial dysfunction due to arrested invasion and excess cytotrophoblast proliferation has been considered a trigger of RPL [4]. The molecular events underlying this abnormality are not well understood. Therefore, study of proteome changes in the placenta of RPL patients will shed light on the molecular pathogenesis of RPL. Interestingly, only a few proteomic studies on placental tissue have been published, and most of them are related to preeclampsia [5, 6]. Moreover, in the single published paper in the field of recurrent pregnancy loss and proteomics, follicular fluid was used by Kim and co-workers as a substitute for the placenta [7]. They indicated that five proteins including complement component C3c, fibrinogen γ, antithrombin, angiotensinogen and hemopexin precursor are altered in follicular fluid from patients with RPL as compared with normal controls [7]. In a study by Liu et al., the proteome of placental villous tissues of six patients affected by early pregnancy loss were compared with those of six normal controls [8]. Twelve altered proteins were identified in their study, including the proteins involved in cell regulations and processes such as antioxidative defense, differentiation, cell...
proliferation, metabolism, apoptosis, transcription, and proteolysis [8]. Considering the lack of study on the whole placental proteome from the first trimester in human pregnancy and proteome changes during URPL, the aim of the present study was to compare the human placental proteome between URPL and normal late first trimester placentas. To achieve this goal, placental proteins from URPL and gestational week-matched normal cases in the late first trimester of pregnancy were extracted and subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). After staining, the gels were scanned, and the intensities of the spots were determined and compared between URPL and normal cases. Spots with statistically different expression were excised from the gels and identified by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF) technique after in-gel digestion.

Materials and Methods

Placental samples

Five URPL placentas and an equal number of normal gestational week-matched first trimester placentas were collected for this study. The mean age of the URPL and normal women were 30.4 and 29.8 years respectively. The research protocol was approved by the local ethics committee, and written informed consent for use of placentas in the study was obtained from all cases and controls.

URPL cases were diagnosed by a gynecologist based on clinical and laboratory tests. To select URPL patients and exclude RPL ones with known etiology from this study, all patients were evaluated for normal anatomical, hormonal and chromosomal criteria and the absence of infections (toxoplasma, cytomegalovirus, rubella, HIV, Chlamydia, hepatitis B and C), anti-thyroid antibody and anti-phospholipid antibodies (including lupus anticoagulant, anti-cardiolipin and β2-glycoprotein antibodies). URPL cases had at least three previous abortions before 20 weeks of gestation, while normal cases experienced at least two previous successful pregnancies. Normal placentas selected from late first trimester pregnant women who were referred to the legal abortion committee of Shiraz University of Medical Sciences due to indications for abortion (such as heart disease). The mean gestational age of the normal and URPL cases were not statistically different (15.4 ± 3 vs. 14.8 ± 2.6 weeks, respectively; P = 0.69).

After collecting placentas, five different areas of each placenta were punched, pooled and washed in cold normal saline to reduce and eliminate contaminating blood. The weight of each punch was about 500 mg. All placenta samples were stored in liquid nitrogen until extraction.

Protein extraction

Total protein from placental tissues was extracted as previously described [6]. After extraction, the protein concentration was detected with a 2-D Quant kit (Amersham, Little Chalfont, UK). All protein samples were stored at −70 C until performing the tests.

2D-PAGE

The first dimension of 2D-PAGE was done using two 18 cm linear precast immobilized pH gradient (IPG) strips (pH 4–7 and pH 3–10, Bio-Rad, USA) for each sample. In order to minimize the variation, normal and URPL protein extracts were run and stained simultaneously in a twin gel electrophoresis system (SCIE-PLAS, Cambridge, UK) for the second dimension as described previously [6].

Staining, statistical analysis and spot detection

The URPL and normal 2D gels were stained with Coomassie brilliant blue (CBB) [9] or silver nitrate [10]. A 2D ImageScanner and the ImageMaster 2D Platinum software (Pharmacia, Uppsala, Sweden) were used for scanning and spot analyzing, respectively. To avoid variation in analysis, the same parameters were used for each gel. A single master gel image containing all spots was prepared in each group as a reference gel. Spot detection parameters were adjusted to the reference gels. The same parameters and also the same area, including all spots, were used for all gels. The total selected area was considered as 100 percent, and the percentage of intensity (% intensity) for each spot was determined. The mean intensity of each spot in patients and controls was compared by nonparametric Mann-Whitney U test. After manually excising the differently expressed spots from CBB stained gels, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF) technique was used for identification of the tryptic protein spots [6]. Mass spectrometry analysis was performed at the Sir Henry Wellcome Functional Genomics Facility, University of Glasgow, using a 4700 MALDI-TOF/TOF Proteomics Analyzer instrument (Applied Biosystems, Paisley, UK). The MASCOT program search algorithm (http://www.matrixscience.com) was used to search the National Center for Biotechnology Information database (NCBI: http://www.ncbi.nlm.nih.gov). One missed cleavage with trypsin and two modifications (carbamidomethylation of cysteine and oxidation of methionine) were allowed in the search settings. In brief, gels were diced into small pieces, washed with 25 mM ammonium bicarbonate (ABC) for 30 min at room temperature and then washed twice washing in 50% acetonitrile (ACN)/25 mM ammonium bicarbonate for 30 min. Samples were then dehydrated using 100% ACN, and supernatants were discarded. Fresh dithiothreitol (DTT) and iodoacetamide (IDD) were used for reduction and alkylation, respectively. At first, 10 mM DTT in 25 mM ABC was added to samples and incubated at 60 C in a water bath for 60 min. After incubation, supernatants were discarded, and IDD solution (55 mM in 25 mM ABC) was added and incubated at room temperature in the dark for 45 min. Supernatants were discarded, and gel pieces were washed with 25 mM ABC and then with 2 mM ABC/50% ACN, each for 10 min. Gels were then dehydrated with 100% ACN in 60 C for 30 min. Gel pieces were covered with trypsin (10 mg/ml in 25 mM ABC, 5% ACN), and digestion was performed at 37 C overnight. The liquid phase was collected, and the gel pieces were washed for a further 10 min with 50% ACN/25 mM ABC and pooled with the first extract. The peptide extracts were dried and resuspended in 10 μl of 0.1% trifluoroacetic acid (TFA). Statistical confidence limits of 95% were applied for protein. In the present study, MASCOT protein scores of greater than 66 were considered statistically significant (P < 0.05).

Relative-quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from five URPL and five normal gesta-
tional week-matched first trimester placentas using the guanidinium acid phenol chloroform method. Extracted RNA was treated with DNase to remove the remaining genomic DNA. Reverse transcription of all RNA samples to first-strand cDNA was performed with a RevertAid™ H Minus M-MuLV Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany) according to manufacturer’s protocol. For cDNA synthesis, two micrograms of total RNA and 10 pmol of random hexamer primers (Fermentas, St. Leon-Rot, Germany) were used for each reaction, while 18s rRNA (housekeeping gene) was used as the internal control. Moreover, in order to test the validity of data in each experiment, a sample was used as a calibrator.

To investigate altered RNA expression of three selected differentially expressed proteins (apolipoprotein A-I, prohibitin and calumenin), real-time PCR (Applied Biosystems 7500 Fast Real-Time PCR system, Life Technologies, Carlsbad, CA, USA) was performed using the SYBR Green I Dye method (Applied Biosystems, Paisley, UK). Selection of the proteins was based on both the functional importance and up- or downregulation of the proteins. Moreover, the expression of all other proteins within normal and URPL placentas at the RNA level was also tested using the RT-PCR method. The primer sequences for apoA-I and 18s rRNA were selected from the paper of Vanderlelie and coworkers [11]. Real-time primers for two other genes were designed with the Beacon Designer Software, and their sequences were as follows: calumenin, 5′-CAGAAGAGAGCAAGGAAAAG-3′ (forward) and 5′-CATCCACAGTGACAAAACC-3′ (reverse) resulting in a 78 bp product, and prohibitin, 5′-TATCTTTGACTGCCGTTCTT-3′ (forward) and 5′-AGTGTTGATGTGACATTCTT-3′ (reverse), which produce a 81 bp product. The PCR protocol consisted of a cycle at 95 C for 5 min followed by 40 cycles consisting of 15 sec at 95 C and 45 sec at 57 C as the annealing temperature. The 2^{\Delta\Delta Ct} method was used for quantification of target gene expression. All tests were done in duplicate, and the mean Ct was used for calculations. The 2^{\Delta\Delta Ct} was calculated using the following formulas:

\[ \Delta Ct \text{ gene of interest} = Ct \text{ gene of interest} - Ct \text{ housekeeping gene} \]
\[ \Delta Ct \text{ calibrator} = Ct \text{ calibrator} - Ct \text{ housekeeping gene} \]

\[ \Delta \Delta Ct = \Delta Ct \text{ gene of interest} - \Delta Ct \text{ calibrator} \]
\[ 2^{-\Delta\Delta Ct} = \text{gene fold change} \]

**Western blot analysis**

To confirm the mass results, the Western blot technique was used for detection of one downregulated (Calumenin) and one upregulated (Vitamin D binding protein) protein after transferring proteins from 2D gels onto PVDF membranes and probing with appropriate monoclonal antibodies (Abcam, Cambridge, UK). For Western blotting of each protein, two 2D gels were first simultaneously run in a twin gel electrophoresis system (SCIE-PLAS, Cambridge, UK). In the next step one gel was stained with CBB while the second one was transferred onto a PVDF membrane using a semi-dry transfer system (Amersham, Uppsala, Sweden). The appropriate mouse mAbs (ab72571 for Calumenin and ab23484 for Vitamin D binding protein, Abcam, Al-Ain, UAE) were used for probing of PVDF transferred protein spots. The incubation time for both antibodies was one hour, and the dilution factor was 1/2000. Anti-mouse IgG conjugated with horseradish peroxidase (1/5000, ab97023, Abcam) was used as the secondary antibody. The incubation time for conjugated antibody was one hour, and SIGMAFAST 3′–3′ di-aminobenzidine (DAB) tablets (Sigma, Steinheim, Germany) were used for visualization of the blotted spots. The location of each blotted spot was compared with a manually excised spot from CBB-stained gel (Supplementary Fig. 1: on-line only).

**Statistical analysis**

The mean intensity of each spot in patients and controls was compared by nonparametric Mann-Whitney U test using the Statistical Package for the Social Sciences (SPSS) 11.5 software (SPSS, Chicago, IL, USA). P values below 0.05 were considered significant differences. Unpaired Student’s t-tests were used for quantification analysis of QRT-PCR results.

**Results**

**Identification of differentially expressed proteins**

The whole placental proteomes of five URPL placentas and the same number of late first trimester normal placentas were analyzed in two pH ranges (between 4–7 and 3–10). The percentage of intensity of each spot in the URPL group was determined and compared with the corresponding spot in the normal group using the Mann–Whitney U test. Statistical analysis indicated that among approximately 1270 distinct detected spots in each silver nitrate stained gel, only 19 spots showed statistically different expression in URPL placentas as compared with normal placentas (P < 0.05). All differentially expressed protein spots were located in pH range 4–7. One URPL gel and one normal late first trimester silver-stained gel in pH range 4–7 are presented in Fig. 1.

To characterized differentially expressed spots, gels were stained with CBB. Out of 19 differently expressed spots in the silver-stained gels, twelve distinct spots were successfully picked up from CBB-stained gels and identified using the MALDI TOF/TOF technique. Two out of twelve differentially expressed proteins were downregulated (calumenin, enolase 1), while the remaining ten spots (actin gamma 1 propeptide, cathepsin D prepropeptide, HSP gp96, tubulin beta, tubulin alpha 1, glutathione S-transferase, vitamin D binding protein, prohibitin, actin beta, apolipoprotein A-I) showed increased expression in placentas from URPL patients compared with normal late first trimester placentas (Fig. 1; Tables 1 and 2).

**QRT-PCR analysis**

The results of QRT-PCR analysis are shown in Table 3. As presented in Table 3, expression of apoA-I and prohibitin at the RNA level indicated significant 6.1- and 1.65-folds upregulation (P < 0.001 and P < 0.05, respectively), while calumenin showed significant downregulation (P < 0.01) in URPL placentas as compared with normal placentas.

**Western blot analysis**

Comparison of the location of blotted spots on PVDF membranes with CBB-stained gels confirmed the mass results regarding the two selected spots (Supplementary Fig. 1: on-line only).
While some data has been published about the full-term placental proteome, no study has been done on the first trimester whole placental proteome till now. Moreover, most investigators have focused on preeclampsia and published papers on RPL are extremely rare. Considering the role of abnormal placentation in predisposing patients to miscarriage and unknown etiology of abortion in URPL cases, Table 1. Differentially expressed protein spots in URPL placentas as compared with normal gestational week-matched placentas, identified by the MALDI TOF/TOF/MASS technique

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Mr (Da)</th>
<th>pI</th>
<th>Score</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Actin, gamma 1 propeptide</td>
<td>41837</td>
<td>5.44</td>
<td>192</td>
<td>gi</td>
</tr>
<tr>
<td>14</td>
<td>Cathepsin D prepropeptide</td>
<td>45036.8</td>
<td>6.10</td>
<td>358</td>
<td>gi</td>
</tr>
<tr>
<td>20</td>
<td>Heat shock protein gp 96 precursor</td>
<td>90309.1</td>
<td>4.73</td>
<td>151</td>
<td>gi</td>
</tr>
<tr>
<td>35</td>
<td>Actin, beta</td>
<td>42077.9</td>
<td>5.29</td>
<td>72</td>
<td>gi</td>
</tr>
<tr>
<td>70</td>
<td>Tubulin, beta</td>
<td>48135.1</td>
<td>4.70</td>
<td>239</td>
<td>gi</td>
</tr>
<tr>
<td>78</td>
<td>Apolipoprotein A-I</td>
<td>28061.5</td>
<td>5.27</td>
<td>266</td>
<td>gi</td>
</tr>
<tr>
<td>105</td>
<td>Tubulin alpha 6</td>
<td>50547.7</td>
<td>4.96</td>
<td>373</td>
<td>gi</td>
</tr>
<tr>
<td>106</td>
<td>Enolase 1</td>
<td>47350.4</td>
<td>6.98</td>
<td>126</td>
<td>gi</td>
</tr>
<tr>
<td>129</td>
<td>Glutathione s-transferase</td>
<td>23595.1</td>
<td>5.43</td>
<td>499</td>
<td>gi</td>
</tr>
<tr>
<td>132</td>
<td>Prohibitin</td>
<td>29871</td>
<td>6.07</td>
<td>120</td>
<td>gi</td>
</tr>
<tr>
<td>155</td>
<td>Vitamin D binding protein</td>
<td>54498.6</td>
<td>5.33</td>
<td>228</td>
<td>gi</td>
</tr>
<tr>
<td>190</td>
<td>Calumenin, isoform CRA-C</td>
<td>38141.1</td>
<td>4.47</td>
<td>47</td>
<td>gi</td>
</tr>
</tbody>
</table>

*Spot numbers are the same as the spot labels in Fig. 1. Theoretical/mass (Dalton) or pI. Protein scores of greater than 66 were considered statistically significant (P < 0.05).

Table 2. Comparison of the mean percentage of intensity of differentially expressed spots between URPL and normal gestational week-matched placentas

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Percentage intensity (Mean ± SD)</th>
<th>Expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Actin, gamma 1 propeptide</td>
<td>0.35 ± 0.012</td>
<td>0.194 ± 0.068</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Cathepsin D prepropeptide</td>
<td>0.43 ± 0.029</td>
<td>0.331 ± 0.025</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>Heat shock protein gp 96 precursor</td>
<td>0.32 ± 0.015</td>
<td>0.203 ± 0.031</td>
<td>+</td>
</tr>
<tr>
<td>35</td>
<td>Actin, beta</td>
<td>0.316 ± 0.040</td>
<td>0.177 ± 0.045</td>
<td>+</td>
</tr>
<tr>
<td>70</td>
<td>Tubulin, beta</td>
<td>0.318 ± 0.039</td>
<td>0.224 ± 0.012</td>
<td>+</td>
</tr>
<tr>
<td>78</td>
<td>Apolipoprotein A-I</td>
<td>0.382 ± 0.041</td>
<td>0.277 ± 0.023</td>
<td>+</td>
</tr>
<tr>
<td>105</td>
<td>Tubulin alpha 6</td>
<td>0.239 ± 0.020</td>
<td>0.150 ± 0.025</td>
<td>+</td>
</tr>
<tr>
<td>106</td>
<td>Enolase 1</td>
<td>0.274 ± 0.033</td>
<td>0.393 ± 0.054</td>
<td>–</td>
</tr>
<tr>
<td>129</td>
<td>Glutathione s-transferase</td>
<td>0.174 ± 0.038</td>
<td>0.068 ± 0.033</td>
<td>+</td>
</tr>
<tr>
<td>132</td>
<td>Prohibitin</td>
<td>0.347 ± 0.024</td>
<td>0.260 ± 0.031</td>
<td>+</td>
</tr>
<tr>
<td>155</td>
<td>Vitamin D binding protein</td>
<td>0.310 ± 0.099</td>
<td>0.173 ± 0.012</td>
<td>+</td>
</tr>
<tr>
<td>190</td>
<td>Calumenin, isoform CRA-C</td>
<td>0.086 ± 0.009</td>
<td>0.151 ± 0.047</td>
<td>–</td>
</tr>
</tbody>
</table>

* Up- and downregulation of spots in URPL placentas as compared with normal placentas is indicated by + and –, respectively. Exact P-value calculated by Mann-Whitney U test.

Table 3. QRT-PCR analysis of Calumenin, ApoA-I and Prohibitin gene in URPL placentas as compared with normal placentas

<table>
<thead>
<tr>
<th>RNA expression (ΔCt)</th>
<th>2−ΔΔCt</th>
<th>% intensity at protein level</th>
</tr>
</thead>
<tbody>
<tr>
<td>URPL (n=5) Mean ± SEM</td>
<td>Normal (n=5) Mean ± SEM</td>
<td>URPL (n=5) Mean ± SD</td>
</tr>
<tr>
<td>Calumenin</td>
<td>17.53 ± 0.46</td>
<td>16.54 ± 1.05</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>18.02 ± 0.83</td>
<td>20.70 ± 0.35</td>
</tr>
<tr>
<td>Prohibitin</td>
<td>15.51 ± 0.61</td>
<td>16.22 ± 0.58</td>
</tr>
</tbody>
</table>

* The 2−ΔΔCt value is presented for the URPL/normal ratio. * P < 0.01; ** P < 0.001; *** P < 0.05.

Discussion

While some data has been published about the full-term placental proteome, no study has been done on the first trimester whole placental proteome till now. Moreover, most investigators have focused on preeclampsia and published papers on RPL are extremely rare. Considering the role of abnormal placentation in predisposing patients to miscarriage and unknown etiology of abortion in URPL cases,
The aim of the present study was comparison of the whole placental proteome between normal late first trimester placentas human and URPL placentas to determine the probable protein changes that may participate in URPL etiology.

The results of the present study showed that the proteins exhibiting altered expression belonged to different functional classes of proteins including cytoskeletal proteins (actin gamma 1 propeptide, actin beta, tubulin alpha and beta), transport/cargo proteins (vitamin D binding protein, apolipoprotein A-I), endoplasmic reticulum proteins (HSP gp96 precursor, calumenin), a glycolysis protein (enolase 1), migration proteins (cathepsin D prepropeptide, prohibitin) and an antioxidant protein (glutathione S-transferase). As indicated in Table 2, among the 12 differentially expressed proteins, calumenin and enolase 1 showed significant downregulation in URPL cases as compared with normal late first trimester placentas. Calumenin is a well-conserved Ca\(^{2+}\) binding protein with a pleiotropic role that is located at the lumen of the endoplasmic reticulum [12]. It has been reported that calumenin is highly expressed by placental cells [13], and through its interaction with other proteins, it participates in several physiological functions including inhibition of coagulation and thrombosis through inhibition of the activity of vitamin k-dependent c-carboxylation [14] and prevention of atherosclerosis [14, 15]. Therefore, downregulation of calumenin in URPL placentas may lead to activation of coagulation and thrombosis, which are considered to be important events in induction of recurrent abortion. On the other hand, endothelial dysfunction, which is proposed to be the main triggering factor for RPL, leads to the development of atherosclerosis lesions via several steps [16]. Interestingly, the role of calumenin in inhibition of atherosclerosis has been reported [14]. Therefore, downregulation of calumenin may be considered one of the factors that predispose URPL placentas to atherosclerosis through induction of endothelial dysfunction [17]. In line with downregulation of calumenin at the protein level, a significant decrease in the expression of calumenin at the RNA level was also observed in URPL placentas as compared with normal cases (Table 3, P < 0.01).

Enolase 1 is a glycolytic enzyme that is downregulated in the URPL placenta. It has been shown that in the late trimester, the placenta is under hypoxic pressure, and therefore, overexpression of genes encoding for glycolytic enzymes is an adoptive response to compensate hypoxia [18]. Interestingly, the results of this study showed that the levels of enolase 1 in URPL placentas were significantly less than in normal placentas. Hence, in the absence of enolase 1 overexpression in the first trimester placentas, hypoxia would not be well tolerated and would eventually lead to URPL.

All differentially overexpressed proteins (Tables 1 and 2) have been previously detected as placental tissue or placental tissue cell line-derived proteins [6, 8, 19–22]. Moreover, the expression of all proteins within normal and URPL placentas at the RNA level was also conformed in this study using the RT-PCR method. Placentation requires trophoblast invasion into the endometrium during early pregnancy, and arrested invasion due to endothelial dysfunction is thought to trigger URPL [4]. Most of the reported overexpressed proteins in this study seem be well match this scenario. In this respect, it has been shown that cathepsin D activates several proteases, including matrix metalloproteases [23]. Production of vasoindhibins, which may inhibit vasodilation, angiogenesis and mediated vascular regression in endothelial cells, is triggered by proteolysis of prolactin by Cathepsin D or matrix metalloproteases [24]. Therefore, overexpression of cathepsin D could predispose pregnant women to URPL by stimulation of vascular regression. In addition, endothelial dysfunction in URPL may be explained by overexpression of apolipoprotein A-I, which is involved in

![Two representative silver-stained 2D-PAGE gels (15%, pH 4-7 linear). Differentially expressed spots are numbered. A: Normal gestational week-matched placenta proteome. B: URPL placenta proteome. C: Magnified view of differentially expressed spots.](image-url)
cholaer transfer to the fetal side [25, 26]. Accordingly, both the relation between high concentration of apolipoprotein A-I and maternal endothelial dysfunction and upregulation of apolipoprotein A-I in preeclamptic placentas have been reported [6, 27]. Therefore, overexpression of apolipoprotein A-I in URPL placentas may result in damage and endothelial dysfunction, which are both seen in RPL placentas. Interestingly, overexpression of Apo-A-I mRNA by about 6-folds was observed in URPL placentas, which confirms our results obtained at the protein level (Table 3, P < 0.001).

Prohibitin is another upregulated protein in the URPL placenta. The prohibitin family includes highly conserved and broadly distributed proteins, which are expressed in the nucleus, lipid rafts and mitochondria [28]. Within mitochondria, prohibitins appear to function as chaperones in cell cycle control [29]. Recently, Schleicher et al. indicated the critical role of prohibitins in regulation of cell proliferation and increasing the angiogenesis capacity of endothelial cells [30]. Hence, it may be concluded that the overexpression of prohibitins in URPL placentas is a mechanism to compensate for endothelial dysfunction and angiogenesis defects that are seen in RPL placentas. The result of real-time PCR showed 1.65-folds over-expression of prohibitin at the RNA level (Table 3, P < 0.05), which is in line with our finding at the protein level.

Exaggerated cytotrophoblast proliferation is also thought to trigger URPL. Excess cytotrophoblast proliferation may have been supported by overexpression of cytoskeleton proteins including, actins and tubulins. Along with our findings, a link between altered cytoskeleton protein expression and a defect in placentation has also been reported [31]. Vitamin D binding protein (DBP) is a highly polymorphic and pleiotropic serum protein [32]. While the major function of DBP is the transport of vitamin D sterols, DBP also participates in transport of fatty acids and inhibition of actin polymerization [32]. Interestingly, DBP is found to be highly expressed on the surface of cytotrophoblast cells [33]. Considering the upregulation of cytoskeleton proteins in the URPL placentas, overexpression of DBP in the URPL placentas may be considered a mechanism for scavenging the actin filaments and control of excess cytotrophoblast proliferation.

Stress response proteins can protect cells or tissues from damage and adverse environmental conditions. Overexpression of Hsp gp96 and glutathione S-transferase (GST) under stress conditions in URPL placentas compared with normal placentas seems to be a normal physiological response to protect the placenta from adverse effects of stress that are present in the placentas of patients with URPL. In addition, the relation between GST gene polymorphisms and RPL has also been reported [34].

In spite of the absence of any published data regarding the placental proteome changes during URPL, some parts of our findings are confirmed by the results obtained from proteome studies in preeclamptic placentas. Among them, upregulation of prohibitin and cytoskeleton proteins in preeclampsia [35], glutathione S-transferase in a BeWo cell line under hypoxic conditions [36], and apolipoprotein A-I, actin gamma 1 propeptide and heat shock protein gp96 precursor in preeclampsia are remarkable [6]. Considering the fact that endothelial dysfunction is the main pathologic phenomenon in both preeclampsia and RPL, the similarity of protein alterations in both diseases is not unlikely. However, the possibility of induction of the abovementioned changes as a result of stress subsequent to abortion in URPL cases should not be disregarded.

In summary, the data of the present study introduce a group of 12 altered proteins in URPL patients for the first time. Most of these proteins may be involved in the endothelial dysfunction that is the hallmark of RPL. Though the present study introduces new proteins that might be used as diagnostic markers and might also be beneficial in therapy of URPL, repeating this study with a larger sample size is recommended.

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