Increased levels of pregnancy-associated plasma protein-A2 in the serum of pre-eclamptic patients

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Pregnancy-associated plasma protein-A (PAPP-A) is a well-known first trimester serum marker of pathological pregnancies. Since serum PAPP-A is reduced in the first trimester of pregnancies with fetal trisomies, PAPP-A in combination with other markers has been used for non-invasive early detection of trisomies (Wald et al., 1996). It is also reported that maternal serum PAPP-A is reduced in various complicated pregnancies in which fetal growth is restricted (Dugoff et al., 2005). Although there are reports that maternal serum PAPP-A is elevated in pre-eclamptic pregnancy (Hughes et al., 1980), some discrepancy still remains (Bersinger et al., 2002; Smith et al., 2002).

Introduction

Pregnancy-associated plasma protein-A (PAPP-A) and -A2 (PAPP-A and -A2) are proteases that cleave insulin-like growth factor-binding proteins (IGFBPs), resulting in local activation of IGF signaling pathways. Here, we examined PAPP-A and -A2 mRNA and protein levels in placenta and maternal sera from women with pre-eclampsia and compared them with samples from uncomplicated pregnancy. PAPP-A2 but not PAPP-A mRNA and protein were elevated in pre-eclamptic placenta ($P < 0.01$). PAPP-A2 is normally produced in placental syncytiotrophoblaster cells and maternal decidua. PAPP-A2 in syncytiotrophoblaster cells was dramatically increased in pre-eclampsia. Maternal serum concentrations of PAPP-A2 but not PAPP-A were also significantly elevated in pre-eclampsia as compared with uncomplicated pregnancy. mRNA levels of IGFBP5, a specific substrate for PAPP-A2 protease activity, were also significantly increased, suggesting a potential role for IGFBP5 in fetal and placental growth suppression during pre-eclampsia. However, IGFBP5 protein levels were not increased in placenta from pre-eclampsia, possibly due to cleavage by up-regulated PAPP-A2. These data might imply that PAPP-A2 may be up-regulated in pre-eclamptic pregnancy to compensate for IGFBP5-mediated suppression of the IGF pathway, although final birthweights are still low in pre-eclamptic pregnancy.

Keywords: IGFBP5; PAPP-A; PAPP-A2; placenta; pre-eclampsia

Uncertainty of specificity of the anti-PAPP-A antibodies possibly underlies this discrepancy.

PAPP-A2 was the first protein identified as having a similar structure to PAPP-A (Farr et al., 2000; Page et al., 2001). Both PAPP-A and -A2 are involved in the insulin-like growth factor (IGF) pathway (Overgaard et al., 2001). PAPP-A is a protease that cleaves IGF-binding protein (IGFBP)4 and IGFBP5, leading to the activation of IGF-I and II, whereas PAPP-A2 has been reported to cleave only IGFBP5 (Overgaard et al., 2001). Both enzymes are expressed in wide-range of tissues, but abundantly in placental syncytiotrophoblasts and the pregnant uterus (Overgaard et al., 1999, Page et al., 2001). Thus, PAPP-A and -A2 are likely local regulators of IGF bioavailability through the cleavage of IGFBPs (Boldt and Conover, 2007). However, the exact role in IGF regulation and function during pregnancy remains unknown.

We have previously reported the results of gene expression profiling of placental tissue from women with and without pre-eclampsia using whole-genome oligonucleotide microarrays (Nishizawa et al., 2007). Our analyses subsequently identified a total of 137 genes with significantly altered expression in pre-eclampsia. Among these 137 genes, PAPP-A2 was identified as one of the genes up-regulated in pre-eclampsia. In our current study, we compared the expression of PAPP-A2 RNA and protein in women with uncomplicated pregnancies and women with severe pre-eclampsia. Our findings indicate

†These authors contributed equally to this work.
that not only is PAPP-A2 significantly overexpressed in pre-eclamptic placental tissue, it is also abundantly secreted into the maternal serum in patients with preeclampsia.

Materials and Methods

Samples
All the clinical samples were collected at the Department of Obstetrics and Gynecology, Fujita Health University Hospital, Japan. Placental biopsy samples were obtained during Caesarean sections from both normotensive patients and those with pre-eclampsia (n = 21). Pre-eclampsia was defined by a blood pressure of >160/110 mmHg, and by proteinuria of more than 2 g in a 24 h collection. Normotensive subjects (n = 21) were matched for maternal age, gestational age and maternal body mass index at pre-pregnancy. Clinical details of these subjects are presented in Table I (Pryor-Koishi et al., 2007). All the data except for microarray data were derived from this sample population.

To avoid any possible effects of labor on PAPP-A2 expression, only placental samples that were obtained through Caesarean section from women who had not undergone labor were included in the study. A central area of chorionic tissue was then dissected, and the maternal decidua and amniotic membranes were removed. After vigorous washing of the maternal blood by saline, tissues were immediately frozen in liquid nitrogen and stored until use. Maternal blood samples were obtained either at the time of diagnosis or during surgery. Serum samples were stored at -80°C until use. Informed consent was obtained from each patient and this study was approved by the Ethical Review Board for Clinical Studies at Fujita Health University.

Real-time RT–PCR
Total RNA was extracted from choriionic villous tissue samples using an RNeasy mini-kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. The quality of the RNA samples was determined by electrophoresis through denaturing agarose gels and staining with ethidium bromide. RNA was also quantified and evaluated for purity by UV spectrophotometry. Details of the microarray experiments have been described in a previous report (Nishizawa et al., 2007). Data analysis was performed using GeneSpring GX software version 7 (Agilent Technologies).

To quantify PAPP-A, PAPP-A2 and IGFBP5 gene expression levels, we performed real-time RT–PCR analyses using an ABI PRISM 7700 Sequence Detection System (Perkin–Elmer, Foster City, CA, USA). A Superscript First-strand Synthesis System for RT–PCR (Invitrogen, Grand Island, NY, USA) using random primers was employed to produce single strand cDNA from total RNA. PCR primers and TaqMan probes (Hs00545593_s1, Hs00535718_m1, Hs00181213_m1) were obtained from Applied Biosoys GmbH (Weiterstadt, Germany). The housekeeping gene 18S rRNA (Hs99999901_s1) was used to normalize mRNA concentrations, since expression of other genes widely used as controls is often regulated by estrogen levels. RT–PCRs were performed in triplicate using a TaqMan EZ RT–PCR Kit (Perkin–Elmer) in a final volume of 25 μl. The cycling conditions were 2 min at 50°C, 30 min at 60°C and 1 min at 95°C for RT, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C for PCR amplification.

Western blot analysis
Total cell lysates from placental tissue were prepared using T-PER (Pierce, Rockford, IL, USA) and the proteins were separated by SDS–PAGE in 14% Tris–glycine gels (TEFCO, Tokyo, Japan). After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and blocked with 5% skimmed milk powder diluted in Tris-buffered saline (TBS) with 0.05% Tween 20. Goat polyclonal antibodies against human PAPP-A2 (AF1668; R&D, Minneapolis, MN, USA) or human PAPP-A (AF2487; R&D) were used as the primary antibody. Membranes were incubated with diluted antibody preparations overnight at 4°C. After washing the next day, membranes were incubated with horseradish peroxidase (HRP)-conjugated affinity-purified donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. The blots were then developed using SuperSignal (Pierce) and images were captured using light-capture with a cooled Charge Coupled Device (CCD) camera (ATTO, Tokyo, Japan). MagicMark XP Western Protein Standard (Invitrogen) and Precision Plus Protein Standards (BIO-RAD) as well as recombinant human PAPP-A2 (Ser234-Cys1936, R&D) were used as size markers. Mouse monoclonal antibodies against PAPP-A2 (MAB1668; R&D) were also used as the primary antibody for the detection of PAPP-A2 with the aid of HRP-conjugated affinity-purified goat anti-mouse IgG antibody (Pierce). For semi-quantitative analysis, the membranes were reprobed with an anti-β-actin antibody (Sigma–Aldrich, St Louis, MO, USA), which was used as an internal control to indicate the relative loading of the samples.

Ligand blot analysis
Total cell lysates from placental tissue were separated by SDS–PAGE in 14% Tris–glycine gels. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and blocked with 0.2% gelatin diluted in TBS. After washing with TBS with 0.1% Tween 20 (TBST), the membrane was incubated with 10 ng/ml of IGF-I/biotin conjugate (Upstate, Temecula, CA, USA) in TBST containing 0.5% bovine serum albumin overnight at 4°C. The membrane was washed the next day and then incubated with NeutrAvidin-HRP (Pierce) for 1 h at room temperature. The blots were then developed using SuperSignal, and images were captured using Light-Capture with a cooled CCD camera.

Immunostaining
Placental samples were fixed with 10% formaldehyde overnight and then embedded with paraffin. Tissue sections (2 μm) were placed on silane-coated slides and dried in a conventional oven at 60°C for 24 h. After deparaffinization and rehydration, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Polyclonal goat anti-human PAPP-A2 was used for detection (AF1668; R&D). Bound antibodies were detected with a peroxidase-based method using Simple Stain MAX-PO (Nichirei, Tokyo, Japan) with 3,3-diaminobenzidine as a substrate. Non-specific goat IgG (Zymed, Rockford, IL, USA) and the proteins were separated by SDS–PAGE in 14% Tris–glycine gels. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and blocked with 0.2% gelatin diluted in TBS. After washing with TBS with 0.1% Tween 20 (TBST), the membrane was incubated with 10 ng/ml of IGF-I/biotin conjugate (Upstate, Temecula, CA, USA) in TBST containing 0.5% bovine serum albumin overnight at 4°C. The membrane was washed the next day and then incubated with NeutrAvidin-HRP (Pierce) for 1 h at room temperature. The blots were then developed using SuperSignal, and images were captured using Light-Capture with a cooled CCD camera.

Table 1. Clinical parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal pregnancy (n = 21)</th>
<th>Severe pre-eclampsia (n = 21)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (year)</td>
<td>29.1 ± 4.1</td>
<td>30.3 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational age at sampling of serum (weeks)</td>
<td>32.2 ± 4.0</td>
<td>33.6 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>39.2 ± 1.3</td>
<td>34.1 ± 3.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>110.3 ± 10.6</td>
<td>166.5 ± 14.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>66.3 ± 7.1</td>
<td>101.4 ± 15.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Proteinuria (%)</td>
<td>0</td>
<td>100</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Body mass index**</td>
<td>21.1 ± 2.3</td>
<td>23.1 ± 4.9</td>
<td>NS</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>2989.4 ± 356.0</td>
<td>1709.5 ± 673.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>593.3 ± 91.6</td>
<td>325.2 ± 112.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

NS, not statistically significant.
*≥2 g in a 24 h collection. **Pre-pregnancy.
**Enzyme-linked immunosorbent assay**

PAPP-A2 concentrations were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) in which monoclonal rat antibodies against human PAPP-A2 (MAB 1668; R&D) were used for capture, and polyclonal goat anti-human PAPP-A2 antibodies were used for detection (AF1668; R&D). Capture antibodies (1 μg/ml) were bound to the well by an overnight incubation in 50 mM carbonate–bicarbonate buffer (pH 9.6). The wells were then blocked for 1 h with phosphate-buffered saline (PBS) containing 5% bovine serum albumin. After washing with PBS containing 0.05% Tween 20 (PBST), diluted serum samples were loaded into the wells. Incubations were performed at room temperature for 2 h, followed by five washes with PBST and a 1 h incubation with the detection antibody (66.7 ng/ml), diluted in PBST. After five further washes, HRP-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) (Jackson ImmunoResearch Laboratories) was used as the secondary antibody for detection (1:10 000 dilution). O-phenylene-diamine was then added as a substrate for colorimetric assays at 492 nm. A dilution series of recombinant human PAPP-A2 was used to establish the standard curves. All samples were run in duplicate. The intra- and inter-assay coefficients of variation were <5 and 7%, respectively. The detection limit for recombinant PAPP-A2 was ~1.25 ng/well. Calibration was linear up to 20 ng/well.

For the determination of the serum concentrations of PAPP-A, we used the Quantikine Human PAPP-A Immunoassay (R&D) according to the manufacturer’s protocol. Anti-PAPP-A and -PAPP-A2 antibodies did not show significant cross-reactivity with each other. The DuoSet ELISA Development System was also used for detection and quantification of human IGFBP5 (R&D).

**Statistical analysis**

Intergroup comparisons were made using Student’s t-test or one-way analysis of variance method, and *P* < 0.05 were considered statistically significant. Correlations were evaluated with linear straight-line regression. In significant difference tests, *P*-values were calculated with the *z* conversion of Fisher’s *r*. *P* < 0.05 were considered statistically significant.

**Results**

**Up-regulation of PAPP-A2 mRNA in pre-eclamptic placenta**

We previously performed gene expression profiling of placental tissue derived from pregnant women with or without pre-eclampsia using whole-genome oligonucleotide microarrays. A total of 137 genes were identified as genes differentially expressed between uncomplicated and pre-eclamptic pregnancies (Nishizawa et al., 2007). Among these 137 genes, we focused on the PAPP-A2 gene as it was one of the most significantly up-regulated genes in pre-eclamptic placentas (Fig. 1A). In contrast, expression of the PAPP-A gene, which has been well documented to be dysregulated in placentas from women with a subset of complicated pregnancies, was not significantly changed in our samples (Fig. 1A).

We performed real-time RT–PCR for both PAPP-A and -A2 using an increased number of samples to validate our measurement and rule out sampling errors in the microarray screenings. A total of 21 placenta samples from severe pre-eclampsia and 21 from uncomplicated pregnancy were analyzed. In this larger set of samples, the over-expression of the PAPP-A2 in pre-eclampsia was again found to be statistically significant, whereas the expression of the PAPP-A was not significantly changed (Fig. 1B).

**Overproduction of PAPP-A2 protein in pre-eclamptic placenta**

To confirm the increased expression of PAPP-A2 protein in pre-eclamptic placental tissue, we performed western blotting using anti-PAPP-A2 antibodies. Although the molecular weight of PAPP-A2 is 199 kDa based upon its amino acid sequence, the size of PAPP-A2 mature protein is predicted to be 172 kDa due to proteolytic cleavage (Overgaard et al., 2001). However, results from placental sample total cell lysates from showed a larger PAPP-A2 protein product (~250 kDa) (Fig. 2A, upper panel). To confirm that the ~250 kDa band was derived from PAPP-A2, we used another antibody against the PAPP-A2, which produced a band with similar size (Fig. 2B). PAPP-A protein is known to form a disulfide-bound 2:2 complex with the eosinophilic major basic protein (Glerup et al., 2005). In fact, a 200 kDa PAPP-A monomer was detected under reducing conditions (Fig. 2A, middle panel).

To evaluate the protein content of PAPP-A2 and -A, the western blots were subjected to semi-quantitative analyses. Using β-actin as an internal control, we validated the placental overproduction of PAPP-A2 protein in pre-eclamptic tissues (Fig. 2A). In contrast, PAPP-A levels in pre-eclamptic placentas were similar to those in placentas from women with uncomplicated pregnancy.

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Figure 1: Differential expression of PAPP-A and -A2 between placental tissue samples from pre-eclampsia and uncomplicated pregnancy. (A) Mean values of the signal intensity in microarray data for PAPP-A and -A2. *P* < 0.05, Student’s t-test. (B) Quantitative PCR analysis of PAPP-A and -A2. Data obtained from uncomplicated pregnancy (open bars) versus severe pre-eclampsia (grey bars) were compared. Each bar represents the mean value. Error bars indicate the standard deviation. *P*-values calculated using Student’s t-test.
Increased PAPP-A2 protein is produced by syncytiotrophoblasts in pre-eclamptic placenta

To localize the overproduction of the PAPP-A2 protein within the placental tissue, we performed histological examinations of placental sections immunostained with an anti-PAPP-A2 antibody. The polyclonal anti-PAPP-A2 antibody that we utilized yielded strong signals in the cytoplasm of syncytiotrophoblast cells in the chorionic villi of uncomplicated term pregnancies (Fig. 3A). This observation suggested that the main source of PAPP-A2 production was the syncytiotrophoblast cells in uncomplicated pregnancy. This finding was similar to previous reports on cell type-specific PAPP-A protein expression (Bonno et al., 1994). However, the signals were negative neither in cytotrophoblasts nor in extravillous trophoblasts (Fig. 3E and F). In pre-eclamptic placenta, PAPP-A2 protein signals in the syncytiotrophoblast cells were much stronger than those detected in uncomplicated pregnancies (Fig. 3B).

Use of non-specific goat IgG or the secondary antibody alone resulted in negative staining in all of these tissue areas in both subject groups (Supplementary Figure A). As further confirmation of the specificity of PAPP-A2 protein immunolocalization, we performed an immunoblotting experiment using recombinant PAPP-A2 prior to immunostaining and observed a concentration-dependent decrease in the signals (Supplementary Figures B–D). When we used recombinant PAPP-A protein in place of PAPP-A2 in this control experiment, the signals did not decrease, suggesting that the staining was specific for PAPP-A2 and not derived from antibody cross-reactivity with PAPP-A.

In maternal decidua, some of the cells are also immunoreactive for PAPP-A2, as has been reported for PAPP-A (Fig. 3C) (Bonno et al., 1994). These cells might be invasive cytotrophoblasts or stromal cells. In addition, the signals in these cells showed similar intensities between uncomplicated and pre-eclamptic tissues (Fig. 3C and D).

High concentration of PAPP-A2 protein in maternal sera in pre-eclampsia

We next determined PAPP-A2 protein levels in maternal sera from patients with uncomplicated pregnancy and pre-eclampsia. We established a sandwich ELISA system for the evaluation of the serum concentrations of PAPP-A2. Serum PAPP-A2 levels were detected at significantly higher levels in our patients with pre-eclampsia than in patients with uncomplicated pregnancy (Fig. 4A, left). Thus, measurement of PAPP-A2 levels may be potentially instrumental for diagnosis of pre-eclampsia, since the pre-eclampsia cut-off value of 39.32 ng/μl allowed for both high sensitivity (95.0%) and high specificity (85.7%). We also determined the serum concentrations of PAPP-A. However, PAPP-A levels did not correlate well with the presence of pre-eclampsia (Fig. 4A, right).

To determine whether PAPP-A2 serum levels reflected the severity of pre-eclampsia and could serve as a reliable prognostic marker for patients, patients with pre-eclampsia were divided into two groups: seven with early onset (earlier than 32 weeks gestation) and 14 with late onset (32 weeks gestation or later). When we compared the PAPP-A2 levels between these groups, no significant differences were observed (data not shown). However, in uncomplicated pregnancy, PAPP-A2 levels were found to be low in early gestation and significantly elevated with increasing gestational age (1st trimester; n = 17, 2nd trimester; n = 10, 3rd trimester; n = 11) (Fig. 4B, left). Based on the fact that the subjects with early onset pre-eclampsia often develop a more severe phenotype, these data suggest that PAPP-A2 serum protein levels relative to standard for each gestational age may reflect disease severity. Indeed, when we compared the PAPP-A2 levels corrected for gestational age (i.e. expressed as a ratio of the standard at each gestational age) significant differences were observed between early and late onset groups (Fig. 4B, right).

As another assessment of a possible association between PAPP-A2 serum protein and severity of pre-eclampsia, we determined whether maternal serum PAPP-A2 levels were correlated birthweight. We calculated normalized birthweight by division of the individual birthweights in this study by the standard birthweight at a particular gestational week (Ogawa et al., 1998). Maternal serum PAPP-A2 levels were significantly and inversely correlated with normalized birthweight in this study (r = −0.502, P < 0.01) (Fig. 4C). However, no correlation was observed when the analyses were performed separately for pre-eclampsia and control groups (r = −0.374, P = 0.116 for pre-eclampsia, r = 0.133, P = 0.593 for...
control). This finding suggests that the correlation between PAPP-A2 levels and birthweight outcomes simply reflects the presence of the disease.

We also examined a possible correlation between maternal serum PAPP-A2 levels and placental weight. Similar to the data on birthweight outcomes, maternal serum PAPP-A2 levels were inversely

Figure 3: PAPP-A2 immunostaining on placental tissue sections. (A, B) Chorionic villi. Prominent staining is apparent in the cytoplasm of syncytiotrophoblast cells. Signal intensity is much stronger in pre-eclampsia (B) as compared with uncomplicated pregnancy (A). (C, D) Maternal deciduas. A subset of cells, invasive cytotrophoblasts or stromal cells of deciduas, was found to be positive for PAPP-A2. The signal intensity is similar between uncomplicated pregnancy (C) and pre-eclampsia (D). (E, F) Chorionic villi at early gestation (8 weeks). Cytotrophoblasts and extravillous trophoblasts were found to be negative in clear contrast to positive staining in syncytiotrophoblast cells.
correlated with the placental weight ($r = -0.617$, $P < 0.01$) (Fig. 4D). However, no correlation was observed when the analyses were performed separately for pre-eclampsia and control groups ($r = -0.243$, $P = 0.321$ for pre-eclampsia, $r = 0.051$, $P = 0.844$ for control).

**Increased PAPP-A2 protein does not compensate for an impaired IGF pathway in pre-eclampsia**

The dramatic increase in PAPP-A2 protein in the placenta as well as the maternal circulation during pre-eclampsia suggested a dysregulation of the IGF signaling pathway. To examine this possibility, we determined the expression levels of other genes involved in IGF pathway in uncomplicated pregnancy and pre-eclampsia. Up-regulation of IGF2R gene expression and down-regulation of IGFBP2 and IGFBP7 gene expression were statistically significant (Table II). Although the expression of IGFBP5, which is the target protein of the PAPP-A2 protease activity, was non-significantly increased in microarray data, real-time RT–PCR using a larger number of samples demonstrated that the IGFBP5 gene is significantly overexpressed in pre-eclamptic placenta (Fig. 5A).

However, ligand blot analysis indicated that IGFBP5 protein did not show significant increase in pre-eclamptic placenta (Fig. 5B). Immunostaining demonstrated that the IGFBP5 protein was produced in the syncytiotrophoblast cells as well as maternal decidual stroma cells (Fig. 5C and D), but the intensities were similar between placenta from pre-eclampsia and uncomplicated pregnancy. ELISA tests were performed in order to measure IGFBP5 serum concentrations, but in most cases, levels were below the threshold concentration for detection. In the small number of samples that could be measured, 

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**Table II.** Microarray analysis of IGF-associated genes.

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy (n = 4)</th>
<th>Severe pre-eclampsia (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1 (NM_000618)</td>
<td>1.22 ± 0.65</td>
<td>0.83 ± 0.49</td>
<td>0.29</td>
</tr>
<tr>
<td>IGF2 (NM_000612)</td>
<td>1.04 ± 0.28</td>
<td>1.33 ± 0.58</td>
<td>0.22</td>
</tr>
<tr>
<td>IGF1R (NM_000875)</td>
<td>1.03 ± 0.17</td>
<td>1.09 ± 0.20</td>
<td>0.57</td>
</tr>
<tr>
<td>IGF2R (NM_000876)</td>
<td>0.65 ± 0.11</td>
<td>1.01 ± 0.23</td>
<td>0.014</td>
</tr>
<tr>
<td>IGFBP1 (NM_000596)</td>
<td>2.71 ± 2.74</td>
<td>3.34 ± 2.10</td>
<td>0.70</td>
</tr>
<tr>
<td>IGFBP2 (NM_000597)</td>
<td>1.77 ± 0.67</td>
<td>1.04 ± 0.29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IGFBP3 (BC000013)</td>
<td>0.96 ± 0.13</td>
<td>1.12 ± 0.45</td>
<td>0.34</td>
</tr>
<tr>
<td>IGFBP4 (NM_001552)</td>
<td>0.90 ± 0.14</td>
<td>1.10 ± 0.34</td>
<td>0.16</td>
</tr>
<tr>
<td>IGFBP5 (NM_000599)</td>
<td>1.07 ± 0.26</td>
<td>1.24 ± 0.60</td>
<td>0.46</td>
</tr>
<tr>
<td>IGFBP6 (NM_002178)</td>
<td>1.31 ± 0.54</td>
<td>1.65 ± 0.54</td>
<td>0.34</td>
</tr>
<tr>
<td>IGFBP7 (NM_001553)</td>
<td>1.44 ± 0.56</td>
<td>0.93 ± 0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>IGFALS (NM_004970)</td>
<td>0.95 ± 0.21</td>
<td>0.96 ± 0.27</td>
<td>0.91</td>
</tr>
</tbody>
</table>
IGFBP5 levels were not significantly different between eight serum samples from pre-eclampsia and six from uncomplicated pregnancy (20.1 ± 34.8 versus 68.8 ± 60.6 ng/ml). Together, these results indicate that IGFBP5 is similar between pre-eclampsia and uncomplicated pregnancy at the protein level in spite of up-regulation at the RNA level.

**Discussion**

The goals of this study were to identify a novel diagnostic and prognostic marker for pre-eclampsia and to explore the underlying disease etiology. To date, several proteins that are elevated in the maternal sera, including activin A/inhibin A (Muttukrishna et al., 1997; Cuckle et al., 1998; Silver et al., 1999), leptin (McCarty et al., 1999), soluble Fms-related tyrosine kinase (FLT1) (Levine et al., 2004) and endoglin (Venkatesha et al., 2006), have been suggested as possible biological markers of pre-eclampsia. The current study demonstrated that maternal serum PAPP-A2 was significantly elevated in pre-eclamptic pregnancy as compared with uncomplicated pregnancy. Serum PAPP-A2 determination showed good sensitivity and specificity and significant correlation with disease severity, indicating its potential usefulness in detecting pre-eclampsia. However, although the diagnosis of severe pre-eclampsia is readily achievable by current clinical assessments, clinicians are eagerly awaiting the development of biomarkers that can be used during the early phases of apparently uncomplicated pregnancy to predict the subsequent development of pre-eclampsia (Cuckle et al., 1998). Prospective randomized studies should clarify the potential usefulness of PAPP-A2 for the early detection of pre-eclampsia in the near future.

Our immunohistochemical analyses showed that the expression of PAPP-A2 in placenta was detected predominantly in the syncytiotrophoblasts in the chorionic villi, but also in the invasive cytotrophoblasts or stromal cells of the decidua. However, in pre-eclampsia, PAPP-A2 expression only increased in the syncytiotrophoblast cells. This observation suggests an alteration of feto-maternal interaction at their interface might possibly contribute to the etiology for this disease.

We also found that IGFBP5 was also up-regulated in pre-eclampsia. A number of proteins have been reported to be overexpressed in the syncytiotrophoblast cells of pre-eclamptic placenta (Nishizawa et al., 2007). Since IGFBP5 levels were not correlated with those of PAPP-A2 (r = 0.186, P = 0.23), we speculate that the elevation of both mRNA is not likely the result of non-specific overexpression due to hyperfunction of the syncytiotrophoblast cells.

Recently, the IGF signaling pathway has been proposed to be involved in the etiology of pre-eclampsia. It has been well documented that altered IGF-II and IGFBP1 expressions at the feto-maternal interface contributes to shallow trophoblast invasion into the maternal decidua in pre-eclampsia (Giudice et al., 1997; Gratton et al., 2002; Shin et al., 2003). Indeed, our current microarray data showed alterations in the expression levels of several genes related to the IGF pathway. ADAM (a disintegrin and metalloprotease) proteins are also implicated in the regulation of placental and fetal growth through IGFBP3 and IGFBP5 proteolysis (Loechel et al., 2000). Interestingly, ADAM12 was reported to be progressively elevated through gestation in uncomplicated pregnancy, but showed even greater expression in pre-eclampsia placental tissues (Gack et al., 2005). Taken together, IGFBPs, along with the enzymatic modifiers ADAMs or PAPP-A/A2, are likely to contribute to the regulation of placental growth and differentiation through an IGF pathway. This regulatory pathway may be locally impaired in pre-eclampsia at the feto-maternal interface. Mice deficient in PAPP-A is viable, but is

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**Figure 5**: Analyses of IGFBP5, the substrate for PAPP-A2 protease activity. (A) Quantitative RT–PCR analysis of IGFBP5. Data obtained from uncomplicated pregnancy (open bars) versus severe pre-eclampsia (grey bars) were compared. Each bar represents the mean value. Error bars indicate the standard deviation. *P < 0.05, Student’s t-test. (B) Semi-quantitative ligand blot analysis for IGFBPs (upper panel). The same blot was reprobed for an anti-β-actin antibody (lower panel). Lanes 1–3, placenta tissue lysates from pre-eclampsia patients; lanes 4–6, placentals lysates from uncomplicated pregnancies. Size markers are indicated on the right. (C,D) IGFBP5 immunostaining on placental tissue sections. Significant staining was present in the cytoplasm of stromal cells in maternal decidual (C), while syncytiotrophoblast cells showed much weaker signals (D). Signal intensities were similar between uncomplicated pregnancies (left) and pre-eclampsia (right).
small at birth (Conover et al., 2004). Likewise, PAPP-A2 might also be indispensable for normal fetal growth and development through its contributions to the IGF pathway.

Our patients with pre-eclampsia had outcomes with significantly lower birthweight relative to the uncomplicated pregnancy control group. The weight of pre-eclamptic placenta was also significantly lower than that of control. Furthermore, PAPP-A2 levels were well correlated with both birthweight outcomes and placental weight. Therefore, the up-regulation of PAPP-A2 during pre-eclampsia might be a consequence of slowed fetal or placental growth. However, there was not a good correlation between PAPP-A2 and birthweight, when evaluated separately for patients with pre-eclampsia or uncomplicated pregnancy, suggesting that the correlation between the PAPP-A2 and low-birthweight simply reflected the severity of reduced birthweight in patients with pre-eclampsia.

In conclusion, we demonstrated increased levels of PAPP-A2 both in placenta and maternal sera from women with pre-eclampsia. Although we did not demonstrate alteration of IGFBP5 at the protein level, it still remains possible that dysregulation of IGFBP5/PAPP-A2 pathway locally at the placental feto-maternal interface might underlie the disease etiology. This pathway might play a role in a defect in trophoblast invasion into the maternal decidua leading to placental dysfunction and growth retardation of both the placenta and fetus, and deserves further investigation to elucidate a cause and effect relationship between its dysregulation and the onset of pre-eclampsia.

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Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/

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