and maternal artery remodelling (Burger *et al.*, 2004; Than *et al.*, 2004a,b). The present study, therefore, quantified the mRNA expression of PP13 in the cellular component of blood from pregnant women in the third trimester and early second trimester, and assessed the association between the PP13 expression and the onset of preeclampsia.

MATERIALS AND METHODS

Subjects

This study was performed as part of a series along with previously reported studies (Purwosunu *et al.*, 2007a,b; Purwosunu *et al.*, 2008, 2009). The first part of the study is a case–control study. The study population included preeclamptic and normal pregnant women who were treated at the Department of Obstetrics and Gynecology, University of Indonesia, at Cipto Mangunkusumo National Hospital. The subjects were recruited between December 2005 and February 2006. Preeclampsia and control group consisted of 24 and 22 cases respectively. The control group included pregnant women with no preexisting diseases or prenatal complications.

The second part of the study was designed as a prospective cohort study in early pregnant women (gestational weeks 15–20). Of the 683 women enrolled, 23, who had incomplete information regarding the outcome, whose pregnancy ended before 20 weeks or who experienced stillbirth, were excluded. Among the remaining 660 women, 62 developed preeclampsia (mild preeclampsia: 26 cases; severe preeclampsia: 24 cases; hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome: 12 cases). Some of the samples were used for the other study and were unavailable for this study. mRNA from 41 subjects were then available in the preeclampsia group (mild preeclampsia: 20 cases; severe preeclampsia: 14 cases; HELLP syndrome: 7 cases). Each case was matched with three controls of a similar gestational age at the time of blood testing, maternal weight and fetal gender. Therefore, a total of 41 women who developed preeclampsia and 123 controls with a normal course of pregnancy were enrolled. No special management or treatment other than prenatal care was administered before the clinical signs of preeclampsia. If abnormalities of blood pressure and/or proteinuria were found, the patients were recommended for admission to the hospital.

Mild and severe preeclampsia and HELLP syndrome were defined as described in a previous report (ACOG, 2002; Purwosunu *et al.*, 2007b). In brief, preeclampsia was defined as gestational hypertension (systolic pressure $\geq$140 mmHg or diastolic blood pressure $\geq$90 mmHg on more than or equal to two occasions after gestational week 20) with proteinuria ($\geq$0.3 g/day). Severe preeclampsia was defined by the presence of more than or equal to one of the following: (1) severe gestational hypertension (systolic pressure $\geq$160 mmHg or diastolic blood pressure $\geq$110 mmHg on $\geq$2 occasions after gestational week 20) or (2) severe proteinuria ($\geq$5 g protein in a 24-h urine specimen or $\geq$3 g in two random urine samples collected $\geq$4 h apart). Infants who were determined to be small for gestational age (SGA) were defined as having birth weight $\leq$2.0 SD below the mean for the gestational age. The control group included pregnant women with no preexisting medical diseases or prenatal complications.

All women provided informed consent to participate in the study, which was approved by the Research Ethics Committee of both University of Indonesia and Showa University.

Processing of blood samples

The blood samples (2.5 mL) were collected in PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland), which contained a solution for lysing nonnucleated erythrocytes and stabilizing the RNA of nucleated cells. The samples were mixed well and kept at room temperature for 3 h, and then were stored at −20 °C until they were transported to Japan. A molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo. RNA extraction was performed according to protocols described elsewhere(Okazaki *et al.*, 2007). In brief, cellular component samples were centrifuged twice at 4000 g for 10 min at room temperature in order to remove the entire supernatant and any mRNA present in residual plasma. The pellet was then washed and centrifuged at 4000 g for 10 min and the supernatant was removed. In this step, the contamination of cell-free RNA of maternal plasma was completely removed. The pellet was then re-suspended and incubated in an optimized buffer solution containing proteinase K to digest the protein. A second round of centrifugation was performed to remove any residual cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube. Then, 100% ethanol was added to the supernatant to adjust the binding conditions, and the resultant lysate was applied to a PAXgene spin column (PreAnalytiX), resulting in selective binding of RNA to the silica-gel membrane of the spin column. After the column was washed three times, pure RNA was eluted in 80 μL of RNase-free water.

Real-time quantitative RT-PCR

Reverse transcription of the mRNA was performed using an Omniscript RT Kit (Qiagen). Real-time quantitative PCR was then performed using a QuantiTect Probe PCR Kit (Qiagen). Reverse transcription was performed according to the manufacturer's instructions. cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions using a 2-μL aliquot of cDNA and the kit's components in a reaction volume of 20 μL. TaqMan PCR analysis for PP13 was performed using pre-developed and commercially available primers and probe set (Cat # Hs00747811_m1; Applied Biosystems, Foster City, CA). The thermal cycling protocol used for PCR was

DOI: 10.1002/pd

as follows: initial denaturation at 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 15 s and annealing at 60 °C for 1 min. The amplification data were collected and analysed with an ABI Prism 7900T Sequence Detector and the SDS 2.3 software program (Applied Biosystems). Each sample was analysed in duplicate, and multiple negative water blanks were included in every analysis. As an initial step, the PCR assay was verified to be specific to mRNA and not to genomic DNA. We also confirmed that PP13 expression was negative in the cellular component of three non-pregnant women, indicating that it was specific to pregnancy. The quantification of gene expression was performed with investigators blinded to the outcome of pregnancy. The amounts of mRNA samples were expressed in terms of copies per mL of the initial whole blood volume by a method reported elsewhere (Okazaki *et al.*, 2007; Sekizawa *et al.*, 2009).

Statistical analysis

In the first part of the study, 24 women with a diagnosis of preeclampsia were matched with 22 controls for gestational age and fetal gender. The gestational age was calculated by ultrasonographic measurements at 11 weeks' gestation. Non-parametric descriptive statistics were calculated. The median mRNA values of PP13 and relative multiple of median (MoM) for each group was calculated and analysed using the Mann–Whitney *U* test.

In the prospective cohort study, as a second part of the study, the data were matched for gestational age at the time of blood drawing and fetal gender in a 1 : 3 case–control design. The gestational age was calculated by ultrasonographic measurements at 11 weeks' gestation. The distributions of demographic characteristics and mRNA for PP13 concentrations were analysed after conversion in MoM. The regression line of the LogPP13 versus gestational age yielded a p-value of 0.017 ($F = 1.25$, $R^2 = 0.031$). MoM values were retrospectively stratified according to the severity of preeclampsia and development of HELLP syndrome. Kruskal Wallis and Dunn tests were used for comparisons among and between groups. The detection rate (DR) and false-positive rate (FPR) were calculated for PP13 RNA using a univariate ROC curve.

RESULTS

Comparison between symptomatic patients and normal controls in the third trimester

The demographics of the study subjects are reported in Table 1. No differences were observed in the maternal age, body mass index (BMI) and gestational age of blood drawing. PP13 RNA levels were lower in the preeclampsia cases than those in controls (p-value <0.001; Mann–Withney *U* test). After MoM conversion, PP13 RNA was 1.00 ± 0.34 for the controls and 0.33 ± 0.19 for the preeclampsia cases (p-value <0.001; Mann–Withney *U* test; Figure 1).

Prospective cohort study of asymptomatic pregnant women during the early second trimester

The demographics of the study subjects are sumarized in Table 2. No differences were observed in the maternal age, BMI, systolic and diastolic blood pressures and gestational age at the time of blood drawing. Among the 41 cases of the preeclampsia group, 20 cases had

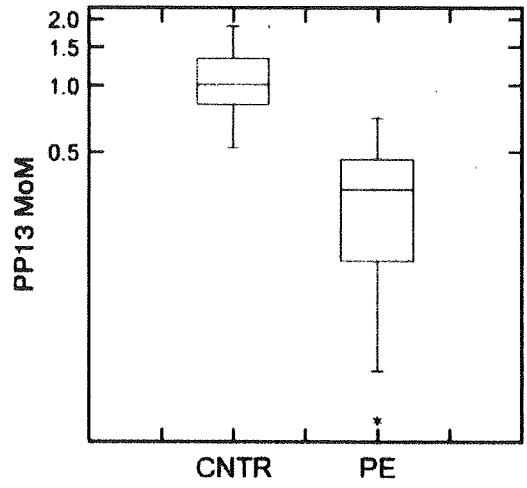

Figure 1—Concentration of PP13 mRNA in the cellular component of the blood from third-trimester pregnant women in the control and preeclampsia groups. PP13 concentrations were expressed by the MoM values

Table 1—Demographics of pregnant women with and without preeclampsia during the third trimester

Variable	Control ($n = 22$)	Preeclampsia ($n = 24$)	p-value[a]
Age (years)	27 (16–42)	27 (18–37)	0.834
BMI	22 (20–28)	23.5 (16–32)	0.839
Systolic blood pressure (mmHg)	115 (100–125)	160 (130–200)	—
Diastolic blood pressure (mmHg)	75 (70–85)	100 (90–140)	—
Birthweight (g)	3250 (2500–4200)	2875 (2000–3500)	0.019
Proteinuria (g/day)	0.2 (0.1–0.4)	5 (1.5–8.0)	—
%Nullipara	50%	41.7%	
Gestational week at the time of blood test	38.5 (35–40)	39 (36–41)	0.665

[a] Mann–Whitney *U* test.

mild preeclampsia, 14 had severe preeclampsia and 7 had HELLP syndrome. After MoM conversion, PP13 RNA was 1.00 ± 2.31 in the controls and 0.56 ± 1.92 in the preeclampsia cases, and the level in the controls was significantly higher than that in the preeclampsia group (p-value = 0.008, Mann–Whitney U Test; Figure 2). Figure 3 shows the PP13 RNA distribution in controls and cases stratified according to severity. The only significant difference was found for control versus HELLP syndrome cases. An ROC curve of PP13 in predicting preeclampsia yielded a DR of 24 and 31% at an FPR of 5 and 10% respectively (Figure 4), corresponding to the values of MoM PP13 of 0.28 and 0.41 respectively. The AUC was 0.735 (95%CI 0.505–0.965). For predicing HELLP syndrome, the DR was 28 and 43% at the same cut-off points of FPR, corresponding to the values of MoM PP13 of 0.27 and 0.43 respectively.

DISCUSSION

PP13 is one of the proteins specifically synthesized by the placenta. Although the function of PP13 is not clear, PP13 is associated with implantation and maternal artery remodelling(Burger *et al.*, 2004), and PP13 prevents erythrocyte adhesion in areas with reduced blood

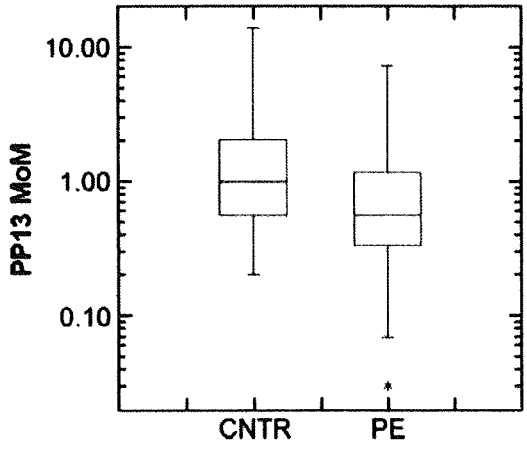

Figure 2—Concentration of PP13 mRNA in cellular component of the blood from second-trimester pregnant women in the control and preeclampsia groups. PP13 concentrations were expressed by the MoM values

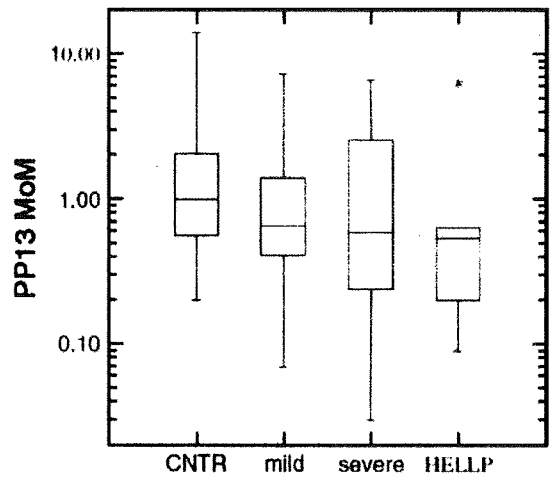

Figure 3—Concentration of PP13 mRNA in cellular component of the blood from second-trimester pregnant women in the control and in preeclampsia stratified according to severity. PP13 concentrations were expressed by the MoM values. p-value = 0.047, Kruskal Wallis Test. Significant comparisons have been made for control versus HELLP by the Dunn test

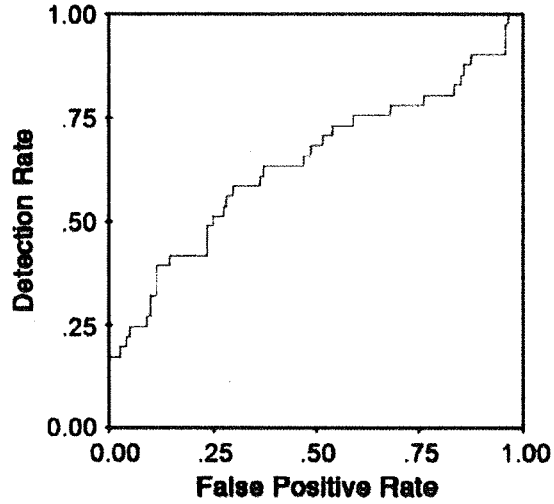

Figure 4—ROC curve showing the performance of PP13 in predicting preeclampsia. AUC = 0.735 (95%CI = 0.505–0.965), p-value = 0.037. We used a non-parametric estimation of the AUC. This method is quite similar to the Mann–Whitney U statistic and provides an unbiased non-parametric estimator for the area

Table 2—Demographics of the asymptomatic pregnant women during the early second trimester

Variable	Control (n = 123)	Preeclampsia (n = 41)	p-value[a]
Age (years)	28 (17–43)	29.5 (19–42)	0.297
BMI	22.7 (13.79–34.22)	23.14 (17.42–33.33)	
Systolic blood pressure (mmHg)	100 (90–120)	100 (90–120)	0.849
Diastolic blood pressure (mmHg)	70 (60–80)	70 (60–80)	0.854
Birthweight (g)	3190 (2500–4080)	2430 (1780–3290)	<0.001
%Nullipara	32.5	31.7	
Gestational week at the time of blood test	17 (15–22)	17 (15–21)	0.528
Gestational week at delivery	38.1 (34–41)	36.5 (32–40)	<0.001

[a] Mann–Whitney U test.

Prenat Diagn 2009; **29**: 1231–1236.
DOI: 10.1002/pd

flow such as intervillous space (Burger *et al.*, 2004; Than *et al.*, 2004a,b). Furthermore, PP13 is suggested to have special immune functions at the feto-maternal interface (Than *et al.*, 2004a,b). An immunohistochemical study indicated that PP13 is localized in the brush border membrane of syncytiotrophoblasts (Than *et al.*, 2004b). However, the function of PP13 in normal pregnancy and its role in the pathogenesis of preeclampsia has not yet been elucidated. A previous study quantified the expression of PP13 mRNA by both syncytiotrophoblasts and EVTs microdissected from normal and preeclamptic placentas. PP13 RNA is predominantly expressed in the syncytiotrophoblasts in comparison to EVTs and the expression by syncytiotrophoblasts in the preeclamptic placenta was significantly lower than that in the normal placenta (Sekizawa *et al.*, 2009). Furthermore, the expression of PP13 mRNA was assessed in the first-trimester trophoblasts from residual samples of chorionic villus sampling (CVS) obtained for fetal karyotyping. In this study, 5 out of 90 cases subsequently developed preeclampsia at later gestation. The PP13 expression of the first-trimester trophoblasts from women who developed PE later was significantly lower than that from normal cases (Sekizawa *et al.*, 2009). This previous study directly proved that the decreased RNA expression of PP13 in the trophoblasts during the first trimester was associated with the pathophysiology of preeclampsia.

The present study assessed the PP13 RNA expression in pregnant women with and without preeclampsia during the third trimester. This revealed that the mRNA expression of PP13 was significantly decreased in the cellular components of blood from pregnant women with preeclampsia, in comparison to normal blood. These results would be associated with the finding that the mRNA expression of PP13 in the preeclamptic placenta was lower than that from normal placenta. This indicates that the analysis of cellular RNA in maternal blood will permit the evaluation of placental pathophysiological alterations, and therefore RNA analysis of PP13 will make it possible to predict preeclampsia.

Next, the possibility of predicting preeclampsia was assessed in blood samples during the early second trimester. The results showed that the PP13 expression was significantly decreased in the cellular component of blood from asymptomatic pregnant women who subsequently developed PE during later gestation. This result is also associated with the finding of decreased placental PP13 production, which was revealed in our previous PP13 analysis of CVS tissue (Sekizawa *et al.*, 2009). The blood sampling was performed at approximately 20 weeks before the onset of clinical symptoms of preeclampsia. At this early gestational age, the pathophysiological alterations of placenta were estimated by analyzing the cellular RNA of the maternal blood. In the study of prediction for preeclampsia, an ROC curve of PP13 RNA yielded a DR of 24 and 31% at an FPR of 5 and 10% respectively. For predicting HELLP syndrome, the DR was 28 and 43% at the same cut-off points of FPR. The AUC was 0.735 (95%CI 0.505–0.965). Using a PP13 protein assay during the first trimester, Chafetz *et al.* reported that the AUC was 0.91 for preeclampsia (Chafetz *et al.*, 2007). The efficiency of this cellular PP13 RNA level may be inferior to the protein assay. One of the reasons is likely that the MoM standard deviation was large in this study series. The other reason is that the study subjects are from early second trimester. Subsequent studies should evaluate the mRNA expression levels in the first trimester samples to compare the efficiency to the protein assay. A panel of mRNA markers in maternal plasma, such as vascular endothelial growth factor receptor 1 (FLT-1), endoglin and plasminogen activator inhibitor-1, is able to predict pregnant women who will develop preeclampsia during later gestation (Purwosunu *et al.*, 2009). If some other markers, such as FLT-1 and endoglin, were combined to the PP13 RNA level, then the prediction from cellular RNA in maternal blood would be more efficient. In this study, preeclampsia occurred in 9% of the patients, a higher frequency than that seen in the published literature. No previous major studies have clarified the prevalence of preeclampsia in Indonesian populations, and this higher prevalence could confound the result.

In conclusion, a decrease in the PP13 mRNA expression was observed in the cellular component of blood from both preeclamptic patients during the third trimester and asympyomatic pregnant women during the early second trimester who develop preeclampsia during later gestation. These findings indicate that an alteration in the PP13 mRNA expression in the placenta may therefore be associated with the pathogenesis of preeclampsia, and that this marker could potentially be one of the key markers to predict the clinical onset of preeclampsia.

ACKNOWLEDGEMENTS

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Nos. 20591930 and 20591308); Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare of Japan.

REFERENCES

ACOG Committee on Obestetric Practice. 2002. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet* **77**: 67–75.

Burger O, Pick E, Zwickel J, *et al.* 2004. Placental protein 13 (PP-13): effects on cultured trophoblasts, and its detection in human body fluids in normal and pathological pregnancies. *Placenta* **25**: 608–622.

Chafetz I, Kuhnreich I, Sammar M, *et al.* 2007. First-trimester placental protein 13 screening for preeclampsia and intrauterine growth restriction. *Am J Obstet Gynecol* **197**: 35.e1–35.e7.

Dugoff L, Hobbins JC, Malone FD, *et al.* 2004. First-trimester maternal serum PAPP-A and free-beta subunit human chorionic gonadotropin concentrations and nuchal translucency are associated with obstetric complications: a population-based screening study (the FASTER Trial). *Am J Obstet Gynecol* **191**: 1446–1451.

Melchiorre K, Wormald B, Leslie K, Bhide A, Thilaganathan B. 2008. First-trimester uterine artery Doppler indices in term and preterm pre-eclampsia. *Ultrasound Obstet Gynecol* **32**: 133–137.

Okazaki S, Sekizawa A, Purwosunu Y, Farina A, Wibowo N, Okai T. 2007. Placenta-derived, cellular messenger RNA expression in

the maternal blood of preeclamptic women. *Obstet Gynecol* **110**: 1130–1136.

Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T. 2006. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. *J Med Genet* **43**: e47.

Poon LL, Leung TN, Lau TK, Lo YM. 2000. Presence of fetal RNA in maternal plasma. *Clin Chem* **46**: 1832–1834.

Purwosunu Y, Sekizawa A, Farina A, *et al.* 2007a. Cell-free mRNA concentrations of CRH, PLAC1, and selectin-P are increased in the plasma of pregnant women with preeclampsia. *Prenat Diagn* **27**: 772–777.

Purwosunu Y, Sekizawa A, Koide K, *et al.* 2007b. Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. *Clin Chem* **53**: 399–404.

Purwosunu Y, Sekizawa A, Farina A, *et al.* 2008. Evaluation of physiological alterations of the placenta through analysis of cell-free messenger ribonucleic acid concentrations of angiogenic factors. *Am J Obstet Gynecol* **198**: 124e1–124e7.

Purwosunu Y, Sekizawa A, Okazaki S, *et al.* 2009. Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma. *Am J Obstet Gynecol* **200**: 386.e1–e7.

Redman CW, Sargent IL. 2005. Latest advances in understanding preeclampsia. *Science* **308**: 1592–1594.

Romero R, Kusanovic JP, Than NG, *et al.* 2008. First-trimester maternal serum PP13 in the risk assessment for preeclampsia. *Am J Obstet Gynecol* **199**: 122.e1–e11.

Sekizawa A, Purwosunu Y, Yoshimura S, *et al.* 2009. PP13 mRNA expression in trophoblasts from preeclamptic placentas. *Reprod Sci* **16**: 408–413.

Sibai B, Dekker G, Kupferminc M. 2005. Pre-eclampsia. *Lancet* **365**: 785–799.

Than NG, Bohn H, Toth P, Bersinger N, Grudzinskas JG, Bischof P. 2004a. From basic research to clinical application of placental proteins–a workshop report. *Placenta* **25**(Suppl A): S109–S111.

Than NG, Pick E, Bellyei S, *et al.* 2004b. Functional analyses of placental protein 13/galectin-13. *Eur J Biochem* **271**: 1065–1078.

Prenat Diagn 2009; **29**: 1231–1236.
DOI: 10.1002/pd

DOI: 10.1111/j.1471-0528.2010.02491.x
www.bjog.org

Prediction of pre-eclampsia by an analysis of placenta-derived cellular mRNA in the blood of pregnant women at 15–20 weeks of gestation

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Accepted 14 December 2009. Published Online 29 January 2010.

Objective A panel of cellular mRNA markers was used to predict the occurrence of pre-eclampsia in pregnant women at 15–20 weeks of gestation.

Design Prospective cohort study.

Setting The Department of Obstetrics and Gynaecology, University of Indonesia, Cipto Mangunkusumo National Hospital, Indonesia.

Sample Peripheral blood samples from asymptomatic pregnant women.

Methods Among 660 women, 62 developed pre-eclampsia at later gestation (pre-eclampsia group) and each case was matched with five controls. Therefore, the RNA expression levels in the cellular component of maternal blood in 62 women with pre-eclampsia were compared with those in 310 controls.

Main outcome measures The cellular RNA expression levels of genes related to angiogenesis and oxidative stress were compared between pre-eclampsia and control groups. A receiver operating characteristic (ROC) curve was used to analyse the sensitivity of each available marker. A logistic regression analysis was performed to calculate the odds for each woman to be classified as a case.

Results The univariate ROC analysis identified soluble vascular endothelial growth factor receptor-1 (Flt-1) and endoglin (ENG) as the markers with the highest sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, placental growth factor (PlGF) and parity. The relative ROC curve yielded a sensitivity of 66% at a 10% 1 – specificity rate with an area under the curve of 0.884 ($P < 0.001$).

Conclusion A panel of cellular mRNA markers in maternal blood can predict the development of pre-eclampsia long before clinical onset.

Keywords Cellular RNA, endoglin, prediction, pre-eclampsia, vascular endothelial growth factor receptor-1.

Please cite this paper as: Sekizawa A, Purwosunu Y, Farina A, Shimizu H, Nakamura M, Wibowo N, Rizzo N, Okai T. Prediction of pre-eclampsia by an analysis of placenta-derived cellular mRNA in the blood of pregnant women at 15–20 weeks of gestation. BJOG 2010; DOI: 10.1111/j.1471-0528.2010.02491.x.

Introduction

Despite advances in perinatal care, pre-eclampsia (PE) is the most common cause of maternal and perinatal mortality and morbidity worldwide.[1] Recently, anti-angiogenic factors, such as soluble vascular endothelial growth factor receptor-1 (sFlt-1) and soluble endoglin (sENG), which are both produced in the placenta, have been shown to play important roles in the pathogenesis of PE.[2–5]

Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor and causes vasodilatation through the production of nitric oxide and prostacyclin.[6–8] As Flt-1 combines with VEGF and placental growth factor (PlGF), and the serum level of soluble Flt-1 increases in pregnant women who develop PE,[3] free PlGF and free VEGF in maternal serum decline prior to the development of PE.[4] Another anti-angiogenic factor is endoglin (ENG), which regulates the endothelial nitric oxide synthase activity and local vascular tone.[9] Venkatesha *et al.*[5] have reported that the placenta is a major source of soluble ENG during pregnancy, and that ENG is up-regulated in the pre-eclamptic placenta, releasing soluble ENG into the

maternal circulation, which correlates with the severity of PE.

Although the molecular mechanism regulating the production of Flt-1 and ENG in the placenta is unknown, it has been suggested that hypoxia or oxidative stress of trophoblasts is associated with the production of these factors. Li *et al.*[10] reported the up-regulation of Flt-1 to be associated with increased oxidative stress as a consequence of hypoxia in placental trophoblasts. Haem oxygenase-1 (HO-1) is known to have antioxidant, anti-inflammatory and cytoprotective functions. HO-1 is an oxygen sensor and its expression is inducible under hypoxic conditions.[11] Although low HO-1 levels in the placenta result in an abortion,[12] the up-regulation of HO-1 by adenoviral administration works protectively during pregnancy.[13] Furthermore, PE is associated with diminished placental HO-1 levels.[14] Moreover, the adenoviral overexpression of HO-1 inhibits soluble ENG release in placental villous explants, whilst also inhibiting Flt-1 production in endothelial cells.[15]

Therefore, anti-angiogenic and anti-oxidative factors are considered to play a crucial role in the pathogenesis of PE. These placental alterations in women who develop PE in later gestation are thought to begin during the first trimester, when extravillous trophoblasts remodel into the endothelial cells of the spiral arteries. The *in vivo* alteration of gene expression has been observed in the first-trimester trophoblasts from pregnant women destined to develop PE later, confirming this hypothesis.[16] In this study, tissue samples of villous trophoblasts at the time of fetal karyotype analysis were collected prospectively through chorionic villous sampling (CVS), and the mRNA expression of these genes was assessed. The results revealed that the expression levels of Flt-1, ENG, VEGF and transforming growth factor-β1 (TGFβ1) were significantly higher in the CVS tissues from pregnant women who later developed PE, whereas the levels of PlGF, HO-1 and superoxide dismutase (SOD) were lower.[16] These findings suggest that the genes associated with angiogenesis and reduced anti-oxidant stress play crucial roles in the pathogenesis of PE, and that the measurement of the expression of these factors in maternal blood may enable the prediction of the onset of PE.

Fetal/placental RNA circulates in the maternal plasma and has enabled the development of several promising approaches for the noninvasive evaluation of placental function.[17,18] Subsequently, cell-free RNA concentrations of VEGF, Flt-1 and ENG were assessed in the plasma of women with and without PE.[19] These transcripts increased in the plasma of pre-eclamptic women and correlated positively with disease severity. An additional study of postpartum samples found the mRNA transcripts to decrease rapidly after delivery, thus suggesting that the majority of the transcripts were derived from the placenta/fetus.[19] To demonstrate the possibility of the prediction of PE by cell-free RNA, the expression of seven genes, including Flt-1, ENG and VEGF, was assessed in the plasma of pregnant women between 15 and 20 weeks of gestation. It was found that this panel allowed an 84% prediction rate for PE with a 5% false positive rate at 15–20 weeks of gestation by means of a discriminant analysis model. This finding indicates that the analysis of cell-free RNA is a highly promising method for the evaluation of alterations in placental function.[20]

The expression of placenta-specific genes, such as human placental lactogen (hPL) and human chorionic gonadotrophin (hCG), has also been shown to be detectable in the cellular component of maternal blood, and the mRNA concentrations of hPL and hCG correlate with the protein assay.[17] Furthermore, the cellular mRNA concentration is approximately ten times greater than that of maternal plasma RNA.[17] These findings indicate that some trophoblasts and placental debris circulate in the blood of normal pregnant women, and that the analysis of the cellular component of maternal blood may be more suitable than maternal plasma analysis for the evaluation of alterations in placental function. Therefore, because the gene expression of anti-angiogenic factors and anti-oxidant enzymes is associated with the pathogenesis of PE, the cellular RNA expression in the blood from asymptomatic pregnant women during the early second trimester was analysed to compare the mRNA levels with the clinical outcomes.

Materials and methods

Subjects

This investigation was performed as part of a series together with previously reported studies.[19–22] The investigation was designed as a prospective cohort study in early pregnant women (gestational weeks 15–20) who visited the Department of Obstetrics and Gynaecology, University of Indonesia, Cipto Mangunkusumo National Hospital, Indonesia from mid-2005 to 2006. Singleton pregnant women without any pre-existing medical diseases at screening or antenatal complications at the time of blood drawing were invited to participate in the cohort. The pregnancies were dated by ultrasound, which was performed during the first trimester. All women provided informed consent to participate in the study, which was approved by the Research Ethics Committee of both the University of Indonesia and Showa University.

Of the 683 women enrolled, 23 with incomplete information on outcome, whose pregnancy ended before 20 weeks or who experienced stillbirth were excluded. Among the remaining 660 women, 62 developed PE. Each case was matched with five controls of the same gestational age at the time of blood testing (within 1 week and ranging

from 15 to 20 weeks), maternal weight and fetal gender. Therefore, 62 women who developed PE and 310 controls with a normal course of pregnancy were enrolled in the study. In the control group, women with fetal growth restriction (below −1.5 SD) based on the Japanese fetal growth curve (http://www.jsum.or.jp/committee/diagnostic/diagnostic) were excluded. No special management or treatment other than antenatal care was provided before the clinical signs of PE presented. If abnormalities of blood pressure and/or proteinuria were found, women were recommended to admit themselves to hospital.

Mild and severe PE and haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome was defined as described previously.[22,23] In brief, PE was defined as gestational hypertension (systolic pressure of ≥140 mmHg or diastolic blood pressure of ≥90 mmHg on two or more occasions after gestational week 20) with proteinuria (≥0.3 g/day). Severe PE was defined by the presence of one or more of the following: (i) severe gestational hypertension (systolic pressure of ≥160 mmHg or diastolic blood pressure of ≥110 mmHg on two or more occasions after gestational week 20); or (ii) severe proteinuria (≥5 g protein in a 24-hour urine specimen or ≥3 g in two random urine samples collected ≥4 hours apart).

Processing of blood samples

Peripheral blood samples (2.5 ml) were collected in PAXgene blood RNA tubes (PreAnalytic, Hombrechtikon, Switzerland) and kept at room temperature for 3 hours, and then stored at −20°C until transport to Japan at −20°C. Molecular analysis was performed at the Department of Obstetrics and Gynecology, Showa University School of Medicine, Tokyo, Japan. RNA extraction was performed according to protocols described elsewhere.[24] In brief, cellular component samples were centrifuged twice at 4000 ***g*** for 10 minutes at room temperature in order to remove the entire supernatant and any mRNA present in residual plasma. The pellet was then washed, resuspended and incubated in optimised buffer solution containing proteinase K to digest protein. A second round of centrifugation was performed to remove any residual cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube. Thereafter, 100% ethanol was added to the supernatant to adjust the binding conditions, and the resultant lysate was then applied to a PAXgene spin column (PreAnalytiX; PreAnalytic), resulting in selective binding of RNA to the silica-gel membrane of the spin column. After the column had been washed three times, pure RNA was eluted in 80 μl of RNase-free water.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription of mRNA was performed using an Omniscript RT Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR was then performed using a QuantiTect Probe PCR Kit (Qiagen). RT-PCR was performed according to the manufacturer's instructions. cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit, Qiagen) using a 2-μl aliquot of cDNA and the kit's components in a reaction volume of 20 μl. TaqMan PCR analyses for VEGF, Flt-1, ENG, PlGF, TGFβ1, P-selectin, placenta specific-1 (PLAC1), HO-1 and SOD were performed using predeveloped and commercially available primers and probe sets (Cat # Hs00900054_m1 for VEGF, Cat # Hs01052936_m1 for Flt-1, Cat # Hs00923997_g1 for ENG, Cat # Hs00182176_m1 for PlGF, Cat # Hs0000171257_m1 for TGFβ1, Cat # Hs00174583_m1 for P-selectin, TaqMan Probes for PLAC1 gene have been described previously,[21] Cat # Hs00157965_m1 for HO-1 and Cat# Hs00166575_m1 for SOD; Applied Biosystems, Foster City, CA, USA). The thermal cycling protocol used for PCR was as follows: initial denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 15 seconds and annealing at 60°C for 1 minute. Initially, each PCR assay was confirmed to be specific to mRNA and not to genomic DNA. Amplification data were collected and analysed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analysed in duplicate, and multiple negative water blanks were included in every analysis. Quantification of gene expression was performed by investigators blind to the outcome of the pregnancy. The amounts of mRNA samples were expressed in term of copies per millilitre by the method reported elsewhere.[22]

Statistical analysis

The distributions of the demographic characteristics and logarithmic mRNA concentrations were analysed after conversion to multiples of the median (MoMs). MoMs were also adjusted for the body mass index (BMI). Median values were stratified retrospectively according to the severity of PE and the development of HELLP syndrome. The non-parametric analysis of variance (Kruskal–Wallis test) and Dunn *post hoc* test were used for comparisons among and between groups. The sensitivity and 1 − specificity rate (false positive rate) were calculated for each available marker using a univariate receiver operating characteristic (ROC) curve. A multivariate analysis was performed using logistic regression to calculate the odds for each woman for classification as a case. The logistic output was adjusted for the incidence of PE in the general population (2%) by calculation of the sampling fraction, as described by Collett.[25] Finally, a ROC curve for the calculation of multivariate sensitivity was built using, as the test variable, the calculated odds for PE by a logistic regression analysis for each woman in the series.

Results

The demographics of the pregnant women from the control and PE groups are shown in Table 1. Although no differences were observed in the maternal age, BMI, gestational age at blood drawing and blood pressure at the time of blood drawing between groups, significant differences were noted in birth weight and gestational age at delivery, and these findings were consistent with those reported in the existing literature.

The values of the available markers are reported in Figure 1A–G. The mRNA expression levels of Flt-1, ENG, P-selectin and PLAC1 were higher in the PE group than in the controls, but those of PlGF and HO-1 were lower. TGFβ1, VEGF and SOD did not reach a significant value in the comparison of PE cases versus controls (Kruskal–Wallis test), and were therefore excluded from any further analysis. For all the other markers, a significant difference was found in the comparisons of HELLP cases versus controls and, for some, including Flt-1, ENG and PlGF, a significant difference was found for any generated subgroup (mild PE, severe PE, HELLP) according with the severity (Dunn *post hoc* test). A univariate analysis identified Flt-1 and ENG as markers with the highest sensitivity. Table 2 reports the output of the ROC curve analysis for those markers with a significant difference in PE cases versus controls, together with the actual MoMs for each marker associated with the given sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, PlGF and parity (Table 3). Only the final model is presented, which was obtained by stepwise logistic regression. After adjusting for the incidence of PE (2% or 1:50), the mean quoted odds (obtained by solving the logistic equation having the odds as a dependent variable for each of the subjects in the study) for PE was 1:50 (0.02) for controls and 1:7 (0.14) for women who developed PE. A ROC curve obtained using the estimated score for PE as the test variable yielded a sensitivity of 66% at a 10% 1 – specificity rate with an area under the curve of 0.884 (0.844–0.922, 95% CI; $P < 0.001$; Figure 2), and therefore with a reasonable discrimination to identify cases. Again, sensitivity and the odds for each woman for classification as PE were correlated with the severity (sensitivity of 50% for mild PE, 70% for severe PE and 75% for HELLP; odds of 1:12 for mild PE and 1:5 for both severe PE and HELLP).

Discussion

The present study examined prospectively mRNA expression in the cellular component of maternal blood samples from

Table 1. Demographic characteristics. Data are expressed as the median (minimum–maximum)

	Controls (n = 310)	Mild PE (n = 26)	Severe PE (n = 24)	HELLP (n = 12)	P value*,**
% Nulliparous	59.8	65.4	87.5	91.7	0.008***
Maternal age (years)	28 (15–43)	32 (20–40)	27.5 (19–42)	24.5 (20–35)	0.065
GA at blood test (weeks)	17 (15–20)	18 (15–20)	16 (15–18)	17.5 (15–20)	0.479
BMI at blood test	22.94 (13.79–34.22)	24.93 (19.31–33.33)	21.93 (17.42–29.48)	21.71 (18.39–30.30)	0.150
SBP (mmHg) at blood test	110 (90–130)	110 (90–125)	100 (90–120)	100 (90–130)	0.388
DBP (mmHg) at blood test	70 (60–85)	70 (60–85)	70 (60–80)	70 (60–85)	0.625
Birth weight (g)	3195 (2600–4080)	2775 (1900–3920)	2500 (2100–3900)	2400 (1800–3130)	<0.001***
Birth weight centiles	50 (14–94)	22 (1.5–92)	16 (1.5–55)	10 (1–90)	<0.001
GA at delivery (weeks)	38 (37–41)	37 (33–40)	37 (34–40)	36 (32–40)	<0.001***

*Chi-squared test.
**Kruskall–Wallis and Dunn test.
Significant comparisons: ***control versus severe PE and HELLP.
BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; HELLP, haemolysis, elevated liver enzymes and low platelets; PE, pre-eclampsia; SBP, systolic blood pressure.

Figure 1. (A–I) Box-and-whisker plots of the distribution of vascular endothelial growth factor receptor-1 (Flt-1) (A), endoglin (B), vascular endothelial growth factor (VEGF) (C), transforming growth factor-β1 (TGFβ1) (D), haem oxygenase-1 (HO-1) (E), placental growth factor (PlGF) (F), superoxide dismutase (SOD) (G), P -selectin (H) and placenta specific-1 (PLAC1) (I) mRNA levels in controls and women with pre-eclampsia, stratified in accordance with the severity of pre-eclampsia [mild and severe pre-eclampsia, and haemolysis, elevated liver enzymes, low platelet (HELLP) syndrome]. The medians are indicated by a line inside each box, and the 75th and 25th percentiles by the box limits; the upper and lower bars represent the 10th and 90th percentiles, respectively. The y-axes represent multiples of the median (MoMs) of each gene expression. Asterisks (*) indicate all specimens above or below the 90th or 10th percentile specimens. CT, control; HELLP, HELLP syndrome; mild, mild pre-eclampsia; severe, severe pre-eclampsia

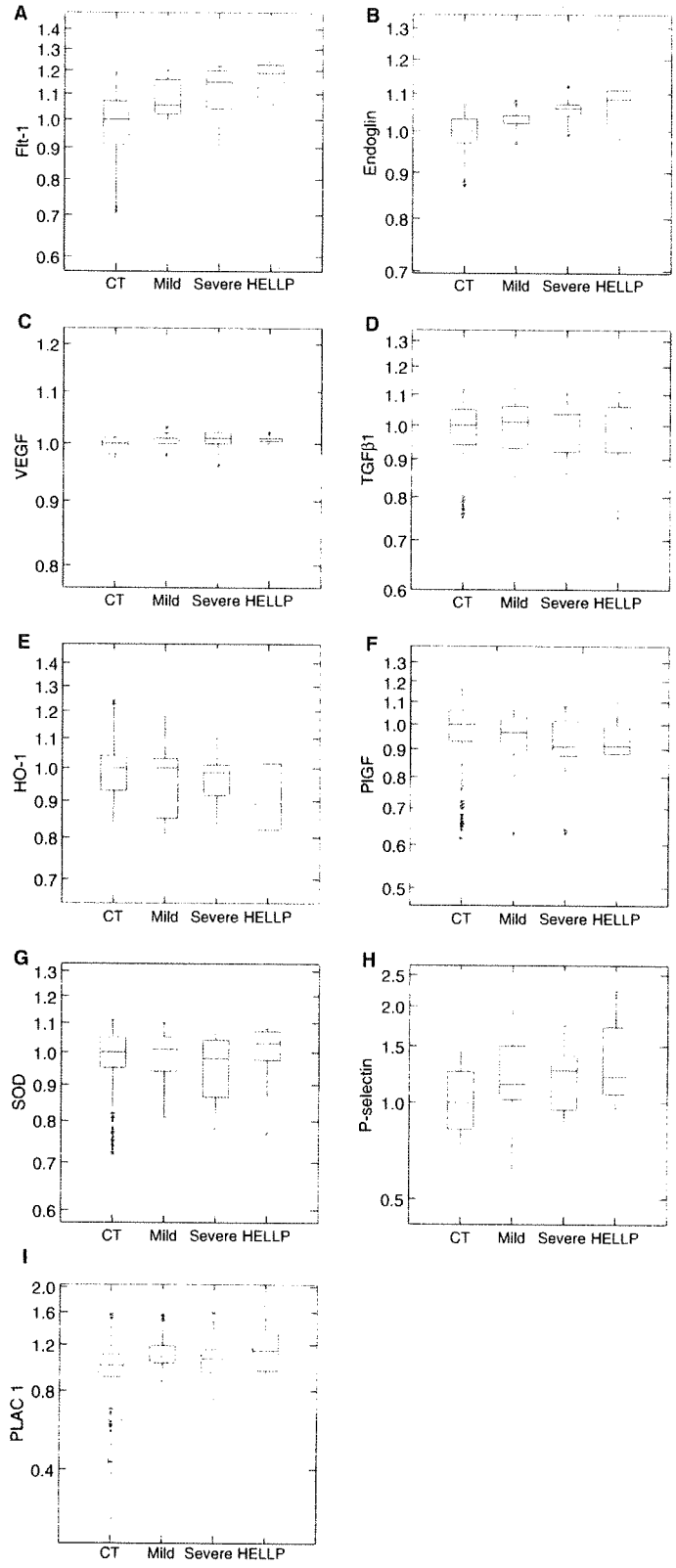

Table 2. Receiver operating characteristic (ROC) curve for each marker

mRNA	AUC	*P* value	Lower 95% bound	Upper 95% bound	Sensitivity (%) at 5% 1 – specificity	Sensitivity (%) at 10% 1 – specificity	MoM cut-off at 5% 1 – specificity	MoM cut-off at 10% 1 – specificity
P-selectin	0.665	0.039	0.588	0.742	18.2	29.1	1.61	1.45
PLAC1	0.631	0.044	0.545	0.717	20	20	1.31	1.26
Flt1	0.806	0.032	0.744	0.868	43.6	52.7	1.11	1.13
Endoglin	0.840	0.029	0.773	0.879	47.3	50.9	1.13	1.15
HO-1	0.588	0.042	0.502	0.675	8.1	14.5	0.84	0.87
PlGF	0.627	0.003	0.547	0.708	24.2	25.8	0.69	0.84

Sensitivity is shown at different cut-off values of 5% and 10% 1 – specificity.
AUC, area under the curve; HO-1, haem oxygenase-1; PLAC1, placenta specific-1; PlGF, placental growth factor.

Table 3. Logistic regression output for plasma cellular RNA levels

Variable	Odds ratio	95% CI		*P* value
		lower	upper	
Flt-1	2.760	1.910	3.988	<0.001
Endoglin	3.214	2.190	4.718	<0.001
PlGF	0.611	0.455	0.821	0.001
Parity (primi or pluri versus nulli)	2.822	1.316	6.052	0.008
Constant (ln)	–7.583			<0.001

The variables were expressed as multiples of the median (MoMs) and categorised at four levels (<25th, 25th–50th, 50th–75th and >75th percentiles).
Flt-1, vascular endothelial growth factor receptor-1; PlGF, placental growth factor.

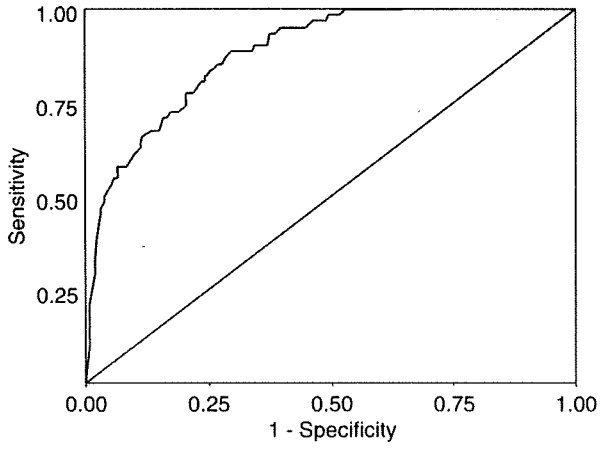

Figure 2. Receiver operating characteristic (ROC) curve obtained by plotting the values of the estimated odds for pre-eclampsia. A multivariate model consisting of endoglin (ENG), vascular endothelial growth factor receptor-1 (Flt-1), placental growth factor (PlGF) and parity was used to generate the ROC curve.

women at gestational weeks 15–20. The study assessed nine species of gene expression associated with angiogenesis and oxidative stress, which coded for factors thought to be important in the pathogenesis of PE. The mRNA levels were compared with the clinical outcomes. As a result, the mRNA expression levels of ENG, Flt-1, P-selectin and PLAC1 were found to be significantly higher in the PE group than in the controls, whereas PlGF and HO-1 levels were lower in the PE group. The blood samples were obtained at an average of 17 weeks, which is almost 20 weeks before the onset of PE. At this gestational age, the mRNA expression of anti-angiogenic factors and anti-oxidants is already altered in pregnant women who subsequently develop PE. These factors play a crucial role in the pathogenesis of PE, and the analysis of cellular components of maternal blood for these transcripts may allow for the prediction of PE.

Although many tests have been proposed for the prediction of PE, the results have been inconsistent and contradictory.[26–28] The present study has demonstrated that a panel of cellular RNA markers quantified long before clinical onset predicts PE occurrence with a degree of accuracy comparable with previous reports, including uterine artery Doppler velocimetry with or without demographic and biochemical parameters.[26] The univariate analysis showed Flt-1 and ENG to be the markers with the highest sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, PlGF and parity. The ROC curve yielded a sensitivity of 66% at a 10% 1 – specificity rate with an area under the curve of 0.884. These results indicate that cellular RNA in maternal blood can be used to assess the pathophysiological alterations which occur in pregnant women who later develop PE.

The cell-free RNA in maternal plasma was assessed in a previous study. The expression of seven transcripts was assessed in the plasma of pregnant women at gestational weeks 15–20.[20] The target genes were Flt-1, ENG, VEGF, plasminogen activator inhibitor-1 (PAI-1), tissue-type

plasminogen activator (tPA), PLAC1 and P-selectin. In the statistical univariate analysis, Flt-1 showed the highest degree of discrimination, followed by ENG, PAI-1, P-selectin, tPA, VEGF and PLAC1. The best multivariate model was obtained by the combination of all markers. A ROC curve yielded a sensitivity of 84% (95% CI, 71.8–91.5) at a 5% 1 – specificity rate with an area under the curve of 0.927 ($P < 0.001$).[20] It has been suggested previously that cellular RNA analysis is not as useful as plasma RNA analysis.[17] In our previous study of the hPL gene, the coefficient of variation of our cellular RNA analysis was approximately 20%; it was not calculated in the current study and this is a significant limitation of this study.[17] However, as cellular RNA can be preserved in the PAXgene blood RNA tube, we have confirmed that the RNA is stable below −20°C at least for 1 year. Moreover, the blood processing of cellular RNA is much easier than that of plasma RNA. In addition, the expression levels of hPL and hCG are approximately ten times higher than those in plasma RNA.[17] Therefore, we suggest that the analysis of plasma cellular RNA is a promising method for the evaluation of the pathophysiological alterations occurring in pregnant women who later develop PE. In both the previous cell-free RNA study and the present cellular RNA study, the levels of Flt-1 and ENG were two of the best predictors for PE. This indicates that these anti-angiogenic factors play a crucial role in the pathogenesis of PE.

The origin of the cellular RNA seen in maternal blood has not been resolved. In our previous study, hPL and hCG expression levels in the cellular components of maternal blood correlated with the corresponding protein levels.[17,24] This finding suggests that some trophoblasts or placental debris circulate in maternal blood. The half-times of hPL expression in cellular and plasma RNA were 203.8 and 32.2 minutes, respectively. Therefore, the half-time of cellular RNA is much longer than that of plasma RNA, and cellular RNA is not removed from the maternal circulation rapidly after delivery. The RNA originating from circulating trophoblasts in maternal blood could be detected for several months, as fetal nucleated erythrocytes reportedly circulate in maternal blood for 3 months after delivery.[29] These findings suggest that the levels of trophoblast-derived RNA in the cellular RNA in maternal blood could reflect the pathophysiological alterations of the placenta. However, real-time evaluation of placental function through cellular RNA may be inferior to the evaluation of cell-free RNA.

Another study assessed the mRNA expression of trophoblasts obtained from CVS at week 11. The expression levels of Flt-1, ENG and VEGF in CVS tissue obtained from pregnant women who later developed PE were higher, and those of PlGF and HO-1 were lower, than those of normal pregnancies.[16] These findings indicate that the up-regulation of anti-angiogenic factors and the down-regulation of anti-oxidant factors have already occurred in first-trimester trophoblasts,[16] and that the alterations could be evaluated by the analysis of cellular RNA. All of these findings support the hypothesis that some mRNA expression of Flt-1, ENG, PlGF, VEGF and HO-1 is derived from circulating trophoblasts, and that the alteration of these mRNA levels may reflect mRNA alterations associated with the pathogenesis of PE in the placenta. Therefore, it is suggested that the evaluation of cellular mRNA may allow for the indirect monitoring of placental function.

In this study, PE occurred in 9% of pregnant women, a higher frequency than seen in the published literature. No previous large studies have clarified the prevalence of PE in Indonesian populations, and this higher prevalence could confound the result. Furthermore, because the case number of gestational hypertension or early-onset-type PE was not sufficient for statistical analysis, women with hypertension in pregnancy were excluded and those with early-onset PE were not analysed separately. Further study in more developed countries is needed to confirm the predictive efficiency of cellular RNA in maternal blood.

In conclusion, the current study has demonstrated that Flt-1 and ENG expression increases in the cellular RNA in the blood from pregnant women who develop PE, whereas HO-1 and PlGF expression decreases. These alterations increase with the severity of the clinical symptoms of PE at later gestation. Furthermore, an analysis of the expression of these transcripts allows the accurate detection of high-risk pregnant women who are likely to develop PE in populations at low risk for the development of PE.

Disclosure of interest

None.

Contribution to authorship

AS, TO, YP and AF designed the research and approved the final, submitted version. AS, MN, HS, NW and YP collected, analysed and interpreted the data, and drafted the manuscript. AF and NR performed the statistical analysis.

Details of ethics approval

Approved by the ethics committee of Showa University, #86, and by the University of Indonesia, #92a/PT02.FK/2006.

Funding

AS received a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (No. 20591930), and a Grant for Child Health and Development (20C-1) and a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan. AF received a grant from the Italian Ministero dell'Università e della Ricerca—PRIN 2008.

Acknowledgements

None. ■

References

1 Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 2005;308:1592–4.
2 Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, *et al.* Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 2006;355:992–1005.
3 Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, *et al.* Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004;350:672–83.
4 Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003;111:649–58.
5 Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006;12:642–9.
6 Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 2001;280:C1358–66.
7 He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. *J Biol Chem* 1999;274:25130–5.
8 He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* 1999;13:537–45.
9 Toporsian M, Gros R, Kabir MG, Vera S, Govindaraju K, Eidelman DH, *et al.* A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. *Circ Res* 2005;96:684–92.
10 Li H, Gu B, Zhang Y, Lewis DF, Wang Y. Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta. *Placenta* 2005;26:210–7.
11 De Marco CS, Caniggia I. Mechanisms of oxygen sensing in human trophoblast cells. *Placenta* 2002;23(Suppl. A):S58–68.
12 Zenclussen AC, Sollwedel A, Bertoja AZ, Gerlof K, Zenclussen ML, Woiciechowsky C, *et al.* Heme oxygenase as a therapeutic target in immunological pregnancy complications. *Int Immunopharmacol* 2005;5:41–51.
13 Zenclussen ML, Anegon I, Bertoja AZ, Chauveau C, Vogt K, Gerlof K, *et al.* Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. *J Reprod Immunol* 2006;69:35–52.
14 Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) and soluble Flt-1 by oxygen – a review. *Placenta* 2000;21(Suppl. A):S16–24.
15 Cudmore M, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, *et al.* Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. *Circulation* 2007;115:1789–97.
16 Farina A, Sekizawa A, DeSanctis P, Purwosunu Y, Okai T, Cha DH, *et al.* Gene expression in chorionic villous samples at 11 weeks' gestation from women destined to develop preeclampsia. *Prenat Diagn* 2008;28:956–61.
17 Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. *J Med Genet* 2006;43:e47.
18 Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;46:1832–4.
19 Purwosunu Y, Sekizawa A, Farina A, Wibowo N, Koide K, Okazaki S, *et al.* Evaluation of physiological alterations of the placenta through analysis of cell-free messenger ribonucleic acid concentrations of angiogenic factors. *Am J Obstet Gynecol* 2008;198:124.e1–7.
20 Purwosunu Y, Sekizawa A, Okazaki S, Farina A, Wibowo N, Nakamura M, *et al.* Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma. *Am J Obstet Gynecol* 2009;200:386.e1–7.
21 Purwosunu Y, Sekizawa A, Farina A, Wibowo N, Okazaki S, Nakamura M, *et al.* Cell-free mRNA concentrations of CRH, PLAC1, and selectin-P are increased in the plasma of pregnant women with preeclampsia. *Prenat Diagn* 2007;27:772–7.
22 Purwosunu Y, Sekizawa A, Koide K, Farina A, Wibowo N, Wiknjosastro GH, *et al.* Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. *Clin Chem* 2007;53:399–404.
23 ACOG Practice Bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet* 2002;77:67–75.
24 Okazaki S, Sekizawa A, Purwosunu Y, Farina A, Wibowo N, Okai T. Placenta-derived, cellular messenger RNA expression in the maternal blood of preeclamptic women. *Obstet Gynecol* 2007;110:1130–6.
25 Collett D, editor. *Modelling Binary Data*, 2nd edn. Boca Raton, FL: Chapman & Hall/CRC; 2003.
26 Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. *Obstet Gynecol* 2004;104:1367–91.
27 Espinoza J, Romero R, Nien JK, Gomez R, Kusanovic JP, Gontalves LF, *et al.* Identification of patients at risk for early onset and/or severe preeclampsia with the use of uterine artery Doppler velocimetry and placental growth factor. *Am J Obstet Gynecol* 2007;196:326.e1–13.
28 Farag K, Hassan I, Ledger WL. Prediction of preeclampsia: can it be achieved? *Obstet Gynecol Surv* 2004;59:464–82; quiz 485.
29 Sekizawa A, Purwosunu Y, Matsuoka R, Koide K, Okazaki S, Farina A, *et al.* Recent advances in non-invasive prenatal DNA diagnosis through analysis of maternal blood. *J Obstet Gynaecol Res* 2007;33:747–64.

OBSTETRICS

Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma

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OBJECTIVE: The purpose of this study was to predict the occurrence of preeclampsia in a series of patients at gestational week 15-20 weeks, with the use of a panel of messenger RNA markers.

STUDY DESIGN: Data from 62 patients with preeclampsia who were asymptomatic at the time of blood testing and 310 control subjects were analyzed. Multivariable analysis was performed with discriminant analysis.

RESULTS: Univariable analysis identified vascular endothelial growth factor receptor 1 as the marker with the highest detection rate; placenta-specific 1 with the lowest. Mean estimated score for preeclampsia was 9.4 for control subjects and 72.5 for subjects who experienced preeclampsia. A receiver operating characteristic curve that was obtained with the estimated score for preeclampsia as a test variable yielded a detection rate of 84% (95% CI, 71.8-91.5) at a 5% false-positive rate with an area under the curve of 0.927 ($P < .001$). Again, detection rate and score for each patient for classification as preeclamptic correlated with severity.

CONCLUSION: A panel of messenger RNA is able to detect subjects who will experience preeclampsia.

Key words: cell-free mRNA, endoglin, prediction, preeclampsia, plasma RNA, VEGF

Cite this article as: Purwosunu Y, Sekizawa A, Okazaki S, et al. Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma. Am J Obstet Gynecol 2009;200:386.e1-386.e7.

Preeclampsia arises as a complication in 3-7% of pregnancies and remains 1 of the main causes of maternal and fetal death and morbidity. Because preeclampsia is known to have a long preclinical phase before clinically manifesting in later gestation, clinical prediction offers the possibility of redirecting maternal and prenatal care in high-risk pregnancies.[1]

Analogous to the discovery of circulating RNA in the plasma of patients with cancer, the discovery of circulating fetal/placental RNA in maternal plasma has enabled the development of several promising approaches for noninvasive evaluation of placental function.[2,3] We quantified messenger RNA (mRNA) expressions of human chorionic gonadotropin and human placental lactogen in maternal plasma.[4] The mRNA levels of human chorionic gonadotropin and human placental lactogen were correlated with the corresponding protein concentrations.[4] Evaluation of placental mRNA levels in maternal plasma therefore may allow indirect monitoring of placental function. Ng et al[5] recently demonstrated increased plasma concentrations of corticotrophin-releasing hormone (CRH) mRNA among pregnant women with preeclampsia. Farina et al[6] reported that plasma CRH mRNA correlates with clinical severity of preeclampsia.

To identify candidate genes for which mRNA expression in maternal plasma reflects placental gene expressions (including pathophysiologic alterations in preeclampsia), we conducted a microarray analysis of villous trophoblasts.[7] We then selected several target genes that are produced mainly by the placenta and that show increased protein concentrations in patients with preeclampsia. Based on the result, mRNA levels of plasminogen activator inhibitor-1 (SERPINE1), tissue-type plasminogen activator (PLAT), vascular endothelial growth factor (VEGFA), VEGFA receptor 1 (FLT1), endoglin, placenta-specific 1 (PLAC1) and selectin P (SELP) were assessed in the plasma of women with and without preeclampsia.[8-10] Expressions of all 8 genes were found to be increased in the plasma of patients with preeclampsia. All

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Received July 15, 2008; revised Sept. 16, 2008; accepted Nov. 18, 2008.

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This study was supported in part by research Grants from the Japan Association of Obstetricians and Gynecologists Ogyaa Donation Foundation Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Nos. 20591930 [A.S.] and 20791169 [S.O.]); Grant for Child Health and Development (20C-1), and Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare of Japan (A.S.) and the Takeda Science Foundation (Y.P.).

 • doi: 10.1016/j.ajog.2008.11.035

TABLE 1
Characteristics of control subjects and women with preeclampsia at enrolment and characteristics of their infants[a]

Characteristic	Control subjects (n = 310)[b]	Women with preeclampsia (n = 62)	*P* value[c]
Women			
Age (y)[d]	27.90 ± 5.37	28.90 ± 5.65	.278
Height (cm)[d]	154 ± 5.89	155 ± 6.42	.328
Weight (kg)[d]	55.58 ± 9.17	56.71 ± 9.58	.557
Body mass index[d,e]	27.37 ± 3.45	23.51 ± 3.75	.180
Systolic blood pressure (mm Hg)[d]	105 ± 9.6	106 ± 10.6	.479
Diastolic blood pressure (mm Hg)[d]	69 ± 7.1	69 ± 7.5	.933
Primigravida (n)	268 (86.4%)	19 (30.6%)	< .0002
Gestational age at enrolment (wk)[d]	17.3 ± 2.3	17.3 ± 2.2	.977
Current smoker (%)	0.9	3.2	.164
Previous hypertension (%)	1.4	3.2	.283
Previous preeclampsia (%)	1.4	0	1
Previous preterm delivery (%)	1.1	0	1
Previous fetal growth restriction (%)	0.9	1.6	.476

[a] Enrolment in this study was at gestational week 15-20. Probability values are given only for significant differences in comparison with control subjects; [b] Women with no preexisting medical diseases or antenatal complications were the control subjects; [c] Student *t* test or χ^2 test; [d] Data are given as mean ± SD; [e] Body mass index: weight in kilograms divided by the square of height in meters.

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expressions correlated positively with disease severity. An additional study of postpartum samples found that all expressions decreased rapidly after delivery, which indicates that most of these transcripts were derived from the placenta/fetus.[8-10] Furthermore, these findings also raised the question of whether those cell-free mRNAs have any significance or role in the development of preeclampsia and indicate pathologic alterations of the placenta in early pregnancy. No previous studies have explored cell-free mRNA concentrations in early gestation of pregnant women who subsequently experienced preeclampsia. In the present study, we quantified these mRNA expressions of placenta-derived genes in maternal plasma from women at gestational weeks 15-20 who were asymptomatic at the time of blood testing and assessed the possibility of predicting preeclampsia.

MATERIALS AND METHODS

Subjects

The study was designed as a prospective cohort study in early pregnant women (gestational weeks 15-20) who visited the Department of Obstetrics and Gynaecology, University of Indonesia, at Cipto Mangunkusumo National Hospital, from mid 2005-2006. All women provided informed consent to participate in the study that was approved by the Institutional Research Ethics Committee.

Of the 683 women who were enrolled, we excluded 23 women who had incomplete information about outcome, whose pregnancy ended at < 20 weeks, or who experienced stillbirth. Among the remaining 660 women, 62 women experienced preeclampsia. Each case was matched with 5 control subjects for the same gestational age at the time of blood testing, maternal weight, and fetal gender. We therefore enrolled 62 women who experienced preeclampsia and 310 control subjects with a normal course of pregnancy. We did not do any special management or treatment other than antenatal care and before clinical sign of preeclampsia. If abnormalities of blood pressure and/or proteinuria were found, the patients were recommended to be admitted to the hospital.

Mild and severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome were defined as described in a previous report.[11] In brief, *preeclampsia* was defined as gestational hypertension (systolic pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg on ≥ 2 occasions after gestational week 20) with proteinuria (> 0.3 g/d). *Severe preeclampsia* was defined by the presence of ≥ 1 of the following occurrences: (1) severe gestational hypertension (systolic pressure > 160 mm Hg or diastolic blood pressure > 110 mm Hg on ≥2 occasions after gestational week 20) or (2) severe proteinuria (≥ 5 g protein in a 24-hour urine specimen or ≥ 3 g in 2 random urine samples that were collected ≥ 4 hours apart). *Fetal growth restriction* was defined as birth weight of ≥ 2.0 SD below the mean expected weight for gestational age. The control group included pregnant women with no preexisting medical diseases or antenatal complications.

RNA extraction and real-time quantitative reverse transcription–polymerase chain reaction (PCR)

Processing of blood samples has been described previously.[4] In brief, 7-mL peripheral blood samples were collected in EDTA-containing tubes and centrifuged at 1600*g* for 10 minutes at 4°C twice. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo, Japan. Total RNA was extracted from 1.6 mL of harvested plasma. The plasma was mixed with 2 mL of Trizol LS reagent (Invitrogen, Carlsbad, CA) and 0.4 mL of chloroform. This mixture was centrifuged at 12,000*g* for 15 minutes at 4°C, then the aqueous layer was transferred to new tubes. After 1 volume of 700 mL/L ethanol was added to 1 volume of aqueous layer, the mixture was applied to a QIAamp MinElute Virus col-

TABLE 2
Characteristics of patients with preeclampsia (n = 62) at later gestation and characteristics of their infants

Characteristic[a]	Mild preeclampsia (n = 26)	Severe preeclampsia (n = 24)	HELLP syndrome (n = 12)
Women			
Age (y)[b]	31.5 (20-40)	27.5 (19-42)	24.5 (20-35)
Body mass index[b,c]	24.6 (19.3-33.3)	21.9 (17.4-29.4)	21.7 (18.4-30.3)
Systolic blood pressure (mm Hg)[b]	150 (130-160)	175 (160-195)	185 (160-180)
Diastolic blood pressure (mm Hg)[b]	90 (90-100)	115 (110-195)	110 (100-140)
Proteinuria (g/24h)[b]	0.75 (0.3-6.4)	6 (5.0-8.2)	5.55 (3.8-9.5)
Primigravida (%)	8 (30.7%)	7 (29.1%)	5 (41.6%)
Infants			
Birthweight (g)[b]	2775 (1500-3290)	2475 (1740-3900)	2400 (1600-3130)
Gestational age at delivery (wk)[b]	37.45 (33-40)	37.2 (34-40)	36.4 (34-40)
Small for gestational age, < 10th percentile (n)	8 (30.7%)	11 (45.8%)	7 (58.3%)

[a] Measurement on admission to the hospital; [b] Values represent median (minimum-maximum); [c] Body mass index: weight in kilograms divided by the square of height in meters.

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umn (Qiagen, Hilden, Germany) and processed according to the recommendations of the manufacturer. Total RNA was eluted with 20 μL of RNase-free water and directly reverse-transcripted with an Omniscript RT kit (Qiagen) in accordance with the instructions of the manufacturer. After this, complementary DNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit; Qiagen) with a 2-μL aliquot of complementary DNA and the kit components in a reaction volume of 20 μL. TaqMan PCR analyses for SERPINE1, PLAT, VEGFA, FLT1, endoglin, PLAC1, and SELP were performed with predeveloped and commercially available primers and probe sets (Cat # Hs00167155_m1 for SERPINE1, Cat # Hs00263492_m1 for PLAT, Cat # Hs00900054_m1 for VEGFA, Cat # Hs01052936_m1 for FLT1, Cat # Hs00923997_g1 for endoglin, and Cat # Hs00174583_m1 for SELP; Applied Biosystems, Foster City, CA). Primers and TaqMan-probes for PLAC1 gene have been described previously.[12] As an initial step, we verified that each PCR assay was specific to mRNA and not to genomic DNA. Amplification data were collected and analyzed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. The following thermal profile was used: 15 minutes of denaturation at 95°C, followed by 15 seconds of annealing at 94°C and 1 minute of extension at 60°C. Quantification of gene expression was performed with investigators who were blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in terms of copies per milliliter. To quantify mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described.[10]

TABLE 3
Cases and control subjects: mean (SD)

MRNA species (Log10 scale)[a]	Control subjects (n = 310)	Women with preeclampsia (n = 62)	*P* value[b]
PLAT	1.17 (0.42)	2.17 (0.52)	< .001
SERPINE1	2.27 (0.39)	2.66 (0.57)	< .001
FLT1	1.90 (0.32)	2.39 (0.32)	< .001
VEGFA	3.25 (0.27)	3.77 (0.38)	.030
Endoglin	3.66 (0.31)	3.99 (0.47)	< .001
SELP	2.42 (0.69)	2.93 (0.85)	.029
PLAC1	3.52 (0.67)	3.94 (0.86)	.040

Power ranged between 0.94 and 1 at a given type I error of 0.05.

[a] Expression levels of SERPINE1, PLAT, VEGFA, FLT1, endoglin, PLAC1, and SELP are expressed as copies per milliliter; [b] Student *t* test.

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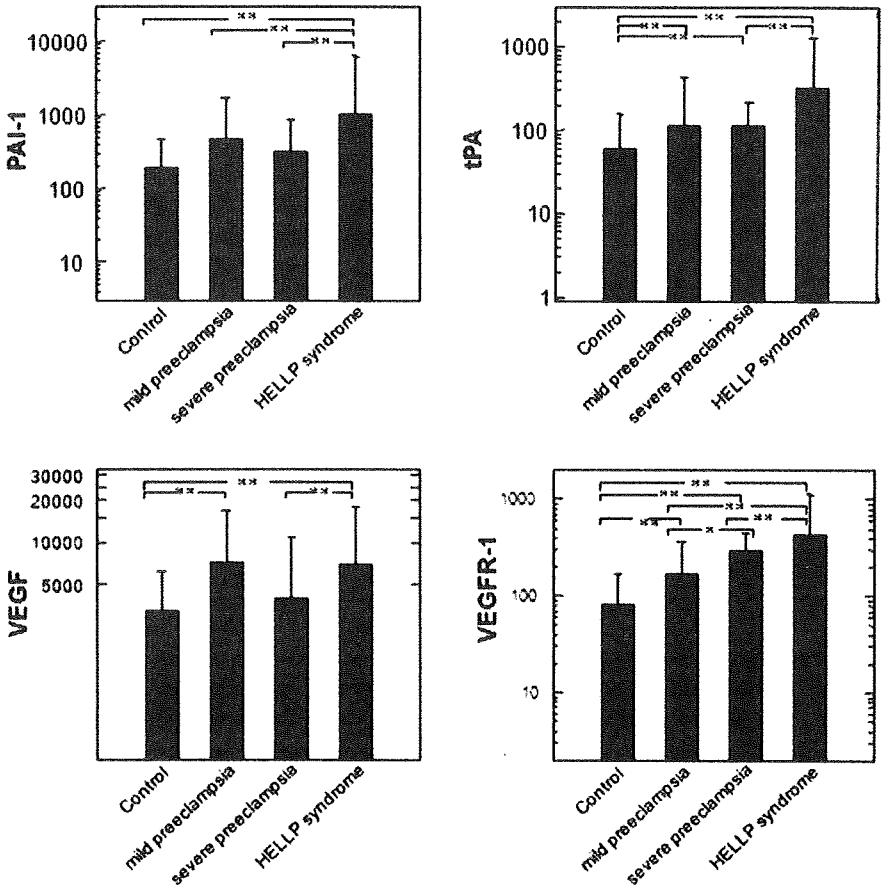
Statistical analysis

Power analysis was performed by means of PASS statistical software (PASS, Rotterdam, The Netherlands). Distributions of demographic characteristics and mRNA concentrations were analyzed by the Student *t* test and $\chi 2$ test. Mean values of the variables of interest were strat-

FIGURE 1
Messenger RNA levels



Mean (±SD mRNA levels of **A**, SERPINE1; **B**, PLAT); **C**, VEGFA; **D**, FLT1; **E**, endoglin; **F**, PLAC1, and **G**, SELP in control subjects and women with preeclampsia; the levels have been stratified in accordance with the severity of preeclampsia (mild and severe preeclampsia and HELLP syndrome). The *double asterisks* denote $P < .01$ by Scheffe post hoc test; the *single asterisk* denotes $P < .05$.

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ified retrospectively according to severity of preeclampsia and development of HELLP syndrome. Analysis of variance and relative Scheffe test were used for comparisons. Detection rate and false-positive rate were calculated for each available marker with a univariable receiver operating characteristic (ROC) curve. Discriminant analysis was used as a multivariable tool with the purpose of pulling together the detection rate of the whole set of markers. Discriminant analysis is useful to build a predictive model of group membership that was based on observed characteristics for each case. The procedure generates a discriminant function that provides the best discrimination between groups (affected vs controls) and is able to assign to each group membership a mutually exclusive score from 0-100 for belonging to the control or affected group. Because discriminant analysis needs a parametric distribution of the markers, the data were converted into Log10 scale and analyzed by Kolmogorov-Smirnov and Shapiro tests. Finally, an ROC curve for the calculation of multivariable detection rate was built with the use of the calculated score of preeclampsia for each patient in the series.

RESULTS

Table 1 shows the clinical characteristics of the pregnant women from control and preeclampsia groups. Preeclampsia occurred in 62 of 683 patients (9%), which is a higher frequency than that seen in the published literature. Although no differences were observed in age, body mass index, smoking status, gestational age at blood drawing, and blood pressure and proteinuria at the time of blood drawing between groups, significant differences were noted in birthweight, frequency of fetal growth restriction, and gestational age at delivery, which was consistent with the existing literature.[13,14] Table 2 shows detailed background of preeclampsia groups. Table 3 shows mean values and relative comparisons for each mRNA. All 7 kinds of mRNA expressions were increased significantly in the preeclampsia group. In the preeclampsia group, 26 (41.9%), 24 (38.7%) and 12 (19.4%) cases showed mild preeclampsia, severe preeclampsia, and HELLP syndrome, respectively. Figure 1 shows comparisons among control subjects, mild and severe preeclampsia, and HELLP syndrome. Almost all comparisons among groups revealed significant differences at the probability value of $< .01$. All mRNAs showed a tendency to increase according to preeclampsia severity. Only VEGFA and PLAC1 did not show any clear correlation with the severity, although concentrations were lower in control subjects than in subjects with HELLP syndrome.

We evaluated the matrix of correlation for patients with preeclampsia and identified SELP, PLAT, and SERPINE1 as the cluster of markers with the strongest associations ($P < .01$), followed by the cluster of endoglin, VEGFA, and PLAC1 ($P < .05$). Table 4 shows ROC outputs for each marker. Univariable ROC curves show FLT1 as the mRNA with the highest degree of discrimination, followed by endoglin, SERPINE1, SELP, PLAT, VEGFA, and PLAC1. All markers displayed very significant probability values. Detection rate at 5% false-positive rate ranged between 17.7% and 58.0% (Table 4). When stratified according to severity, the highest detection rate was found for HELLP syndrome (Table 5). The power of the ROC curve was 83% at a given type I error of 0.05.

TABLE 4
Output of univariable ROC curve for each available marker and multivariable ROC curve with the use of the discriminant score of the development of preeclampsia as a test variable

Variable	Area under the curve	SEM	*P* value	95% CI for area under the curve		Detection rate at 5% false-positive rate (%)	95% CI for detection rate (%)	
				Lower	Upper		Lower	Upper
FLT1	0.846	0.032	< .001	0.783	0.909	58.0	44.8	70.2
Endoglin	0.756	0.038	< .001	0.683	0.830	43.5	31.2	56.6
SERPINE1	0.732	0.037	< .001	0.660	0.805	29.0	18.5	42.1
SELP	0.727	0.033	< .001	0.662	.0792	24.2	14.5	37.0
PLAT	0.686	0.042	< .001	0.604	0.768	33.9	22.6	47.1
VEGFA	0.651	0.042	< .001	0.568	0.734	29.0	18.5	42.1
PLAC1	0.645	0.040	< .001	0.567	0.723	17.7	9.6	29.9
Score	0.927	0.025	< .001	0.877	0.976	84.0	71.8	91.5

Under nonparametric assumption
Null hypothesis: true area = 0.5

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In the multivariable model, degree of correlation among markers was taken into account. Mean estimated score for preeclampsia (range, 0-100) was 9.4 for control subjects and 72.5 for preeclampsia cases, which demonstrates that this multimarker model was able to assign a very different score for preeclampsia in those subjects who were destined to have preeclampsia, compared with those who remained control subjects for the entire pregnancy. An ROC curve that was generated by the actual score for preeclampsia that was calculated for each subject yielded a global detection rate of 83.9% and 88.7% at false-positive rates of 5% and 10%, respectively, with an area under the curve of 0.927 ($P < .001$; Figure 2). Finally, the score of each patient for classification of preeclampsia was correlated with severity. In fact, mean scores were 56.3 for mild preeclampsia, 79.3 for severe preeclampsia, and 93.7 for HELLP syndrome.

Comment

The present study, as an extension of the earlier study, prospectively examined maternal blood samples from women at gestational weeks 15-20. Because our preliminary study showed that the mRNA level of CRH was too low to quantify in the early mid-trimester samples, we eliminated CRH quantification in this study. We then assessed 7 kinds of mRNA expressions and compared them with clinical outcomes. Although standard deviations were large for each gene, all of these mRNA expressions were increased in the plasma from pregnant women who experienced preeclampsia later. The mRNA expression levels of PLAT and SERPINE1 were increased 8.9- and 8.0-fold in the preeclampsia group. The results revealed that these gene expressions are increased not only in the third trimester but also in the early second trimester. Because these cell-free mRNA expressions in plasma were cleared rapidly after delivery,[8-10] cell-

TABLE 5
Output of multivariable ROC curve with the use of the discriminant score of the development of preeclampsia as a test variable, according to the severity of the preeclampsia

Variable	Area under the curve	SEM	*P* value	95% CI for area under the curve		Detection rate at 5% false-positive rate (%)	95% CI for detection rate (%)	
				Lower	Upper		Lower	Upper
Mild preeclampsia (n = 26)	0.837	0.055	< .001	0.728	0.945	65.4	44.3	82.0
Severe preeclampsia (n = 24)	0.989	0.005	< .001	0.980	0.998	95.8	76.8	99.7
HELLP syndrome (n = 12)	0.997	0.004	< .001	0.991	1.000	100	69.8	100

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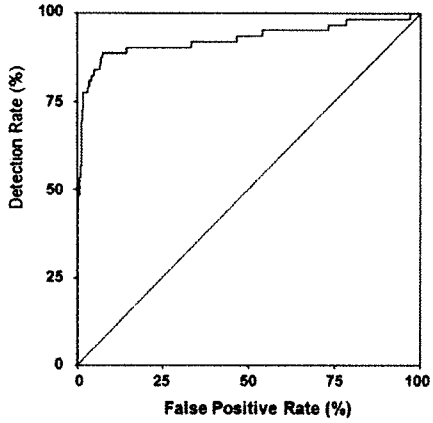
FIGURE 2
Multivariable ROC curve



Multivariable receiver operating characteristic (ROC) curve that was obtained with the discriminant score as a test variable for the prediction of preeclampsia.

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free mRNA analyses allow the evaluation of placental pathophysiologic alterations. This approach thus could have significant clinical value and should lead to the development of real-time monitoring of placental function.

Furthermore, we assessed the possibility of prediction by analyzing these transcripts in plasma. Although many tests have been proposed for the prediction of preeclampsia, results have been inconsistent and contradictory.[15-17] However, in the present study, a panel of mRNAs were dosed long before clinical onset properly predicted preeclampsia occurrence with a degree of accuracy comparable or substantially higher than that reported for Doppler ultrasonography with or without demographic and biochemical parameters.[15] Univariable analysis showed FLT1 as the marker with the highest detection rate and PLAC1 with the lowest detection rate. The best multivariable model was obtained by the combination of all markers. An ROC curve yielded a detection rate of 84% at 5% false-positive rate with an area under the curve of 0.927 ($P < .001$). To the best of our knowledge, this result offers the best prediction of preeclampsia in low-risk populations so far. Furthermore, this study also observed that the score, as generated by discriminant analysis of the development of any form of preeclampsia (mild, severe, or HELLP syndrome) that was analyzed long before clinical onset, is proportional to the degree of severity that was observed later in pregnancy. In fact, patients who had mild preeclampsia had a mean score for preeclampsia of 53, compared with 97.3 for those who had HELLP syndrome.

In the previous study of plasma mRNA from patients with preeclampsia, SERPINE1 and PLAT expressions were the most closely correlated with disease severity. However, the present study revealed that the area under the curve was highest for FLT1, followed by endoglin. These findings suggest that antiangiogenic factors such as FLT1 and endoglin play more critical roles in the earlier steps of preeclampsia development than do SERPINE1 and PLAT, whereas SERPINE1 and PLAT play important roles in the final steps of clinical manifestations of proteinuria and hypertension. FLT1 and endoglin recently have been reported to play important roles in the pathophysiologic condition of preeclampsia.[18] Protein concentrations of FLT1 and endoglin in plasma are increased before the onset of preeclampsia and correlate with disease severity.[18,19] Overexpression of FLT1 in pregnant rats results in a preeclampsia-like phenotype. Overexpression of soluble endoglin in rodents by means of adenoviral vectors also leads to increased vascular permeability and induction of modest hypertension without significant proteinuria.[18,19] Furthermore, adenoviral-mediated overexpression of both FLT1 and endoglin causes severe vascular damage, nephrotic-range proteinuria, severe hypertension, and a syndrome similar to HELLP syndrome.[20] These reports have indicated that FLT1 and endoglin from the placenta induce severe maternal endothelial dysfunction. This is concordant with the present finding that FLT1 and endoglin are likely to offer the best predictors among genes that are derived from the placenta. FLT1 and endoglin are suggested as the main factors that cause preeclampsia at gestational weeks 15-20; expressions in the placenta can be evaluated through the analysis of cell-free mRNA in plasma from pregnant women.

Although the reason that those mRNA expressions increased cannot be explained in this article, the increased mRNA expression may reflected mRNA alterations that are associated with the pathogenesis of preeclampsia in the placenta. Evaluation of cell-free mRNA may allow indirect monitoring of placental function.[4] Further research of other placental mRNA expression to resolve the mechanism that regulates the trophoblasts during early gestation may elucidate the pathogenesis of preeclampsia. Several other hypotheses that are related to increased plasma RNA have been reported,[5] but the exact mechanism that produces the increase of those mRNA expressions in maternal plasma requires further investigation.

In view of stability of mRNA in maternal plasma, Ng et al[2] have shown that placental mRNAs are very stable in maternal plasma. This stability may suggest the practicality of the mRNA marker in maternal plasma for clinical use. In this study, preeclampsia occurred in 9% of patients, which is a higher frequency than seen in the published literature. No previous large studies have clarified the prevalence of preeclampsia in Indonesia populations; this higher prevalence could confound the result. Furthermore, because the case number of gestational hypertension or early onset type preeclampsia is not enough to analyze statistically, we did not include patients of with hypertension in pregnancy and did not analyze those patients with early onset of preeclampsia separately.

In conclusion, we demonstrated that mRNA expression levels of FLT1, VEGFA, endoglin, PLAT, SERPINE1, PLAC1, and SELP are increased in plasma from pregnant women at gestational weeks 15-20 who subsequently experience preeclampsia and that alterations in placental function can be evaluated through analyses of plasma mRNA in pregnant women at early gestation. Furthermore, in populations that are at low risk of preeclampsia, a panel of these mRNA expressions allows accurate detection of high-risk pregnant women who are likely to experience preeclampsia. ■

REFERENCES

1. MacKay A, Berg C, Atrash H. Pregnancy-related mortality from preeclampsia and eclampsia. Obstet Gynecol 2001;97:533-8.

2. Ng EK, Tsui NB, Lau TK, et al. mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003; 100:4748-53.

3. Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. Clin Chem 2000;46:1832-4.

4. Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. J Med Genet 2006;43:e47.

5. Ng EK, Leung TN, Tsui NB, et al. The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. Clin Chem 2003; 49:727-31.

6. Farina A, Chan CW, Chiu RW, et al. Circulating corticotropin-releasing hormone mRNA in maternal plasma: relationship with gestational age and severity of preeclampsia. Clin Chem 2004;50:1851-4.

7. Sekizawa A. [Detection of the damage of placental villous trophoblasts and the possible pathogenesis of preeclampsia]. Acta Obst Gynaec Jpn 2003;55:830-7.

8. Purwosunu Y, Sekizawa A, Farina A, et al. Evaluation of physiological alterations of the placenta through analysis of cell-free messenger ribonucleic acid concentrations of angiogenic factors. Am J Obstet Gynecol 2008;198: 124.e1-7.

9. Purwosunu Y, Sekizawa A, Farina A, et al. Cell-free mRNA concentrations of CRH, PLAC1, and selectin-P are increased in the plasma of pregnant women with preeclampsia. Prenat Diagn 2007;27:772-7.

10. Purwosunu Y, Sekizawa A, Koide K, et al. Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. Clin Chem 2007;53:399-404.

11. American College of Obstetricians and Gynecologists. ACOG practice bulletin no.: 33. Diagnosis and management of preeclampsia and eclampsia. Int J Gynaecol Obstet 2002;77: 67-75.

12. Fujito N, Samura O, Miharu N, Tanigawa M, Hyodo M, Kudo Y. Increased plasma mRnas of placenta-specific 1 (PLAC1) and glial cells-missing 1 (GCM1) in mothers with pre-eclampsia. Hiroshima J Med Sci 2006;55:9-15.

13. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005;308:1592-4.

14. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet 2005;365:785-99.

15. Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. Obstet Gynecol 2004;104:1367-91.

16. Farag K, Hassan I, Ledger WL. Prediction of preeclampsia: can it be achieved? Obstet Gynecol Surv 2004;59:464-82; quiz 485.

17. Espinoza J, Romero R, Nien J, et al. Identification of patients at risk for early onset and/or severe preeclampsia with the use of uterine artery Doppler velocimetry and placental growth factor. Am J Obstet Gynecol 2007;196: 326.e1-13.

18. Levine RJ, Lam C, Qian C, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006;355:992-1005.

19. Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004;350:672-83.

20. Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med 2006;12:642-9.