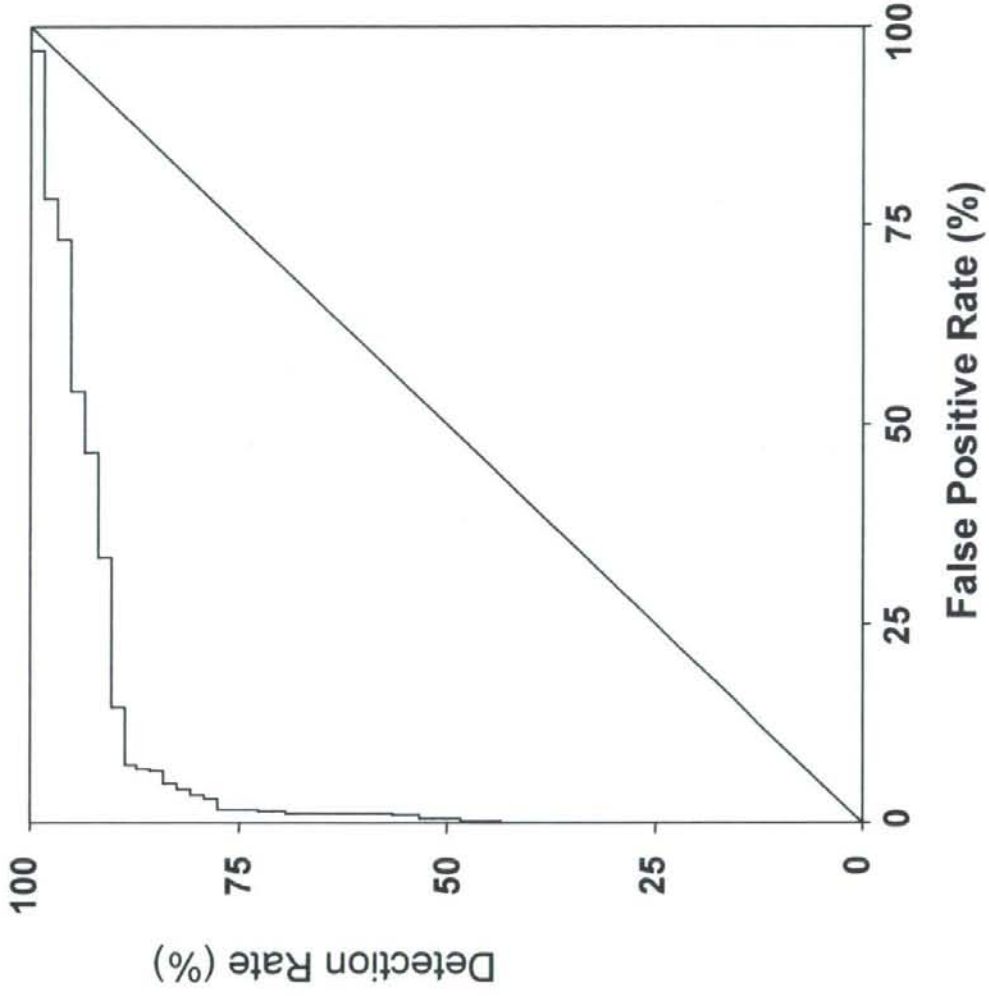
Figure 2


Detection Rate (%)
100
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False Positive Rate (%)

PREDICTION OF PREECLAMPSIA BY ANALYSIS OF CELL-FREE MESSENGER RNA IN MATERNAL PLASMA

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Condensation

Highly sensitive prediction of preeclampsia is possible by analyzing plasma RNA from pregnant women at 15-20 weeks of gestation

Title: Prediction of preeclampsia by analysis of cell-free messenger mRNA in maternal plasma

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ABSTRACT

Objective: To predict occurrence of preeclampsia in a series of patients at gestational week 15-20 using a panel of mRNA markers.

Study design: Data from 62 preeclamptic patients asymptomatic at the time of blood testing and 310 controls were analyzed. Multivariable analysis was performed using discriminant analysis.

Results: Univariable analysis identified vascular endothelial growth factor receptor 1 as the marker with the highest detection rate, and placenta-specific-1 with the lowest. Mean estimated score for preeclampsia was 9.4 for controls and 72.5 for subjects who developed preeclampsia. An ROC curve obtained using the estimated score for preeclampsia as test variable yielded a DR of 84% (95%CI 71.8-91.5) at a 5% false-positive rate with an area under the curve of 0.927 ($p<0.001$). Again, DR and score for each patient for classification as preeclamptic correlated with severity.

Conclusion: A panel of mRNA is able to detect subjects who will develop preeclampsia.

Key words: Prediction; Preeclampsia; plasma RNA, cell-free RNA, VEGF, endoglin

INTRODUCTION

Preeclampsia arises as a complication in 3-7% of pregnancies and remains one of main causes of maternal and fetal mortality and morbidity. Since 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 non-invasive evaluation of placental function [2, 3]. We quantified mRNA expressions of human chorionic gonadotropin (hCG) and human placental lactogen (hPL) in maternal plasma[4]. The mRNA levels of hCG and hPL were correlated with the corresponding protein concentrations[4]. Evaluation of placental mRNA levels in maternal plasma may therefore allow indirect monitoring of placental function. Ng et al. recently demonstrated increased plasma concentrations of corticotrophin-releasing hormone (CRH) mRNA among pregnant women with preeclampsia[5]. Farina et al. reported that plasma CRH mRNA correlates with clinical severity of preeclampsia [6].

To identify candidate genes for which mRNA expression in maternal plasma reflects placental gene expressions, including pathophysiological alterations in preeclampsia, we conducted a microarray analysis of villous trophoblasts [7]. We then selected several target genes that are mainly produced 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 (ENG), 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 preeclamptic patients. All expressions correlated positively with disease severity. An additional study of postpartum samples found that all expressions decreased rapidly after delivery, indicating that the majority 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 pathological alterations of the placenta in early pregnancy. No previous studies have explored cell-free mRNA concentrations in early gestation of pregnant women who subsequently developed preeclampsia. In the present study, we therefore quantified

these mRNA expressions of placenta-derived genes in maternal plasma from women at gestational week 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 week 15-20) who visited the Department of Obstetrics and Gynaecology, University of Indonesia, at Cipto Mangunkusumo National Hospital, Indonesia from mid-2005 to 2006. All women provided informed consent to participate in the study, which was approved by the Institutional Research Ethics Committee.

Of the 683 women enrolled, we excluded 23 who had incomplete information about outcome, whose pregnancy ended before 20 weeks, or who experienced stillbirth. Among the remaining 660 women, 62 developed preeclampsia. Each case was matched with 5 controls of same GA at the time of blood testing, maternal weight and foetal gender. We therefore enrolled 62 women who developed preeclampsia and 310 controls 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 admit 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 mmHg or diastolic blood pressure >90 mmHg on ≥2 occasions after gestational week 20) with proteinuria (>0.3 g/day). Severe preeclampsia was defined by the presence of ≥1 of the following: 1) severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >110 mmHg on ≥2 occasions after gestational week 20); or 2) severe proteinuria (≥5 g protein in a 24-h urine specimen or ≥3 g in two random urine samples collected ≥4 h apart). Fetal growth restriction was defined as birth weight ≥2.0 SD below the mean expected weight for GA. The control group included pregnant women with no preexisting medical diseases or antenatal complications.

RNA extraction and real-time quantitative reverse transcription-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 min 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 min 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 column (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 using an Omniscript RT kit (Qiagen) in accordance with the instructions of the manufacturer. After this, 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 components in a reaction volume of 20 μL. TaqMan PCR analyses for SERPINE1, PLAT, VEGFA, FLT1, ENG, PLAC1 and SELP were performed using 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 ENG, 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 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. The thermal profile used was as follows: 15 min of denaturation at 95 °C, followed by 15 s of annealing at 94 °C and 1 min of extension at 60 °C. Quantification of gene expression was performed with investigators blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in term of copies per millilitre. To quantify mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described [10]..

Statistical analysis

Power analysis was performed by means of PASS software. Distributions of demographic characteristics and mRNA concentrations were analysed by Student t-tests and $\chi 2$ tests. Mean values of the variables of interest were retrospectively stratified according to severity of preeclampsia and development of HELLP syndrome. ANOVA and relative Scheffe test were used for comparisons. Detection rate (DR) and false-positive rate (FPR) were calculated for each available marker using a univariable receiver operating characteristic (ROC) curve. Discriminant analysis was used as a multivariable tool with the purpose of pulling together the DR of the whole set of

markers. Discriminant analysis is useful to build a predictive model of group membership based on observed characteristics for each case. The procedure generates a discriminant function that provides the best discrimination between groups (affected vs. controls), able to assign to each group membership a mutually exclusive score from 0 to 100 for belonging to the control or affected group. Since discriminant analysis needs a parametric distribution of the markers, the data were converted into Log10 scale and analysed by Kolmogorov-Smirnov and Shapiro test. Finally, a ROC curve for the calculation of multivariable DR was built using the calculated score of preeclampsia for each patient in the series.

RESULTS

Table 1 describes clinical characteristics of pregnant women from control and preeclampsia groups. Preeclampsia occurred in 62 of 683 patients (9%), 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, blood pressure and proteinuria at the time of blood drawing between groups, significant differences were noted in birth weight, frequency of foetal growth restriction and

gestational age at delivery, 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 significantly increased 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. Figures 1 show comparisons among control, mild and severe preeclampsia, and HELLP syndrome. Almost all comparisons among groups revealed significant differences at the p-value<0.01 level. 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 controls than in HELLP subjects.

We evaluated the matrix of correlation for preeclampsia patients and identified SELP, PLAT and SERPINE1 as the cluster of markers with the strongest associations ($p<0.01$), followed by the cluster of ENG, VEGFA and PLAC1 ($p<0.05$). Table 4 shows ROC outputs for each marker. Univariable ROC curves show FLT1 as the mRNA with the highest degree of discrimination, followed by ENG, SERPINE1, SELP, PLAT, VEGFA and PLAC1. All markers displayed very significant p-values. DR at 5% FPR

ranged between 17.7% and 58.0% (Table 4). When stratified according to severity, the highest DR was found for HELLP syndrome (Table 5). Power of the ROC curve was 83% at a given type I error of 0.05.

In the multivariable model, degree of correlation among markers was taken into account. Mean estimated score for preeclampsia (ranging between 0 and 100) was 9.4 for controls and 72.5 for preeclampsia cases, demonstrating that this multimarker model was able to assign a very different score for preeclampsia in those subjects destined to develop preeclampsia, compared to those who remained controls for the entire pregnancy. An ROC curve, generated using the actual score for preeclampsia calculated for each subject, yielded a global DR of 83.9% and 88.7% at FPRs of 5% and 10%, respectively, with an area under the curve of 0.927 ($p<0.001$) (**Figure 2**). Finally, the score of each patient for classification as 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.

COMMENT

The present study, as an extension of the earlier study, prospectively examined maternal blood samples from women at gestational week 15-20. Since our preliminary study showed that mRNA level of CRH was too low to quantify in the early

mid-trimester samples, we eliminated CRH quantification in this study. We therefore assessed 7 kinds of mRNA expressions and compared them to clinical outcomes. Although standard deviations were large for each gene, all of these mRNA expressions were increased in the plasma from pregnant women who develop 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. Since these cell-free mRNA expressions in plasma were rapidly cleared after delivery [8-10], cell-free mRNA analyses allow the evaluation of placental pathophysiological alterations. This approach could thus 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 analysing these transcripts in plasma. Although many tests have been proposed for prediction of preeclampsia, results have been inconsistent and contradictory [15-17]. However, in the present study, a panel of mRNAs 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 demographical and biochemical parameters [15]. Univariable analysis showed FLT1 as the marker with the

highest DR and PLAC1 with the lowest DR. The best multivariable model was obtained by the combination of all markers. An ROC curve yielded a DR of 84% at 5% FPR with an area under the curve of 0.927 ($p<0.001$). 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 developing any form of preeclampsia (mild, severe or HELLP), analysed long before clinical onset, is proportional to the degree of severity observed later in pregnancy. In fact, patients who developed mild preeclampsia had a mean score for preeclampsia of 53, compared to 97.3 for those who developed HELLP.

In the previous study of plasma mRNA from preeclamptic patients, 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 ENG. These findings suggest that anti-angiogenic factors such as FLT1 and ENG play critical roles in the earlier steps of preeclampsia development than SERPINE1 and PLAT, whereas SERPINE1 and PLAT play important roles in the final steps of clinical manifestations of proteinuria and hypertension. FLT1 and ENG have recently been reported to play important roles in the pathophysiology of preeclampsia[18]. Protein concentrations of FLT1 and ENG in plasma are increased before onset of preeclampsia

and correlate with disease severity[18, 19]. Over-expression of FLT1 in pregnant rats results in a preeclampsia-like phenotype. Overexpression of soluble ENG 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 ENG causes severe vascular damage, nephrotic-range proteinuria, severe hypertension and a syndrome similar to HELLP syndrome [20]. These reports have indicated that FLT1 and ENG from the placenta induce severe maternal endothelial dysfunction. This is concordant with the present finding that FLT1 and ENG are likely to offer the best predictors among genes derived from the placenta. FLT1 and ENG are suggested as the main factors causing preeclampsia at gestational week 15-20 and expressions in the placenta can be evaluated through the analysis of cell-free mRNA in plasma from pregnant women.

Although the reason why those mRNA expressions increased can not be explained in this paper, those increased mRNA expression may reflected mRNA alterations which 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 regulating the trophoblasts during early gestation may elucidate the pathogenesis of

preeclampsia. Several other hypothesis related to increased plasma RNA have been reported[5], but the exact mechanism producing the increase of those mRNA expressions in maternal plasma requires further investigation.

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

In conclusion, we demonstrated that mRNA expression levels of FLT1, VEGFA, ENG, PLAT, SERPINE1, PLAC1 and SELP are increased in plasma from pregnant women at gestational weeks 15-20 who subsequently develop preeclampsia and that alterations in placental function can be evaluated through analyses of plasma mRNA in pregnant women at early gestation. Furthermore, in populations at low risk of

preeclampsia, a panel of these mRNA expressions allows accurate detection of high-risk pregnant women who are likely to develop preeclampsia.

Authorship information

A. Sekizawa, T. Okai, and A. Farina designed the research and approved the final, submitted version. A. Sekizawa, M. Nakamura, S. Okazaki, N. Wibowo, H. Saito and Y. Purwosunu collected, analyzed, interpreted data and drafted the manuscript. A. Farina and N. Rizzo performed statistical analysis.

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