Change in Amniotic Fluid Levels of Multiple Anti-Angiogenic Proteins before Development of Preeclampsia and Intrauterine Growth Restriction

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Context: The cause of preeclampsia remains unknown. Excessive antiangiogenic proteins have been proposed to play a pathogenic role in preeclampsia.

Objective: Our objective was to determine the differences in soluble endoglin (sEndoglin), soluble fms-like tyrosine kinase receptor-1 (sFLT1), leptin, adiponectin, and endothelin 1 concentrations between normal and preeclampsia amniotic fluid (AF). Such results may help us understand the pathophysiology of preeclampsia.

Methods: We performed a nested case-control study. Seventy-one women with preeclampsia were matched to 71 normotensive controls. The preeclamptic women were broken into two subgroups according to the association with fetal intrauterine growth restriction (IUGR). AF concentrations of sEndoglin, sFLT1, leptin, adiponectin, and endothelin 1 were measured by ELISA. Receiver-operating characteristics curve analysis was used to compare the discriminative values of these potential biomarkers. Functional network analysis was performed using MetaCore to reveal the common functions of the interacting proteins.

Results: Increased AF concentrations of sFLT1, sEndoglin, endothelin 1, and leptin were found in women who later developed preeclampsia. sFLT1, sEndoglin, leptin, and adiponectin were significantly higher in the preeclampsia with IUGR than those without IUGR. Leptin has the largest area under the curve (0.753). Network analysis revealed that elevated amniotic proteins are involved in the inflammatory process of the human placenta.

Conclusions: Significant elevation of leptin can be detected in AF 2 months earlier than the appearance of symptoms; thus, it may be used as a predictive marker for preeclampsia. The increase of these antiangiogenic proteins supports the roles of inflammation and oxidative stress in pathogenesis of preeclampsia. **(*J Clin Endocrinol Metab* 95: 1431–1441, 2010)**

Preeclampsia, which occurs in about 3–5% of all pregnancies, is one of the most common causes for maternal mortality and neonatal death (1, 2). Widespread endothelial dysfunctions triggered by placenta-derived antiangiogenic factors are considered to be major mechanisms of preeclampsia, and symptoms resolve after expulsion of the placenta. Preeclampsia is thought to be caused by the maternal systemic response to poor placen-

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Abbreviations: AF, Amniotic fluid; AUC, area under curve; BMI, body mass index; CV, coefficient of variation; IUGR, intrauterine growth restriction; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; ROC, receiver-operating characteristic; sEndoglin, soluble endoglin; sFLT1, soluble fms-like tyrosine kinase receptor-1; SMAD, phosphorylated mothers against decapentaplegic; STAT, signal transducer and activator of transcription; VEGFR, vascular endothelial growth factor receptor.

tal formation. Recently some authors proposed that preeclampsia is not only an endothelial disease but a wide systemic inflammatory disease, which results from oxidative stress rather than hypoxia (3, 4). However, the pathophysiology of preeclampsia is not completely clear.

Endoglin, also named CD105, is a coreceptor of TGF-β1 and -3, which is highly expressed in the cell membranes of vascular endothelium and syncytiotrophoblasts (5, 6). The TGF-β receptors bind TGF-β1 and -3, and endoglin modulates the action of these ligands, acting as an intracellular signaling mediator and regulating transcriptional responses (7). However, soluble endoglin (sEndoglin) is a placenta-derived soluble form of endoglin. This hypoxia-induced protein is associated with the subsequent development of preeclampsia (8).

Soluble fms-like tyrosine kinase 1 (sFLT1), also known as soluble vascular endothelial growth factor receptor (VEGFR)-1, is a truncated form of the VEGFR-1 that is secreted by the human placenta (9). Circulating sFLT1 binds the proangiogenic proteins, vascular endothelial growth factor and placental growth factor, and prevents their interactions with endothelial cell receptors, thereby inducing endothelial dysfunction (10). Recent studies demonstrated that both circulating and placental sFLT1 expression is essential in the pathogenesis of preeclampsia (11–13). In addition, elevated sFLT1 levels have also been identified in intrauterine growth restriction (IUGR) pregnancies (14).

Endothelin-1, a potent endogenous vasoconstrictor peptide, is involved in the regulation of vascular tone in hypertensive states, and increased blood concentrations of endothelin 1 are associated with preeclampsia (15). Thus, endothelin-1 was proposed to be one of the key links between primary placental disorders and the systemic endothelial dysfunction of preeclampsia (16).

Leptin, normally produced by peripheral adipocytes, is also secreted by human amnion cells into amniotic fluid (AF) (17). The human placenta expresses a high amount of leptin mRNA, and the excessive placental leptin release is thought to be secondary to hypoxia in nonobese preeclamptic women (18). Hypoxia is shown to up-regulate placental leptin gene expression (19), and increased placental leptin synthesis is associated with preeclampsia (20). Furthermore, leptin also plays a key role in T cell activation (21).

Adiponectin is an insulin-sensitizing and antiinflammatory protein released by adipocytes. It suppresses endothelial activation, and positive immunostaining for adiponectin has been observed in endothelial cells of preeclamptic placental tissues (22). The role of adiponectin in preeclampsia remains unclear, and serum levels of adiponectin are found to be increased (18, 23, 24) or decreased (22, 25) in patients with preeclampsia. However, the AF concentrations of adiponectin and preeclampsia have not been determined.

The initiating events in preeclampsia, even before preeclampsia onset, are postulated to be reduced uteroplacental perfusion and poor placentation (4, 26). Thus, we hypothesized that changes in these protein levels in AF might reflect the early pathophysiology of preeclampsia before the appearance of clinical manifestations. In the second trimester of pregnancy, the AF concentrations have been described for sFLT1 (27), endothelin-1 (28), and leptin (29), but those of sEndoglin, and adiponectin are yet to be defined. Our study appears to be the first to simultaneously analyze the concentrations of multiple antiangiogenic proteins in AF in an attempt to understand the pathophysiology of preeclampsia.

Subjects and Methods

Study population

From 2002 to 2008, we prospectively stored AF of 7283 pregnant women who underwent genetic amniocentesis at Chang Gung Memorial Hospital, Lin-Kou Medical Center. The various reasons for amniocentesis included advanced maternal age, high levels of maternal Down syndrome serum markers, fetal ultrasound anomalies, previous history of chromosomal anomalies, a balanced chromosomal translocation carrier in either of the parents, thalassemia carriers, and maternal request.

Preeclampsia was defined by gestational hypertension, proteinuria, and hyperuricemia, with the reversal of hypertension and proteinuria after delivery (30). Gestational hypertension is defined as systolic blood pressure of more than 140 mmHg or diastolic blood pressure of more than 90 mm Hg, beginning after 20 wk of gestation in previously normotensive women. Proteinuria is defined as more than 300 mg protein in a 24-h urine collection or more than 2+ on a voided or more than 1+ on a catheterized random urine sample (31). The time of preeclampsia onset is defined as the time of detection of elevated blood pressure or urinary protein measurement leading to the diagnosis of preeclampsia. Fetal IUGR was defined as women with infant birth weight less than the fifth percentile. Percentiles for growth parameters were derived from a reference Taiwanese population (32).

The flow chart of patient enrollment is summarized in Fig. 1. Patients with obesity, multiple pregnancies, gestational or overt diabetes, chronic hypertension with superimposed preeclampsia, renal disease, or any known metabolic disorders (*e.g.* hyperthyroidism, hyperlipidemia) were excluded to avoid any possible bias such as the association of leptin with diabetes (33) or obesity with preeclampsia (34). The obesity defined as body mass index (BMI) greater than 27 kg/m^2 during midtrimester amniocentesis. The exact gestational age, fetal growth, and amniotic fluid index of all cases were all ascertained by serial ultrasonography. At amniocentesis, fetuses with growth restriction, oligohydramnios, or polyhydramnios were all excluded. From the same amniotic fluid bank, we used matching criteria of maternal age,

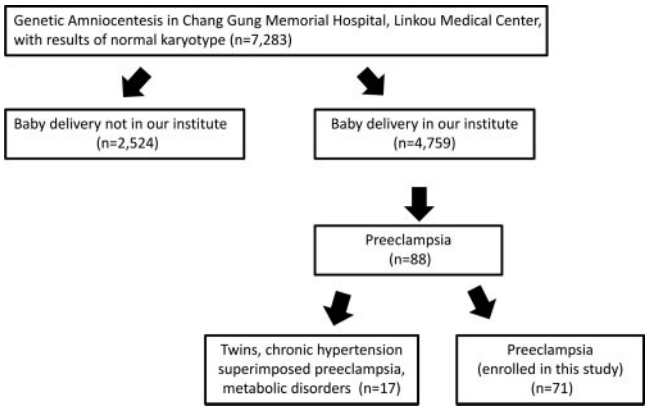

FIG. 1. The flow chart of patient enrollment.

parity, gestational age, and blood pressure during the time of the amniocentesis and BMI to select women who had uncomplicated, normotensive pregnancies free of the aforementioned medical complications and deliveries of healthy babies as the control group. This study was approved by the Ethical Committee of Chang Gung Memorial Hospital (institutional review board no. 93-6366 and 97-2024B).

Procedures of specimen collection

Before amniocentesis was performed, written informed consents were obtained from all participants. In this study, genetic amniocenteses were performed between 16 and 19 gestational weeks at our hospital. Using a transabdominal approach, about 20 ml of AF were aspirated from each patient. After amniocytes were collected via centrifugation for culture and cytogenetic studies, we consecutively stored 4 ml of fresh AF in a −80 C freezer.

Immunoassay procedures

All of the commercially available ELISAs were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) as previously described (35). The AF concentrations of sEndoglin and adiponectin were measured without prior dilution. The minimal detectable limits of the assays for sEndoglin and adiponectin were 0.007 and 0.246 ng/ml, respectively. The intraassay coefficients of variation (CVs) for sEndoglin and adiponectin ELISA were 2.8 and 3.2%, respectively, and the interassay CVs were 6.9 and 7.9%, respectively (n = 20).

Before the AF sFLT1 concentrations were analyzed, AF specimens were diluted to 1:100 in the calibrator diluent buffer that was included in the ELISA kit. The minimal detection limit of the sFLT1 was 3.5 pg/ml, the intraassay CV was 2.6%, and the interassay CVs was 9.8% (n = 20). Before the AF concentrations of leptin were determined, AF samples were diluted to 1:50 with the calibrator diluent buffer. The minimal detection limit of the leptin ELISA was 7.8 pg/ml, the intraassay CVs was 3.3%, and interassay CVs was 5.4% (n = 20).

On the other hand, endothelin-1 in AF needed to be concentrated before the ELISA was performed. Before AF concentration of endothelin-1 were measured, 0.5 ml of each AF was evaporated down in a centrifugal evaporator (minimum drying time 4 h at 37 C) and then reconstituted with 0.125 ml of sample diluent. The minimal detection limit of the endothelin-1 ELISA was 1.0 pg/ml, the intraassay CVs was 4.6%, and the interassay CVs was 6.5% (n = 20).

Statistical analysis

In this study, we used χ^2 tests to compare categorical variables and Pearson's correlation to analyze the relationships of all permutation pairs of antiangiogenic markers. Student *t* tests, one-way ANOVA tests, and *post hoc* Scheffé tests were used to analyze continuous variables (preeclampsia with IUGR, preeclampsia without IUGR, and normal controls). Binary logistic regression with the forward stepwise method was used for multivariate analysis. Receiver-operating characteristic (ROC) curve analysis was used to evaluate the discriminative values of the preeclamptic markers. For all analyses, $P < 0.05$ was considered statistically significant. All statistics were carried out using SPSS version 12 for Windows (SPSS Inc., Chicago, IL).

Bioinformatics analysis

Procedures of networks analysis to elucidate the biologic processes of differentially expressed proteins in amniotic fluid of the patients who later develop preeclampsia were similar to what we have reported (36). Briefly, we used the analyze networks algorithm in MetaCore (GeneGo, St. Joseph, MI) to build the networks from five input genes: adiponectin, endoglin, endothelin-1, FLT1, and leptin (37, 38). MetaCore is a Web-based computational platform designed for systems biology and drug discovery. It includes a curated database of human protein interactions and metabolism; thus, it is useful for analyzing a cluster of genes in the context of regulatory networks and signaling pathways (39). For the network analysis of a group of genes, MetaCore can be used to calculate the statistical significance (*P* value) based on the probability of assembly from a random set of nodes (genes) of the same size as the input list (40).

Results

Characteristics of the patients

One hundred forty-two pregnant women who underwent genetic amniocentesis were enrolled in this study. The preeclampsia group consisted of 71 women who subsequently developed preeclampsia, and the control group consisted of 71 women with uncomplicated pregnancies. There was no significant difference in maternal age, BMI, parity, gestational age at amniocentesis, or smoking behavior between groups. The karyotypes of all enrolled cases were normal. As expected with the nature of this disease, women with preeclampsia had higher blood pressures during delivery, lower gestational weeks at delivery, and lower birth weight (all were $P < 0.05$) than the control women. Likewise, the birth weights of neonates were significant lower in the preeclampsia women with IUGR than the preeclampsia women without IUGR ($P < 0.05$). The onset of preeclampsia was 26.6 ± 3.1 wk (mean ± SD), with a range of 21 to 33 wk. Patients' characteristics are summarized in Table 1.

TABLE 1. Characteristics of the entire study population

	PE without IUGR (n = 39)	PE with IUGR (n = 32)	Control (n = 71)	*P* value
Age (yr)	35.4 ± 3.4	34.6 ± 3.9	35.1 ± 1.4	NS
Nulliparity (%)[#]	17 (43.6)	18 (56.3)	37 (52.1)	NS
BMI at AC	21.9 ± 2.2	21.8 ± 2.3	21.2 ± 3.0	NS
BMI at delivery	27.5 ± 3.1	28.3 ± 2.8	27.2 ± 2.8	NS
SBP at AC (mm Hg)	118.2 ± 13.7	116.8 ± 11.6	115.7 ± 12.1	NS
DBP at AC (mm Hg)	73.3 ± 10.2	69.7 ± 8.2	69.6 ± 8.6	NS
SBP at delivery (mm Hg)	171.6 ± 15.8[a]	168.2 ± 15.3[b]	125.3 ± 10.0[a,b]	<0.001*
DBP at delivery (mm Hg)	102.6 ± 8.8[c]	101.4 ± 10.2[d]	76.9 ± 8.5[c,d]	<0.001*
GA at AC (wk)	17.3 ± 0.9	17.5 ± 0.9	17.8 ± 1.0	NS
GA at delivery (wk)	33.6 ± 2.8[e]	33.3 ± 3.0[f]	38.6 ± 1.2[e,f]	<0.001*
Birth weight (g)	2042 ± 620[g,h]	1470 ± 527[g,h]	3109 ± 333[g,h]	<0.001*
Smoking behavior (%)[#]	2 (5.1)	1 (3.1)	2 (2.8)	NS

The values shown are mean ± SD (Student *t* test). The data were analyzed with Scheffé *post hoc* tests and are presented as mean ± SEM. PE, Preeclampsia; BW, body weight; AC, amniocentesis; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; NS, not significant, defined as $P \geq 0.05$ (*P* values are given only for significant differences).

[#] Data were calculated by χ^2 test; * significant difference by one-way ANOVA test ($P < 0.05$).

[a] $P < 0.001$; [b] $P < 0.001$; [c] $P < 0.001$; [d] $P < 0.001$; [e] $P < 0.001$; [f] $P < 0.001$; [g] $P < 0.001$; [h] $P < 0.001$.

Amniotic fluid sEndoglin, sFLT1, endothelin-1, leptin, and adiponectin concentrations between preeclampsia and control groups

The AF concentrations of sEndoglin, sFLT1, endothelin-1, and leptin were significantly higher in the preeclampsia than in the control groups. However, there were no significant differences in adiponectin concentrations between two groups (Table 2). We also analyzed these proteins in only primigravida (labeled as nulliparity in this study) and identified similar results (Supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). In women with preeclampsia, there was no significant difference in these AF protein levels between nulliparity and multiparity (Supplemental Table 2).

TABLE 2. AF concentrations of leptin, sEng, sFlt-1, endothelin-1, and adiponectin between the PE and control groups

	PE (n = 71)[a]	Control (n = 71)[a]	*P* value
Leptin (ng/ml)	7.11 ± 0.46	4.65 ± 0.33	<0.001
sEndoglin (ng/ml)	0.99 ± 0.82	0.68 ± 0.25	0.001
sFLT1 (pg/ml)	67834 ± 5539	47648 ± 2315	0.001
Endothelin-1 (pg/ml)	57.56 ± 12.55	28 ± 3.43	0.025
Adiponectin (ng/ml)	20.52 ± 2.28	16.09 ± 0.8	NS

PE, Preeclampsia; NS, not significant, defined as $P \geq 0.05$ [*P* values are given only for significant differences (Student t test)].

[a] Results are represented as mean ± SEM.

Amniotic fluid antiangiogenic proteins between the preeclampsia with IUGR and the preeclampsia without IUGR groups

One-way ANOVA revealed significant differences ($P < 0.05$) in the AF concentrations of leptin, sEndoglin, sFLT1, and adiponectin among the preeclampsia with and without IUGR and the control groups (Table 3). In *post hoc* Scheffé tests, sEndoglin and leptin were significantly increased in the preeclampsia with IUGR and the preeclampsia without IUGR groups, compared with the control group (all were $P < 0.05$). There was no significant difference in leptin and sEndoglin between the preeclampsia with IUGR and the preeclampsia without IUGR groups. The AF concentrations of sFLT1 in the preeclampsia with IUGR, but not the preeclampsia without IUGR, were significantly higher than that of the controls ($P = 0.001$). The AF concentrations of adiponectin were significantly higher in the preeclampsia with IUGR group than in either the preeclampsia without IUGR group ($P = 0.008$) or the control group ($P = 0.004$), despite a lack of a significant difference between the preeclampsia without IUGR and control groups.

Correlations among the amniotic fluid concentrations of sEndoglin, sFLT1, endothelin-1, leptin, and adiponectin

To examine the relationships among the five antiangiogenic proteins in pathogenesis of preeclampsia, we performed Pearson's correlation tests (n = 142) on all of the 10 protein pairs (Fig. 2). There were significantly positive correlations of sEndoglin with sFLT1, leptin, and adiponectin (all were $P < 0.001$). sFLT1 was positively correlated with leptin, endothelin-1, sEndoglin, and adi-

TABLE 3. AF concentrations of leptin, sEng, sFlt-1, endothelin-1, and adiponectin among three study groups

	PE without IUGR (n = 39)	PE with IUGR (n = 32)	Control (n = 71)	*P* value
Leptin (ng/ml)	6.83 ± 0.68[a]	7.47 ± 0.68[b]	4.65 ± 0.33[a,b]	<0.001
sEndoglin (ng/ml)	0.99 ± 0.13[c]	0.98 ± 0.09[d]	0.68 ± 0.03[c,d]	0.003
sFLT1 (pg/ml)	59263 ± 7440	78279 ± 8036[e]	47648 ± 2315[e]	0.001
Endothelin-1 (pg/ml)	50.01 ± 17.17	66.76 ± 18.53	28.0 ± 3.43	NS
Adiponectin (ng/ml)	15.3 ± 2.25[f]	26.9 ± 4.01[f,g]	16.09 ± 0.8[g]	0.001

The data were analyzed with Scheffé *post hoc* tests and are presented as mean ± SEM. *P* values are given only for significant differences (one-way ANOVA). PE, Preeclampsia; NS, not significant, defined as $P \geq 0.0$.

[a] $P = 0.002$; [b] $P = 0.01$; [c] $P = 0.02$; [d] $P = 0.02$; [e] $P = 0.001$; [f] $P = 0.008$; [g] $P = 0.004$.

ponectin (all were $P < 0.05$). Also, there were positive correlations between the leptin *vs.* adiponectin pair ($P < 0.001$) and the endothelin-1 *vs.* adiponectin pair ($P < 0.001$). Only two pairs were not significantly correlated: endothelin-1 *vs.* leptin and endothilin-1 *vs.* sEndoglin (data not shown).

Multivariate analysis

The association between antiangiogenic proteins and preeclampsia was further studied by a logistic regression model. Among these proteins, leptin ($P < 0.001$) and sFLT1 ($P = 0.008$) were factors independently associated with preeclampsia occurrence.

ROC curve analyses

We used the ROC curve analyses to evaluate the usefulness of each of the AF proteins for identifying women with subsequent development of preeclampsia from the control women. When we compared the preeclampsia (n = 71) with the control (n = 71) groups, the areas under curve (AUC) of leptin, sEndoglin, sFLT1, endothelin-1, and adiponectin were 0.753 ($P < 0.001$), 0.655 ($P = 0.002$), 0.655 ($P = 0.002$), 0.614 ($P = 0.02$), and 0.499 (P = NS), respectively (Fig. 3A). Only AF leptin is considered to have a discriminative value (AUC > 0.7). When the preeclampsia with IUGR (n = 32) and the control (n = 71) groups were compared, the AUC of leptin, sEndoglin, sFLT1, endothelin-1, and adiponectin were 0.774 ($P < 0.001$), 0.676 ($P = 0.004$), 0.750 ($P < 0.001$), 0.680 ($P = 0.003$), and 0.655 ($P = 0.012$), respectively (Fig. 3B). Both leptin and sFLT1 have potentially discriminative values for the preeclampsia with IUGR cases. However, when the preeclampsia without IUGR (n = 39) and the control (n = 71) groups were analyzed, the AUC of leptin, sEndoglin, sFLT1, endothelin-1, and adiponectin were 0.750 ($P < 0.001$), 0.609 (P = NS), 0.562 (P = NS), 0.611 (P = NS), and 0.374 (P = NS), respectively (Fig. 3C). Only leptin can discriminate the preeclampsia without IUGR cases from the controls. On the other hand, the combined use of any protein pair did not increase the AUC in ROC analyses (Supplemental Table 3).

Functional network

Using MetaCore algorithm for network analysis, we found that leptin, endoglin, FLT1, endothelin-1, and adiponectin interacted in the network of pathways with

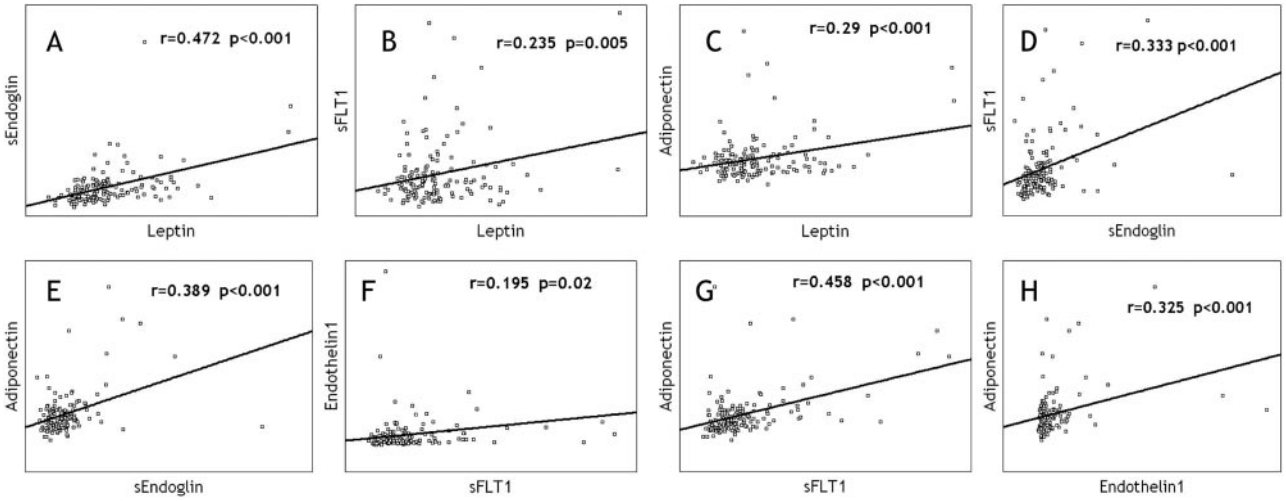

FIG. 2. Significant correlations exist in eight of 10 pairs between amniotic fluid antiangiogenic proteins. Leptin and sEndoglin (A), leptin and sFLT1 (B), leptin and adiponectin (C), sEndoglin and sFLT1 (D), sEndoglin and adiponectin (E), sFLT1 and endothelin 1 (F), sFLT1 and adiponectin (G), and endothelin-1 and adiponectin (H) are shown.

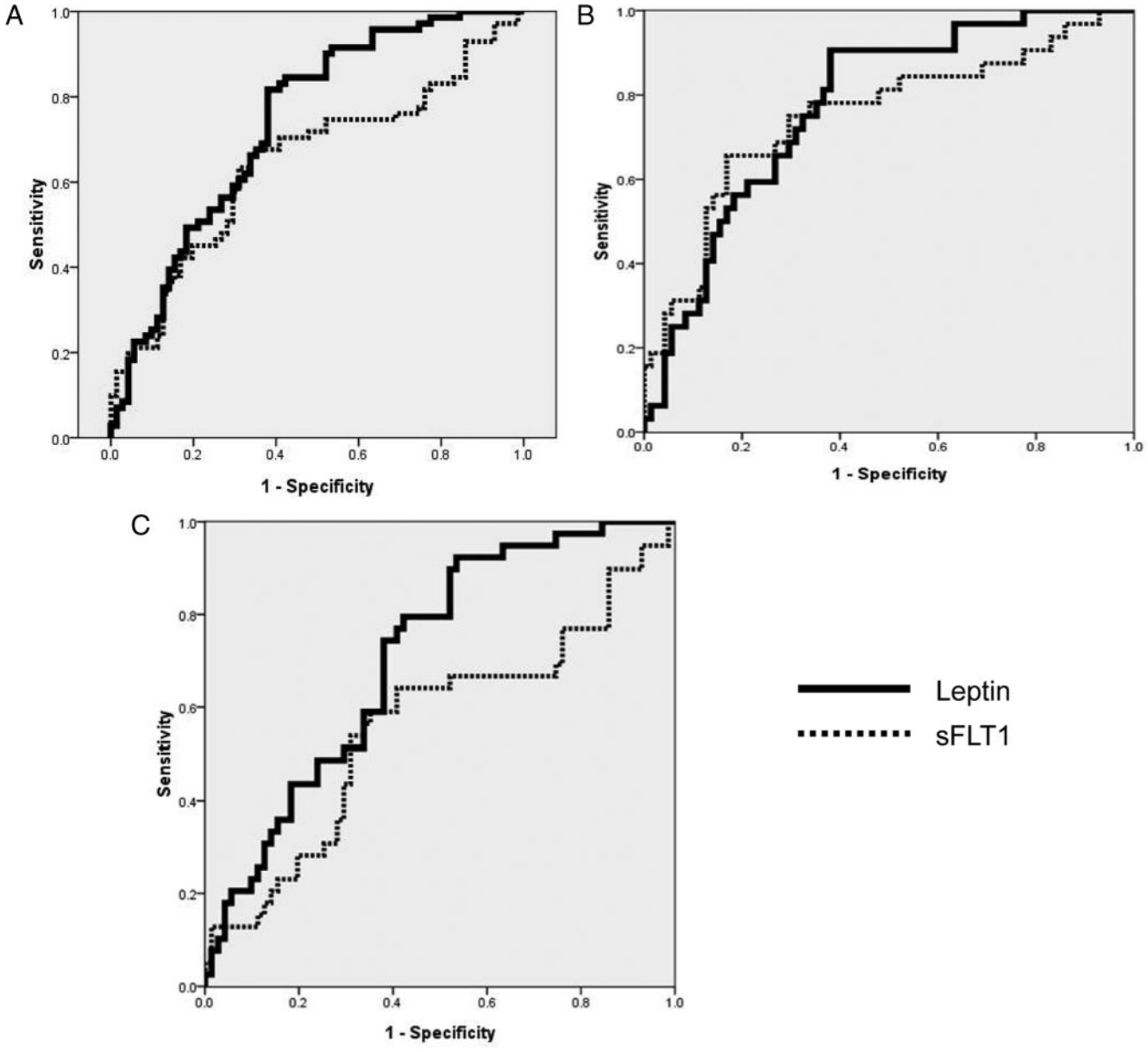

FIG. 3. ROC curve analyses in three comparison groups. ROC curves between the preeclampsia (n = 71) and the control (n = 71) groups (A), ROC curves between the preeclampsia with IUGR (n = 32) and the control (n = 71) groups (B), and ROC curves between the preeclampsia without IUGR (n = 39) and the control (n = 71) groups (C) are shown. AUC and *P* values are described in the text. Only the plots with AUC greater than 0.7 are shown here (leptin and sFLT1).

$P = 1.14 \times 10^{-12}$ (Fig. 4), indicating that the probability of assembly from random sets of nodes (genes) was very low (40). Through functional analyses using the Disease identification and Tissue localization modules of MetaCore Suites, this network was related to inflammation at $P = 8.26 \times 10^{-10}$ and located to placenta at $P = 3.5 \times 10^{-15}$, respectively. Among the genes of this network, there are several inflammation modulating proteins such as signal transducer and activator of transcription (STAT)-3, SMA- and MAD-related protein 3 (SMAD)-3, nuclear factor-κB (NF-κB), and c-Jun N-terminal kinases (JNKs). Results of these analyses further support our hypotheses of these proteins are relevant to pathogenesis of preeclampsia.

Discussion

This study provides the most comprehensive information about multiple AF antiangiogenic protein concentrations in a cohort of 4795 women with complete obstetrical follow-up; among them 88 subsequently developed preeclampsia (Fig. 1). The large amniotic fluid bank, 7283 AF from 2002 to 2008, allowed us to set strict criteria for inclusion of preeclampsia cases, both with IUGR (n = 32)

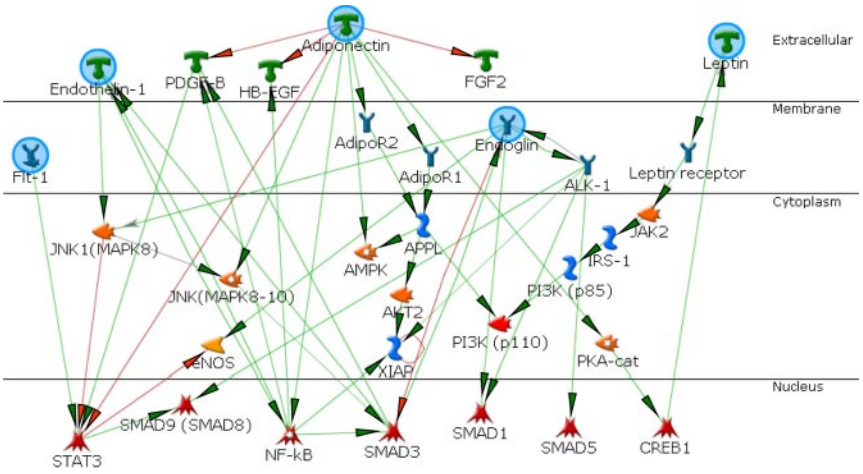

FIG. 4. Functional network analysis of five AF antiangiogenic proteins that are shown as *filled blue circles*. *Green lines* and *arrowheads* indicate stimulation, whereas *red lines* and *arrowheads* indicate inhibition.

and without IUGR (n = 39), and matched control subjects (n = 71). Unfortunately, we did not have maternal serum collected at the time of amniocentesis; hence, we could not compare protein concentrations between AF and serum. We like to point out that our results were derived from an Asia population. In Asia population, the incidence of preeclampsia was around 1.4–2% (41), which is lower than the worldwide incidence of 3–5% (1), and the Taiwanese fetal growth charts (32) are also different from those in Western populations (42).

In our institute, most cases who underwent amniocentesis were because of advanced maternal ages (age > 34 yr). Therefore, in this cohort, about half of the cases who later developed preeclampsia were multipara. Although our institute is a tertiary referral center, most referred cases with severe preeclampsia did not receive amniocentesis in our institute; thus, they were not enrolled in this study. Furthermore, after we excluded the cases who did not deliver in our hospital, our incidence of preeclampsia in the study cohort was 1.9% (88 of 4759), which was within the range of reported incidences in Taiwan (41, 43). So we consider that the excluded cases did not result in bias in the study population.

The main source of antiangiogenic proteins in AF during midtrimester amniocentesis is still unknown. The AF begins to fill in amnion since the 12th day after fertilization. At this stage, the likely mechanism of AF generation is active transport of solute by the amnion into the amniotic space (44). The fetus is not the major producer of AF until midtrimester (45), and fetal urine does not account for the major volume of AF until 20 wk of gestational age (46). Therefore, the AF proteins analyzed in this study are most likely to be derived from the placenta, not the fetus.

Results of this study indicate increased AF levels of leptin, sEndoglin, sFLT1, endothelin-1, and adiponectin were associated with subsequent development of preeclampsia, either with or without IUGR (Tables 2 and 3). Significantly positive correlations were also found among these proteins (Fig. 2), so a network analysis was performed according to the MetaCore database to elucidate common functions of these proteins. Using merely a couple of input genes, Analyze network algorithm of MetaCore was able to reveal additional information that was not present in the original list of root nodes (36, 47). Our network analyses revealed that adiponectin, endothelin-1, FLT1, and endoglin interacted with several inflammation modulating proteins, NF-κB, SMAD3, JNKs, and STAT3, supporting a recent theory that inflammation in the placenta plays a central role in the pathogenesis of preeclampsia (4). In addition to receiving activating signals from adiponectin (38), NF-κB can be activated by endothelin (48) and vice versa (49). SMAD3 up-regulates endothelin-1 (50) and endoglin-1 (51), but it receives negative feedback from endoglin-1 (52). JNKs may play modulating functions in several pathways: JNKs are activated by adiponectin (53), endoglin-1, and endothelin-1 (54), and JNKs can further activate SMAD3 (55) but inhibit STAT3 (56). STAT3 also moderates the effects from multiple pathways: it can be inhibited by both adiponectin (53) and JNKs (56) but activated by FLT1 (VEGFR-1) (57). Furthermore, this functional network also related endoglin-1 (58), endothelin-1, sFLT1, and adiponectin, all through STAT3 (59) to endothelial nitric oxide synthase, further supporting their roles in regulation of vascular tone and hypertension. Nevertheless, the networks derived from our study remain to be validated by *in vitro* studies.

Excessive placental leptin secretion (18, 20) and elevated AF concentrations of leptin (60) are associated with preeclampsia development. Leptin is secreted during acute inflammation (61). Inflammatory cytokines, such as TNF-α and IL-6, are significantly correlated with elevated leptin in women with preeclampsia (62). Results of our study further strengthen the importance of leptin in the pathogenesis of preeclampsia, which is also associated with inflammatory processes. Because the elevation of AF leptin was identified 2 months earlier than the appearance of symptoms, our results further suggest that AF leptin may be used as a predictive biomarker for preeclampsia.

Increased plasma sEndoglin concentrations are detected 2 months before the onset of preeclampsia (8). A reduced placental perfusion leading to hypoxia is thought

to increase endoglin expression in IUGR pregnancies (63). The low placental oxygenation is believed to be responsible for the growth restriction seen in IUGR pregnancies (64, 65). In our study, the sEndoglin concentrations were significantly elevated in both the preeclampsia with and without IUGR groups, but there was no significant difference between preeclampsia with and without IUGR groups. Although Staff *et al.* (66) reported increased AF concentrations of sEndoglin in term preeclamptic pregnancies, our study is the first report of the association between the elevation of AF sEndoglin in early second trimester and subsequent development of preeclampsia (Tables 2 and 3).

Increased placental production and serum level of sFLT1 are thought to be crucial in the pathogenesis of preeclampsia (11, 67–69). Low oxygen conditions stimulate the secretion of sFLT1, and the increased sFLT1 levels exacerbate the oxidative stress that contributes to vascular dysfunction (70, 71). In addition to preeclampsia, previous studies also found an association between increased sFLT1 and fetal growth restriction (72–74). Levine *et al.* (8, 12) first demonstrated that maternal plasma concentrations of sEndoglin and sFLT1 increase 5–8 wk before preeclampsia occurrence, although Smith *et al.* (75) reported that high concentrations of plasma sFLT1 in 10–14 wk of gestation are not associated with the risk of preeclampsia. Park *et al.* (27) did not detect increased sFLT1 in amniotic fluid in preeclampsia women at the time of the midtrimester amniocentesis. Similarly, we did not detect a significant difference between the preeclampsia without IUGR and the control groups (Table 3). However, there was a significant increase in AF sFLT1 concentrations in women with preeclampsia complicated by IUGR, which, by definition, is severe preeclampsia (Table 3). Placental hypoxia and inflammatory stimulation are thought to be the triggers for sFLT1 release from preeclampsia placentas (4, 76). Results of this study further implicate placenta-derived sFLT1 as an important player in the pathogenesis of preeclampsia.

By analyzing the largest ever reported sample size of AF in this study (n = 142), we found significantly increased AF concentrations of endothelin-1 in the women who subsequently developed preeclampsia (Table 2). These results confirm the findings that increased AF concentrations of endothelin-1 in women who later developed preeclampsia in two previous reports, one with nine cases of preeclampsia (28) and another with 12 cases of IUGR (54). A clear trend is that the AF concentrations of endothelin-1 are highest in severe preeclampsia, followed by the mild preeclampsia, and then the control groups (Table 3). We speculate that the complex assay procedures of endothelin-1, especially the concentrating and reconstituting steps, may add variations to the final endothelin 1 concentrations, resulting in a lack of statistical significance on the difference among endothelin-1 concentrations in Table 3.

This study is the first report of increased AF adiponectin concentrations preceding the appearance of clinical symptoms of preeclampsia, although increased plasma adiponectin concentrations have been reported in preeclamptic women (23). Masuyama *et al.* (77) proposed that the role of adiponectin is to maintain endothelial function, and its deficiency leads to endothelial dysfunction or hypertension. Thus, an elevation of circulating adiponectin concentrations might represent a compensatory response to endothelial dysfunction. Significantly elevated concentrations of adiponectin in the preeclampsia with IUGR than the preeclampsia without IUGR groups (Table 3) further indicate that adiponectin levels may reflect the severity of preeclampsia.

In conclusion, the changes of amniotic antiangiogenic proteins, which occur before the onset of clinical symptoms, may shed new insight to our understanding of the pathophysiology of preeclampsia. Network analyses reveal the role of inflammation in the pathophysiology of preeclampsia, supporting such recent hypotheses that preeclampsia may not only result from a hypoxic placenta but may also be induced from a widespread inflammatory response followed by oxidative stress (3, 4). Among the antiangiogenic proteins, an increase in leptin and sEndoglin may occur in the early pathogenesis process of preeclampsia, but it may not be directly associated with its severity. Both sFLT1 and endothelin-1 may be involved in pathogenesis of preeclampsia and reflect its severity. However, increased levels of adiponectin appear to occur later in the pathophysiology of preeclampsia; thus, it is elevated only in severe preeclampsia.

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