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Gene Expression in First Trimester Preeclampsia Placenta

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Abstract

Background—The goal of this study was to further validate eight candidate genes identified in a microarray analysis of first trimester placentas in preeclampsia.

Material and method—Surplus chorionic villus sampling (CVS) specimens of 4 women subsequently diagnosed with preeclampsia (PE) and 8 control women (C) without preeclampsia analyzed previously by microarray and 24 independent additional control samples (AS) were submitted for confirmatory studies by quantitative real-time polymerase chain reaction (qRT-PCR).

Results—Downregulation was significant in FSTL3 in PE as compared to C and AS ($p = .04$). PAEP was downregulated, but the difference was only significant between C and AS ($p = .002$) rather than between PE and either of the control groups. Expression levels for CFH, EPAS1, IGFBP1, MMP12, and SEMA3C were not statistically different among groups, but trends were consistent with microarray results; there was no anti-correlation. S100A8 was not measurable in all samples, probably because different probes and primers were needed.

Conclusions—This study corroborates reduced FSTL3 expression in the first trimester of preeclampsia. Nonsignificant trends in the other genes may require follow-up in studies powered for medium or medium/large effect sizes. qRT-PCR verification of the prior microarray of CVS may support the placental origins of preeclampsia hypothesis. Replication is needed for the candidate genes as potential biomarkers of susceptibility, early detection, and/or individualized care of maternal–infant preeclampsia.

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Keywords

gene expression; preeclampsia; CVS; qRT-PCR; microarray validation; pregnancy complication

Preeclampsia remains a leading cause worldwide of maternal and fetal morbidity and mortality (Duley, 2009; Khan, Wojdyla, Say, Gulmezoglu, & Van Look, 2006). Later life burden in survivors continues with increased occurrence of cardiovascular disease (Irgens, Reisaeter, Irgens, & Lie, 2001). Prevention of preeclampsia is hindered by incomplete knowledge of its etiology. We do know that impaired trophoblast invasion, particularly of myometrial spiral arteries, and their failed physiologic remodeling characterize the placental bed in preeclampsia (Lyall, 2002; Meekins, Pijnenborg, Hanssens, McFadyen, & van Asshe, 1994). Although not specific to preeclampsia, we also know that shallow implantation plays a role in the origins of this pregnancy complication (Roberts, Pearson, Cutler, Lindheimer, & NHLBI Working Group, 2003). Much remains to be elucidated, however, about the precise early molecular maternal–fetal interface in normal and aberrant placentation.

Prior microarray analyses of placental tissues were conducted to identify potential contributors to the pathogenesis of preeclampsia (reviewed in Founds, Dorman, & Conley, 2008). These studies, however, could not differentiate cause from effect of the disease in placentas after delivery in the second or third trimesters. Gene expression studies of candidates in first trimester surplus chorionic villus sampling (CVS) specimens found that vascular endothelial growth factor receptor-1 (VEGFR-1, also known as fms-like tyrosine kinase-1 [Flt-1]), vascular endothelial growth factor (VEGFA), endoglin (Eng), and transforming growth factor-β1 (TGF-β1) were upregulated and placental growth factor (PlGF), heme oxygenase-1 (HO-1), and superoxide dismutase (SOD) were downregulated in preeclampsia compared to control pregnancies (Farina et al., 2008).

To address the gap in unbiased discovery-based global gene expression differences in early placentas of preeclampsia, we conducted a microarray analysis with surplus CVS (Founds et al., 2009). This study was the first to demonstrate the feasibility of obtaining good RNA integrity and successful methods for identifying dysregulated genes and pathways in first trimester preeclampsia versus normal pregnancy. Experimental variability was minimal, such that no statistical transformation of raw microarray signal was required in normalizing data across samples. Enrichment was enhanced by five analytic strategies conducted within the software package used and by pathways analysis (detailed in Curtis, Oresic, & Vidal-Puig, 2005 and Founds et al., 2009). We found 36 differentially expressed genes, including 20 not previously associated with the disease, which represent biologically based candidate genes that may serve as markers of susceptibility or early detection months before onset of clinical disease at mid- or later gestation (Founds et al., 2009). In contrast to Farina's candidate gene study (2008), our microarray analysis of over 53,000 transcripts indicated no evidence of hypoxia gene dysregulation in first trimester preeclampsia.

The current report focuses on the results of quantitative real-time polymerase chain reaction (qRT-PCR) studies we conducted to follow up on a subset of 8 genes selected from among the 36 identified by the microarray analysis of CVS (Table 1 includes gene names, symbols, and known functions; Founds et al., 2009). The number of candidates tested by qRT-PCR was determined by the limited amount of mRNA remaining from the specimens that had undergone the microarray analysis. The most dysregulated known genes were prioritized (S100A8, MMP12, PAEP, EPAS1, IGFBP1, and FSTL3). Others (SEMA3C and CFH) were chosen based on known functions (Table 1) and potential relationships to the two gene networks identified by bioinformatics analysis of the 36 candidate genes. Network 1 included Cancer, Respiratory Disease, and Cellular Movement, whereas Network 2 included

Inflammatory Disease, Cellular Movement, and Hematological System Development and Function. The overall interpretation of these pathways was that impaired immune regulation decreased trophoblast proliferation and invasion in the first trimester of preeclampsia (Founds et al., 2009).

Materials and Method

Surplus CVS Specimens

Samples were selected from a bank of 160 collected in 2001–2005 under protocols approved by the university ethical review board. Clinical CVS is the only available access to first trimester placental biopsies for fetal karyotyping in women who will be of advanced maternal age at delivery or who have family or personal history of genetic abnormality. Surplus tissues were snap frozen within 10 min of clinical CVS procedures; no extra tissue was biopsied for the research. Specimens were stored at −80 °C until pregnancy outcome data became available postpartum.

A total of 36 CVS specimens were submitted for qRT-PCR case control studies. These consisted of mRNA from the 4 preeclampsia (PE) and 8 matched control (C) samples that had been analyzed by microarray, plus 24 unaffected additional samples (AS) analyzed for replication purposes. Matching was based on parity, gestational age at CVS within 3 days, and race. Case sample size represented all the PE samples that occurred in the specimen bank according to the study's inclusion/exclusion criteria, reflecting a relatively low incidence of preeclampsia (~3%) in a low-risk patient population. PE was defined as the new onset of hypertension and proteinuria after 20 weeks' gestation, with systolic blood pressure ≥140 and/or diastolic blood pressure ≥90 on at least two occasions at least 6 hr apart prior to labor and administration of medications, and >300 mg of protein in a 24-hr urine within 72 hr of hypertension (Roberts et al., 2003). PE participants had no underlying medical disorders or other obstetrical complications. C and AS specimens were donated by normotensive women with blood pressure < 140/90, no proteinuria, and no other pregnancy complication or medical disorder. Comprehensive description of the clinical data was previously reported (Founds et al., 2009).

qRT-PCR

Total RNA was optimized by mechanical disruption during homogenization of the frozen specimens and extracted by standard protocols as previously reported (Founds et al., 2009). cDNA was generated from 0.1 μg of total mRNA per sample using the high capacity cDNA reverse transcription kit (Applied Biosystems [ABI]; Foster City, CA). qRT-PCR was conducted using parameters specific for the ABIPRISM 7000 system (ABI). Each gene was evaluated in duplicate for every sample and the average value used for analyses. Pre-designed probe and primer sets were used (Table 1) and ribosomal protein large P0 (RPLP0; ABI assay #Hs99999902) was used as the endogenous control for normalization of the raw data. RPLPO was selected after data generated using the endogenous control plate (ABI) indicated consistent expression of RPLPO and the most appropriate control for the CVS tissue.

Data Analysis

Clinical and demographic characteristics of participants who donated CVS specimens were analyzed by analysis of variance (ANOVA; SPSS 15.0), using $p \leq .05$ as the level of significance.

Relative gene expression was determined by the comparative C_t method (Dharmaraj, 2007). Raw cycle threshold (C_t) values were determined using SDS v1.1 (S100A8 0.20) and SDS

v1.2 ABI software (CFH 0.24, EPAS1 0.14, FSTL3 0.08, IGFBP1 0.03, MMP12 0.14, PAEP 0.07, and SEMA3C 0.16). ΔC_t was calculated using the average of duplicate values for each sample normalized to the average value of RPLPO in the same well (Dharmaraj, 2007). $\Delta C_{t,\ \text{reference}}$ was the C_t value for the calibrator sample value normalized to RPLPO. The calibrator was a referent control CVS specimen from a nulliparous nonsmoker who had characteristics of an average control-group participant (see Results section; Arany, 2008; Dharmaraj, 2007). The difference between ΔC_t and the average calibrator expression value resulted in $\Delta\Delta C_t$. $2^{(-\Delta\Delta Ct)}$ determined the fold change in expression level relative to the calibrator sample. Prior to analyses, qRT-PCR data were screened and all influential data points were removed. Formal tests (Shapiro-Wilk) for normality indicated that fold change distributions by group were not normally distributed, thus nonparametric analyses were implemented. Descriptive information of qRT-PCR data can be found in Table 2. Analyses of fold changes were accomplished by computing exact p values for the multigroup case using Kruskal-Wallis tests, with significance set at $p \leq .05$. Analyses were carried out in Excel and SAS 9.2 (SAS Institute Inc., Cary, North Carolina).

Results

Clinical and Demographic Data

All women sampled were 35–44 years of age, White, and had normal fetal karyotype. Mean maternal age was 38.1 years with no difference between groups ($p = .59$). Mean gestational age was 11.4 weeks and groups were not different ($p = .66$). All mothers in the PE group were nulliparous, as were the majority of the C and AS groups ($p = .51$). PE, C, and AS groups also did not differ by body mass index.

qRT-PCR Data

Relative quantitation of gene expression by qRT-PCR of eight candidates showed that FSTL3 was significantly underexpressed in the PE group (Table 3). Differences in FSTL3 expression occurred between the PE and C groups and between the PE and AS groups (p=.04). There was also a significant difference in PAEP expression overall ($p = .01$), with a significant difference between the C and AS groups ($p = .002$) and non-significant differences between the PE and C groups ($p = .08$) and the PE and AS groups ($p = .46$). S100A8 was reported as “undetermined” by the ABI software with the probes and primers used. Values for the endogenous control RPLPO were consistent among all samples for every gene evaluated (C_t range 23–29). Although not statistically different, trends in expression of the remaining five genes by qRT-PCR were similar to the relative up-/downregulation found in the microarray results (MMP12, CFH, IGFBP1, EPAS1, SEMA3C; Table 3).

Discussion

The results of the current qRT-PCR studies are generally consistent with the findings of our prior microarray analysis of surplus CVS in pregnancies with preeclampsia and unaffected outcomes (Founds et al., 2009). Expression levels for FSTL3 were significantly different between cases and controls (Table 3). Expression levels for six genes (MMP12, PAEP, EPAS1, IGFBP1, CFH, and SEMA3C) trended with the microarray findings, while one gene, S100A8, was not detected in this qRT-PCR analysis. There was no anti-correlation or fold change in the opposite direction between the qRT-PCR results compared with the microarray data for any gene, lending further support to the consistency of the qRT-PCR expression levels with those in the microarray study (Arikawa et al., 2008; de Reyniès et al., 2006).

S100A8 had been relatively highly upregulated in preeclampsia in the prior microarray analysis (Table 3). Stable endogenous control values in every sample of the qRT-PCR substantiates that the most plausible explanation for the indeterminate levels in the current study was use of incorrect pre-designed probe sequences. An alternative assay for S100A8 will be used for follow up of this gene.

Replication of qRT-PCR results between the PE and C groups by the AS group occurred in six genes, but the difference in PAEP between C and AS was unexpected. Reanalysis was conducted by combining C and AS into a single control group for comparison with PE, but the difference remained nonsignificant ($p = .91$).

FSTL3 was downregulated in preeclampsia in the current study. The calculated effect size for expression levels in FSTL3 was 0.6, which is a large effect in the sample sizes tested (Cohen, 1988). This calculation will be used to estimate the required sample size to achieve necessary power in future studies of the candidate genes identified in the CVS microarray analysis (Founds et al., 2009).

FSTL3 is located on chromosome 19p13 and is a member of the follistatin-module protein family, a group of extracellular matrix-associated glycoproteins that regulate morphogenesis, growth, and differentiation factors (Hayette et al., 1998). FSTL3 mRNA is expressed by trophoblast, decidua, and fetal membranes both at early and at term gestation, with the most intense staining detected in the walls of decidual and placental blood vessels (Ciarmela et al., 2003). FSTL3 is a 27 kDa-secreted glycoprotein that acts through paracrine control (Hayette et al., 1998). It binds activin A on trophoblast cells, reducing the ability of activin A to bind to its receptor, thereby altering processes such as cellular growth and differentiation, immune response, and response to injury/wound healing (Biron-Shental et al., 2008).

The current study validates our previous findings of FSTL3 underexpression in first trimester placental tissues of preeclampsia. Controversy exists in the literature, however, in that other researchers found FSTL3 to be overexpressed in the placenta and maternal serum in severe preeclampsia (Pryor-Koishi et al., 2007; Sitras et al., 2009). These contrasting results could be attributed to later gestational age of the placenta at delivery and sampling, effects of disease on the placenta, and effects of labor on placental gene expression (Founds et al., 2008). Hypoxia, a characteristic feature of pre-eclamptic placentas, was shown to enhance FSTL3 expression in cultured human trophoblasts (Biron-Shental et al., 2008). Our CVS microarray analysis in preeclampsia, however, demonstrated no evidence of dysregulated hypoxia gene expression at the average gestational age of 11.4 weeks (Founds et al., 2009). Okamoto and colleagues (2006) also found FSTL3 to be upregulated in placentas of pregnancies complicated by intrauterine growth restriction (IUGR).

Finally, it is reassuring that some of the trends observed in the differential expression of other genes in this qRT-PCR study are indirectly supported by the literature. For example, PAEP was reported to be decreased in the decidua of patients with IUGR, preeclampsia and hemolysis, elevated liver enzymes, and a low platelet count syndrome (HELLP; Jeschke et al., 2005). More-over, circulating IGFBP-1 concentrations were found to be lower in early pregnancy before development of preeclampsia (Anim-Nyame, Hills, Sooranna, Steer, & Johnson, 2000).

Limitations

Although the qRT-PCR studies could not be powered for generalizable findings due to the inherent limitations of sample and patient chart availability, the results in these arduously attained surplus CVS with known outcomes are nonetheless provocative. Trends in some of

the qRT-PCR results may be nonsignificant because of small samples sizes, especially the preeclampsia cases, but expression levels that were found to be statistically different will inform power analyses in future studies. The indications for clinical CVS procedures may impose limitations on relating the research findings to younger women, multiparous women, or to women of diverse racial/ethnic groups.

Conclusion

The qRT-PCR study corroborates reduced FSTL3 expression in the first trimester of preeclampsia pregnancies. Others of the genes that trended with the microarray data could be analyzed in qRT-PCR studies powered for medium or medium/large effect sizes. Larger studies of CVS specimens to replicate and further validate differential gene expression are needed to advance understanding of the pathogenesis and identify bio-markers of preeclampsia. The hypothesis that preeclampsia originates with placental gene expression dysregulation may be reinforced by the qRT-PCR support of the microarray analysis in first trimester tissues. Robust biomarkers of preeclampsia could eventually lead to screening tests for prevention, early detection, and appropriate individualized treatments for women and their offspring, thereby reducing maternal and perinatal morbidity and mortality (Founds, 2009).

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Table 1

Genes Submitted to Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Gene Title	Gene Symbol	Affymetrix Probe set ID	RefSeq	ABI Assay ID	Function[a]
S100 calcium binding protein A8	S100A8	202917_s_at	NM_002964	Hs00374264_g1	Endothelium, immune cell function
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	203789_s_at	NM_006379	Hs00170762_m1	Neural/axonal development, angiogenesis, endothelium, metabolism
Complement factor H /// complement factor H-related 1	CFH	215388_s_at	NM_000186	Hs00164830_m1	Endothelium, immune cell function
			NM_001014975		
			NM_002113		
Follistatin-like 3 (secreted glycoprotein)	FSTL3	203592_s_at	NM_005860	Hs00610505_m1	Insulin/glucose metabolism
Insulin-like growth factor binding protein 1	IGFBP1	205302_at	NM_000596	Hs00236877_m1	Insulin/glucose metabolism
			NM_001013029		
Endothelial PAS domain protein 1	EPAS1	242868_at	NM_001430	Hs01026142_m1	Angiogenesis, endothelium, metabolism
Progestagen-associated endometrial protein (placental protein 14, pregnancy-associated endometrial alpha-2-globulin, alpha uterine protein)	PAEP	206859_s_at	NM_001018049	Hs00171462_m1	Angiogenesis, endothelium
			NM_002571		
Matrix metallopeptidase 12 (macrophage elastase)	MMP12	204580_at	NM_002426	Hs00159178_m1	Common marker between 2 networks

Note. ABI = Applied Biosystems; RefSeq = Reference Sequence database built by National Center for Biotechnology Information (NCBI).

[a]Referenced in Founds et al., 2009.

Table 2

Descriptives of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Data for Eight Candidate Genes

Gene Symbol	Group	Minimum	Maximum	Median	Standard Deviation
SEMA3C	PE	0.08	2.03	1.53	0.86
	C	0.72	6.04	1.97	2.06
	AS	0.31	5.37	1.00	1.34
CFH	PE	0.05	1.42	0.35	0.60
	C	0.04	6.06	0.99	2.28
	AS	0.17	11.84	0.83	3.79
FSTL3	PE	0.47	3.00	0.63	1.22
	C	1.00	9.09	2.35	3.35
	AS	0.79	58.49	3.71	13.66
IGFBP1	PE	0.01	0.17	0.01	0.08
	C	0.01	1.75	0.30	0.70
	AS	0.00	0.35	0.04	0.11
EPAS1	PE	0.25	2.31	1.02	0.92
	C	0.29	2.63	0.95	0.83
	AS	0.30	63.12	0.84	13.58
PAEP	PE	0.09	2.40	1.57	1.09
	C	0.74	40.50	2.92	15.56
	AS	0.01	7.04	0.45	1.71
MMP12	PE	0.49	3.13	0.73	1.25
	C	0.46	42.67	14.96	15.38
	AS	0.30	77.94	3.42	24.94
S100A8	PE, C, AS	Undetermined	Undetermined	Undetermined	—

Note. AS = additional control samples; C = control samples; PE = preeclampsia samples.

Table 3

Comparison of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Values With Prior Microarray Expression for Eight Candidate Genes in Preeclampsia Chorionic Villus Sampling (CVS) Specimens

			qRT-PCR[c]				
Gene Symbol	**Microarray[a]**	**Trend[b]**	**PE (*n* = 4)**	**C (*n* = 8)**	**AS (*n* = 24)**	***p* Value**	**Trend[b]**
S100A8	13.19	↑	Undetermined	Undetermined	Undetermined	—	—
SEMA3C	−8.42	↓	15.50 ± 19.86	22.25 ± 26.28	17.75 ± 29.80	0.50	↓
CFH	−8.45	↓	9.25 ± 19.28	21.14 ± 24.25	18.54 ± 28.14	0.16	↓
FSTL3	−10.05	↓	6.00 ± 19.86	20.06 ± 26.28	20.06 ± 29.80	0.04[d]	↓
IGFBP1	−10.35	↓	12.38 ± 19.47	24.75 ± 25.76	17.44 ± 29.20	0.10	↓
EPAS1	−15.34	↓	16.00 ± 19.86	16.38 ± 26.28	19.63 ± 29.80	0.68	↓/=
PAEP	−15.64	↓	18.63 ± 19.28	27.38 ± 25.45	14.63 ± 28.77	0.01[d]	↓/=
MMP12	−17.18	↓	11.50 ± 19.87	22.63 ± 26.28	18.29 ± 29.80	0.23	↓

Note. AS = additional control samples; C = control samples; PE = preeclampsia samples.

[a] Expression level by J5 score is a gene-specific ratio between the mean difference in expression intensity of a gene in two groups to the average mean group difference of all genes for the entire data set, as previously reported (Founds et al., 2009).

[b] Up-(↑) or down-(↓) regulation or about the same (=) in the PE group as compared to the C or AS group or both. The microarray study included only PE and C groups.

[c] Wilcoxon mean score ± *SD*.

[d] Significant difference, $p \leq .05$.