

Whole-genome microarray and targeted analysis of angiogenesis-regulating gene expression (ENG, FLT1, VEGF, PlGF) in placentas from pre-eclamptic and small-for-gestational-age pregnancies

JOHANNE HOLM TOFT^{1*}, INGRID ALSOS LIAN^{1*}, ADI LAURENTIU TARCA^{2,4}, OFFER EREZ², JIMMY ESPINOZA², IRINA POLIAKOVA EIDE¹, LINE BJØRGE³, CHEN-SUN³, SORIN DRAGHICI⁴, ROBERTO ROMERO², & RIGMOR AUSTGULEN¹

¹Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway, ²Perinatology Research Branch, Division of Intramural Research, National Institute of Child Health and Human Development, Detroit, Michigan, USA, ³Department of Obstetrics and Gynecology, Haukeland University Hospital, Bergen, Norway, and ⁴Computer Science, Wayne State University, Detroit, Michigan, USA

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Abstract

Objective. To compare the placental pathology associated with pre-eclampsia (PE) and/or fetal growth restriction, the transcriptomes of placental tissues from PE and small-for-gestational-age (SGA) pregnancies were explored. In addition, a targeted analysis of angiogenesis-regulating gene expression was performed.

Methods. Whole-genome microarray analysis was performed on placental tissue from gestational age-matched PE ($n = 10$), SGA ($n = 8$) and PE + SGA ($n = 10$) pregnancies. The expression of genes regulating angiogenesis (endoglin (ENG), fms-related tyrosine kinase 1 (FLT1), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF)) was analyzed by quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR).

Results. Microarray analysis did not reveal any significant differences between groups. However, an increased expression of ENG and FLT1 was detected by qRT-PCR in the PE + SGA group.

Conclusions. The placental transcriptome did not differ between groups, although an increased anti-angiogenic gene expression in PE + SGA was observed with qRT-PCR analysis. Based on this, we conclude that although microarray technology may represent a powerful tool in generating new hypothesis in complex fields, it may not be sensitive enough to detect subtle changes in gene expression.

Keywords: pre-eclampsia, small-for-gestational-age, placenta, genome-wide gene expression, ENG, FLT1, angiogenesis

Introduction

The pre-eclamptic syndrome is characterized by maternal manifestations (hypertension and proteinuria), but in some cases, the fetus may also be involved, and pre-eclamptic women have an increased risk of delivering a small-for-gestational-age (SGA) neonate [1]. The placental bed histopathological changes in PE and in SGA pregnancies (without pre-eclamptic manifestations) have several shared characteristics, including abnormal trophoblast invasion and impaired spiral artery remodeling with subsequent placental hypoperfusion

[2]. Severe fetal growth restriction is usually associated with reduced placental weight, villous surface, capillary volume and capillary surface, independent of pre-eclamptic manifestations present [3]. However, isolated PE is generally associated with less placental abnormalities, with the exception of the early-onset (<34 weeks) PE subgroup, which can present extensive placental changes [4]. It has been proposed that an excess of syncytiotrophoblast shedding may evoke a maternal inflammatory response in PE patients. In contrast, shedding appears to occur at physiological levels in SGA pregnancies [5].

Correspondence: Rigmor Austgulen MD, PhD, Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU), Medisinsk teknisk senter, 7489 Trondheim, Norway. Tel: +47 72573561. Fax: +47 72574704. E-mail: rigmor.austgulen@ntnu.no

*Toft and Lian have contributed equally to this work.

A number of studies have aimed at comparing the pathogenesis of the placental failure in PE and SGA pregnancies by studying levels of different angiogenesis-regulating factors. Placental gene expression levels of angiogenesis-inhibiting factors, such as Endoglin (ENG) and Vascular endothelial growth factor receptor fms-related tyrosine kinase 1 (FLT1), have been assessed. As might be expected, increased levels of ENG have been reported in PE [6] and SGA [7]. Similarly, FLT1 levels are increased in PE [8], whereas findings in SGA placentas are diverging; Tsatsaris et al. have reported increased levels of FLT1 [9], in contrast to those finding FLT1 within normal ranges [10]. Conflicting results have also been obtained with regards to proteins promoting angiogenesis, such as Vascular endothelial growth factor (VEGF) and Placental growth factor (PlGF). Placental expression of VEGF and PlGF have both been detected at elevated [11–14] and reduced levels [10,15] in PE and SGA. Thus, existing data does not allow any well-funded discrimination between the placental angiogenesis failure in PE and SGA pregnancies.

Microarray technology provides simultaneous expression analysis of thousands of genes, representing a powerful tool for developing new hypotheses. In recent years a number of microarray-based gene expression analyses have been performed on PE and SGA placentas, but findings reported are not consistent [16–18]. A major part of the divergence should probably be ascribed to pitfalls in the statistical analysis of data [19] since correction for multiple testing (to avoid false positive results) has not been included in most studies. McMinn et al. corrected for multiple testing in their analysis and reported that 409 genes were differentially expressed in pregnancies complicated by fetal growth restriction, relative to normal pregnancies [17]. However, none of the major angiogenesis-regulating factors were among them. The aim of the present study was first to compare the placental diseases underlying PE and SGA pregnancies by analyzing the global gene expression in placental tissues collected from gestational age-matched pregnancies. Secondly, since disturbed angiogenesis may characterize the abnormal placental development in PE and SGA, a targeted (qRT-PCR) analysis of ENG, FLT1, VEGF and PlGF expression was included.

Methods

Study groups

The study population included singleton pregnancies delivering at St Olavs University Hospital (Trondheim, Norway), and Haukeland University Hospital (Bergen, Norway) from 2002 to 2006 in the following groups: women who delivered an SGA

neonate; patients who developed pre-eclampsia and patients who developed pre-eclampsia complicated by an SGA neonate. The study groups were matched for gestational age at delivery. To eliminate labor-associated changes in placental gene expression, placental tissue was collected during caesarean delivery (due to clinical indications) without labor. PE was defined as hypertension (blood pressure of ≥ 140 mmHg systolic or 90 mmHg diastolic), that developed after 20 weeks of gestation and proteinuria (≥ 0.3 g in a 24-h urine collection or one dipstick measurement $\geq 1+$) [20]. Based on a Scandinavian normogram an SGA neonate was defined as a birthweight < 2.5 th percentile according to gestational age at delivery [21]. Women with diabetes, cardiovascular, renal or rheumatologic diseases, or pregnancies with fetal abnormalities (chromosomal or congenital) were excluded. The study was approved by the Norwegian Regional Committee for Medical Research Ethics and given exemption for research purposes by The Human Investigation Committee of Wayne State University. Informed consent was obtained from all participants prior to collection of placental samples.

Placental tissue

Placental biopsies were obtained within 15 min after delivery. The tissue was dissected tangentially from the central region of the maternal side, close to the insertion of the umbilical cord, immediately stabilized in RNAlater solution (Ambion, Austin, Texas, USA) and stored at -80°C in Norway until used. Areas of infarction and hematomas were avoided.

Preparation of total RNA

Tissue homogenization and RNA extraction was performed at Applied Genomics Technology Center at Wayne State University, Detroit, USA, using the Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) method as described in the protocol (<http://www.agtc.wayne.edu:9080/agtc/microarray/TotalRNAIsolation.pdf>). The RNA quality was assessed using the RNA 6000 Nano Assay on the Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA). RNA concentration and purity was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only RNA of high quality was used in the subsequent analyses. Microarray- and qRT-PCR analyses were performed on aliquots from identical RNA samples.

Affymetrix microarray

Both Affymetrix microarray- and qRT-PCR gene expression analyses were performed at the Detroit

Medical Centre, as part of the collaboration between the Norwegian University of Science and Technology (Norway) and the Perinatology Research Branch, Division of Intramural Research, National Institute of Child Health and Human Development (Detroit, USA). The experiments were conducted as described in the Affymetrix GeneChip® Expression Analysis Technical Manual (2004 revision) (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Samples were hybridized to the Affymetrix HG U133 plus 2.0 array and scanned on the Affymetrix GeneChip® scanner 3000. All experiments were performed in accordance with the Minimum Information About a Microarray Experiment (MIAME) guidelines [22].

TaqMan real time polymerase chain reaction

TaqMan® (Applied Biosystems, Foster City, CA, USA) 5'-nuclease real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays was used to measure mRNA expression for membrane-bound isoform of ENG (assay number: Hs00164438_m1), total FLT1 (membrane-bound and soluble isoform, assay number: Hs01052937_m1), VEGF (assay number: Hs00173626_m1) and PIGF (assay number: Hs00182176_m1). Quantitative RT-PCR was performed on the ABI 7900HT Fast Real Time PCR System (Applied Biosystems) in accordance with manufacturer's standard protocol. The qRT-PCR assays were run in triplicates for each sample and each gene to assess technical variability. The number of RT-PCR cycles (Ct) needed to reach a given fluorescence level was recorded for each gene in every sample. A reference gene (RPLPO) was used as internal control. The corrected Ct value (dCt) was modeled using the Generalized Estimating Equations (GEE) approach as illustrated by Fu et al. [23]. An ANOVA-like model was fitted and a *p*-value was derived for each gene to identify genes where at least one group was different than the other two. For genes with a *p* < 0.05, all three comparisons between

groups were performed, and the resulting *p*-values were adjusted for the three tests. ANOVA methods for GEE models were performed using the *geepack* package in R statistical language [24].

Microarray analysis

The pre-processing of Affymetrix microarray data was performed using the Robust Multichip Average (RMA) method [25]. Principal component analysis was performed on the normalized expression values. Differential mRNA expression was tested using a moderated *t*-statistic [26], and *p*-values were corrected with the False Discovery Rate (FDR) method [27]. A significance level of 0.05 was chosen. Both RMA, moderated *t*-test and the FDR procedure are available via the Bioconductor Project (www.r-project.org).

Results

Study groups

The clinical characteristics of the study groups are presented in Table I. Twenty-eight patients fulfilled the criteria for being enrolled in the study and the following study groups were included: (i) PE (*n* = 10); (ii) SGA (*n* = 8); and (iii) PE + SGA (*n* = 10) (Table I). As expected, patients in the SGA and PE + SGA groups had lower birthweight than in the PE group. Blood pressure was higher among patients in the PE and PE + SGA groups than in the SGA group.

Placental gene expression, as assessed by microarray analysis

No significant differences in gene expression were observed between the study groups after correction for multiple comparisons was performed (Table II). Of note there were several genes which had *p*-values < 0.05 prior to the multiple testing

Table I. Clinical information.

	PE <i>n</i> = 10	PE + SGA <i>n</i> = 10	SGA <i>n</i> = 8	<i>p</i> -value
Maternal age (years)	30.3 ± 5.4	30.3 ± 4.9	34.4 ± 5.0	0.11
Gestational age (weeks)	34.2 ± 2.5	33.9 ± 2.0	34.5 ± 3.8	0.85
Birth weight (gram)	2166.6 ± 390.5	1529.7 ± 341.8	1561.5 ± 611.2	<0.05* ¹
Systolic BP	151.5 ± 10.7	148.3 ± 12.0	129.0 ± 15.4	<0.05* ²
Diastolic BP	97.9 ± 6.3	94.1 ± 7.3	70.3 ± 10.4	<0.05* ²
Nulliparous	5/10	5/10	3/8	0.36
Multiparous	5/10	5/10	5/8	0.36

PE, pre-eclampsia; SGA, small-for-gestational-age; BP; blood pressure measured at time for diagnosis; **p* < 0.05 in the comparisons: ¹(PE vs. SGA, PE + SGA vs. PE) and ²(PE vs. SGA, PE + SGA vs. SGA); Data are presented as mean ± SD.

Table II. Comparison between expression of angiogenesis-regulating genes, as assessed by microarray and qRT-PCR.

Microarray (<i>p</i> -values)				qRT-PCR (<i>p</i> -values)			
Gene name	PE + SGA vs. PE	PE + SGA vs. SGA	PE vs. SGA	Global	PE + SGA vs. PE	PE + SGA vs. SGA	PE vs. SGA
				<i>p</i> -value			
ENG	0.999	0.859	0.952	0.033*	0.023	0.019	0.346
FLT1	0.999	0.867	0.930	0.033*	0.046	0.015	0.241
VEGF	0.999	0.940	0.941	0.054	0.067	0.006	0.171
PIGF	0.999	0.909	0.956	0.740	0.945	0.444	0.455

PE, pre-eclampsia; SGA, small-for-gestational-age; gene name, gene names are taken from Entrez-Gene; ENG, Endoglin; FLT1, fms-related tyrosine kinase 1, includes both soluble and membrane-bound isoforms of FLT1; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; *p*-values are adjusted for multiple comparisons; **p* < 0.05.

correction as well as fold changes higher than 2 (data not shown).

Placental gene expression, as assessed by qRT-PCR

Patients in the PE + SGA group had a significantly higher placental expression of the anti-angiogenic factors ENG and FLT1 than patients in the PE and SGA groups (*p* < 0.05), in spite of the non-significant result in the microarray analysis. (Table II, Figure 1). The expression of VEGF and PIGF did not differ significantly between the groups (Table II, Figure 1).

Correlation between microarray and qRT-PCR

By comparing fold change values for ENG, FLT1, VEGF and PIGF obtained by microarray and qRT-PCR analyses, a moderate to high correlation was observed (*r* = 0.645) (Table III, Figure 2).

Discussion

Principal findings

First, microarray analysis did not demonstrate significant differences in the placental transcriptome of patients with PE, SGA and PE + SGA with the current sample size and method for data analysis applied; Second, patients with PE + SGA had significantly higher placental expression of ENG and FLT1 than patients in the PE and SGA groups; Third, there were no differences in placental VEGF and PIGF expression between the study groups; and finally, there was a positive correlation between the microarray results and the qRT-PCR data.

Increased expression of anti-angiogenic genes in PE + SGA placentas

Assessment by qRT-PCR revealed that expression of ENG and FLT1 in placental tissue from PE + SGA

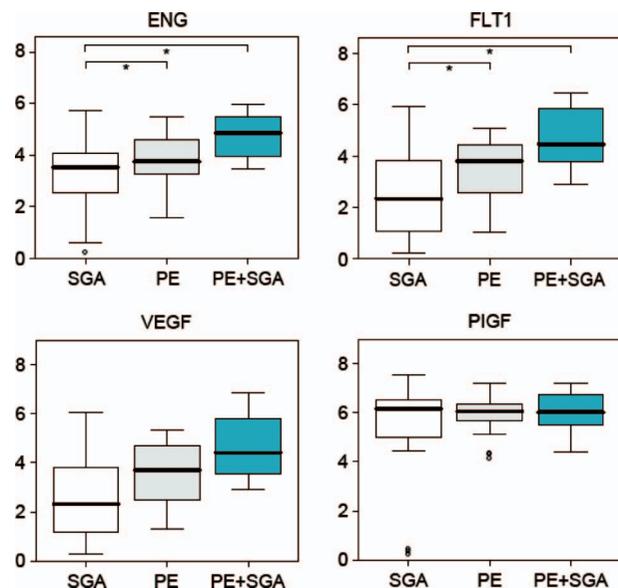


Figure 1. Expression of the angiogenesis-regulating genes ENG, FLT1, VEGF and PIGF assessed by qRT-PCR on placental tissue from pre-eclamptic (PE), small-for-gestational-age (SGA) and PE + SGA pregnancies. The Y-axis shows arbitrary expression units ($-\Delta C_t + C_{nst}$), and each unit is equivalent to a 2-fold change in expression. Significant differences by comparisons between groups are given by *; placentas from PE + SGA pregnancies demonstrated a higher expression of both ENG and FLT1 than placentas from pregnancies complicated by either isolated PE (*p* < 0.05) or SGA (*p* < 0.05).

pregnancies was significantly higher than that detected in isolated PE or SGA pregnancies. This is consistent with previous reports indicating the association of high placental mRNA expression of ENG in both isolated PE and SGA pregnancies [6,7]. However, these reports are based on comparisons between normal placentas and placentas obtained from pregnancies complicated by either PE or SGA. Therefore, the data is in fact not comparable. Similarly, FLT1 expression has also been reported to be elevated in isolated PE and SGA pregnancies compared to normal pregnancies, with PE displaying the highest level of expression [9,10]. We observed a similar tendency (although not

Table III. Fold change expression of angiogenesis-regulating genes, as assessed by microarray and qRT-PCR.

Gene name	Microarray			qRT-PCR		
	PE + SGA vs. PE	PE + SGA vs. SGA	PE vs. SGA	PE + SGA vs. PE	PE + SGA vs. SGA	PE vs. SGA
ENG	1.578	1.951	1.227	2.087	3.179	1.523
FLT1	1.539	2.373	1.540	2.456	4.826	1.965
VEGF	1.279	1.114	0.881	2.130	4.442	2.085
PIGF	0.886	0.946	1.122	1.018	1.550	1.522

PE, pre-eclampsia; SGA, small-for-gestational-age; gene name, gene names are taken from Entrez-Gene; ENG, Endoglin; FLT1, fms-related tyrosine kinase 1, includes both soluble and membrane-bound isoforms of FLT1; VEGF, vascular endothelial growth factor; PIGF, placental growth factor.

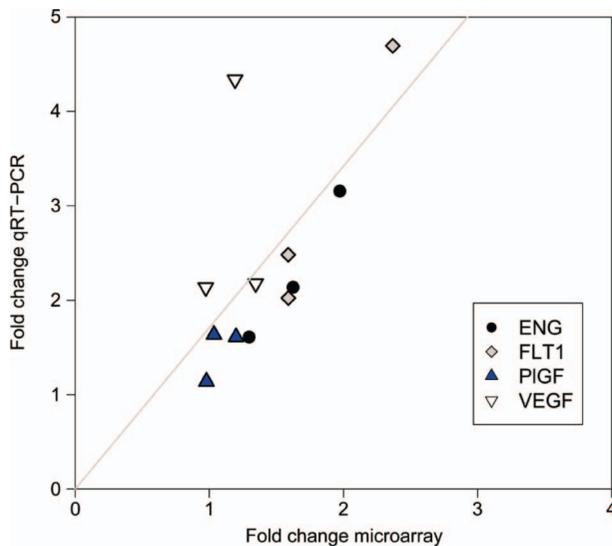


Figure 2. Pearson's correlation coefficient was determined from expression of angiogenesis-regulating genes ENG, FLT1, VEGF and PIGF in placental tissue obtained from pre-eclamptic (PE), small-for-gestational-age (SGA) and PE + SGA pregnancies ($r=0.645$), as assessed by microarray and qRT-PCR analysis. Fold change values for the microarray analysis are given on the X-axis, and qRT-PCR analysis on the Y-axis. When a gene was represented by multiple probe sets in the microarray experiments, the median value of fold change values was determined and used for correlation analysis.

significant) towards a higher expression of FLT1 in PE than in SGA pregnancies (Figure 1). The apparent discrepancy between our results and others' may probably be ascribed to differences in the selection of PE cases and the strict definition of SGA as below the 2.5th percentile. In previous studies, both isolated PE and PE + SGA pregnancies were included in the PE group. When we did a similar grouping, a significantly higher expression of FLT1 was detected in the combined PE group (PE and PE + SGA) than in the isolated SGA group (data not shown). In contrast, placental expression of VEGF and PIGF did not differ between groups, as previously reported [9,15]. In conclusion, angiogenesis-inhibiting gene expression data suggest that separate, or to some extent, overlapping biological phenomena

are underlying the development of PE and isolated fetal growth restriction. Moreover, the combined pathology of PE + SGA is associated with higher expression of the anti-angiogenic genes, suggesting that these genes may be associated with the underlying mechanism of PE + SGA to a larger extent than in isolated PE or SGA.

Lack of differences between PE and SGA placentas in global gene expression

Prior to correction for multiple testing, a number of genes were found differently expressed between groups (data not shown). The positive correlation between fold change values of ENG, FLT1, VEGF and PIGF expression by microarray and qRT-PCR suggests that the microarray data and qRT-PCR data share the direction of change. Correction for multiple testing is required to avoid false positive results, as demonstrated by the diverging findings reported from studies not including such correction of their microarray data [16–18]. McMinn et al. however, did include a correction for multiple testing in their data analysis [17]. They found several differentially expressed genes when comparing SGA placentas to placentas obtained from normal pregnancies. The differences between their study groups are more pronounced and, thus, more robust towards the multiple correction than in the present study, in which different case groups were compared. These results raise additional questions. Do PE, SGA and PE + SGA share the same pathological pathways in the placenta but in a different degree of severity? Could the difference in the placental mRNA expression between these complications be masked by the large number of genes that were studied in the microarray analysis? With the current sample size we had a power of 60% to detect a fold changes of 2 or more at a family wise error $\alpha = 0.05$, for 61% of the genes. About 40 samples per group would have been required to detect a fold change of 2 or more with a power of 80% for 90% of the genes while preserving the same family wise error $\alpha = 0.05$.

Positive correlation in fold change values of microarray and qRT-PCR data

In accordance with the data presented here, others have also reported positive correlations in fold change values between microarray and qRT-PCR data [28], suggesting that both technologies accurately predict the direction of change of mRNA expression (i.e., either up- or down-regulation). Thus, it appears that microarray and qRT-PCR provide results that differ in magnitude and reproducibility, while sharing similar expression patterns. In recent years, there has been increased attention to microarray data analysis and the potential pitfalls associated with data interpretation [29]. Since thousands of genes are tested in parallel, a correction of the raw *p*-values is required to reduce numbers of false positives, implying that over-correction may generate false negatives [30]. Based on this, we conclude that microarray analysis may represent a good approach to explore gene expression patterns in biological tissues, aiming toward new hypothesis for complex physiological or pathological interaction. However, when it comes to targeted comparisons between expression of selected genes, qRT-PCR seems to be a more appropriate choice of method.

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