0031-3998/08/6402-0147
PEDIATRIC RESEARCH
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Vol. 64, No. 2, 2008
Printed in U.S.A.

Effects of Prenatal Tobacco Exposure on Gene Expression Profiling in Umbilical Cord Tissue

NAVEED HUSSAIN, WINFRIED KRUEGER, JONATHAN COVAULT, STEPHEN WALSH, HENRY R. KRANZLER, AND CHERYL ONCKEN

Departments of Pediatrics [N.H.]; Genetics and Developmental Biology [W.K.]; Psychiatry [J.C., H.R.K.]; Community Medicine [S.W.]; Medicine and Obstetrics and Gynecology [C.O.], University of Connecticut School of Medicine, Farmington, Connecticut 06030

ABSTRACT: Maternal smoking doubles the risk of delivering a low birth weight infant. The purpose of this study was to analyze differential gene expression in umbilical cord tissue as a function of maternal smoking, with an emphasis on growth-related genes. We recruited 15 pregnant smokers and 15 women who never smoked during pregnancy to participate. RNA was isolated from umbilical cord tissue collected and snap frozen at the time of delivery. Microarray analysis was performed using the Affymetrix GeneChip Scanner 3000. Six hundred seventy-eight probes corresponding to 545 genes were differentially expressed (*i.e.* had an intensity ratio $>$ ± 1.3 and a corrected significance value $p < 0.005$) in tissue obtained from smokers *versus* nonsmokers. Genes important for fetal growth, angiogenesis, or development of connective tissue matrix were upregulated among smokers. The most highly upregulated gene was *CSH1*, a somatomammotropin gene. Two other somatomammotropin genes (*CSH2* and *CSH-L1*) were also upregulated. The most highly downregulated gene was *APOBEC3A*; other downregulated genes included those that may be important in immune and barrier protection. Validation of the three somatomammotropin genes showed a high correlation between qPCR and microarray expression. We conclude that maternal smoking may be associated with altered gene expression in the offspring. **(*Pediatr Res* 64: 147–153, 2008)**

Although a number of adverse perinatal, neonatal, and childhood health outcomes have been attributed to prenatal tobacco smoke exposure, the most consistent and measurable association is between prenatal tobacco exposure and low birth weight (1). Since low birth weight infants have an exponential increase in mortality rate compared with infants of normal birth weight (2), the public health significance of smoking during pregnancy is substantial. Moreover, low birth weight increases the risk for cardiovascular disease, metabolic syndrome, and type 2 diabetes among adults (3). The exact relationship between low birth weight and the onset of disease in adulthood is unknown. However, there has been growing evidence that environmental factors that cause fetal growth restriction may cause “fetal reprogramming,” and increase the risk of disease in adulthood (3).

Received November 7, 2007; accepted March 8, 2008.

Correspondence: Cheryl Oncken, M.D., Department of Medicine, University of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06030-3940; e-mail: oncken@nso2.uchc.edu

Supported by the State of Connecticut Department of Public Health Tobacco Research Funds, The Patrick and Catherine Weldon Donaghue Medical Research Foundation, and NIH grants RR06192 (University of Connecticut General Clinical Research Center), AA13736, and DA15167.

Supplementary material available online at www.pedresearch.com.

The aim of the present study was to examine the effects of prenatal tobacco exposure on mRNA expression in umbilical cord (UC) tissue. Tobacco smoke contains more than 4000 chemicals, including carcinogens and mutagens, which either alone or in combination may influence gene expression (4). Since the UC is exclusively fetal tissue, it may facilitate an examination of the effects of prenatal tobacco exposure on the fetal vascular system (5). Based on the magnitude of upregulation or downregulation of mRNA expression, we sought to identify the most differentially regulated genes in fetal tissue in relation to maternal smoking. This information may help to understand how smoking causes poor infant outcomes (including low birth weight) and increases the risk of childhood and adult diseases.

MATERIALS AND METHODS

The institutional review board at the University of Connecticut Health Center approved the study. Participants provided written consent before study procedures. Subjects were recruited from the labor and delivery unit at University of Connecticut Health Center. Inclusion criteria included gestational age ≥32 wk and no active maternal infection. We administered a medical history (which elicited information on smoking, other drug use and medication use) before delivery. UC tissue was collected shortly after delivery. Infant outcomes were obtained from chart review.

Tissue collection and RNA isolation. UC tissue midway between infant and placenta was collected within 10 min postpartum, cut into 1.5 cm lengths, flash frozen by immersion in liquid nitrogen and stored at −80°C. A random sample of UC tissue was selected and RNA was isolated using a modified guanidinium isothiocyanate/CsCl procedure (6). The frozen tissue was ground to a fine powder using a mortar and pestle cooled with liquid nitrogen, and (~300 mg) transferred into a tube containing 1 mL of GTC lysis buffer (4 M guanidinium isothiocyanate in 50 mM sodium citrate, TrisHCl or HEPES, 0.1% sodium sarcosyl). Samples were then sheared using a polytron tissue disrupter to reduce the molecular weights of extracellular matrix material and genomic DNA.

For estimation of blood contamination, an aliquot was analyzed spectrophotometrically at a wavelength of 418 nm. The homogenate was then layered on top of a 1.5 mL cushion of CsCl buffer (5.7 M CsCl/10 mM sodium citrate pH 7.0 and 1 mM ethylene diamine tetraacetic acid), and centrifuged at 45,000 rpm, at 22°C, in a SW50 rotor for 23 h. RNA sediment was dissolved in 100 μL H_2O (RNAsefree), concentrated by overnight precipitation at −20°C in the presence of 0.3M NaOAc and 75% ethanol (final concentration) and sedimented for 10 min at 15,000 rpm, at 4°C, with the air dried pellets dissolved at a concentration of ~1 mg/mL. RNA was quantified by spectrophotometry at 260 nm, and purity examined by 260/280 ratio. RNA quality was further examined by electrophoresis on a 1.1% agarose/2.2M formaldehyde gel.

Microarray hybridization. For each sample, 1.5 μg of RNA was converted into biotinylated cRNA and hybridized to arrays following protocols supplied by the array manufacturer (Affymetrix, Santa Clara, CA). The RNA was used for first and second strand cDNA synthesis and the double-stranded cDNA

Abbreviations: cRNA, copy ribonucleic acid; **qPCR,** quantitative polymerase chain reaction; **UC,** umbilical cord was transcribed in the presence of biotinylated ribonucleotides to generate biotinylated cRNA, which was then purified by ion exchange column chromatography. The biotinylated cRNA was fragmented, and hybridized to human U133 Version 2.0 Plus GeneChips (Affymetrix, Santa Clara, CA) without technical replication. The chip includes 54,674 probes covering all currently identified transcripts (over 47,000 transcripts from ~39,000 genes). After 16 h of hybridization at 45°C, the arrays were stained in an Affymetrix Fluidics Station using a two-stage signal amplification protocol based on detection of the biotinylated targets by streptavidin-phycoeritherin (SAPE) according to Affymetrix instructions. The signal was quantified by detection of bound phycoerythericin using an Affymetrix GeneChip Scanner 3000.

Quantitative polymerase chain reaction methodology. Total RNA (100 ng) was reverse transcribed using a commercial kit (High Capacity cDNA Archive, Applied Biosystems Inc. ABI, Foster City, CA) in parallel with calibrator total RNA from pooled human tissues (Universal Reference Total RNA PN636690, Clontech Laboratories, Mountain View, CA). Triplicate TaqMan real-time PCR reactions were prepared with cDNA from 2 ng of RNA and amplified in 384-well plates using an ABI 7900HT Sequence Detection System. Average threshold cycle number (Ct) for each sample was converted to the equivalent amount of the calibrator RNA by use of standard curves on each plate of cDNA prepared from pooled human tissue reference RNA. Relative amounts of target RNAs were normalized to the amount of two ubiquitously expressed genes encoding beta-2 microglobulin (*B2M*), and glyceraldehyde phosphate dehydrogenase (*GAPDH*). Premade and validated TaqMan quantitative polymerase chain reaction (qPCR) probe and primers for each target or reference gene were obtained from Applied Biosystems (Hs01862611_g1 chorionic somatomammotropin hormone 1 [placental lactogen, *CSH1*]; Hs00831897_s1 chorionic somatomammotropin hormone 2 [*CSH2*]; Hs00741469_g1 chorionic somatomammotropin hormone-like [*CSHL1*]; 4326319E-0411004 *B2M*; 4326317E-0411007 *GAPDH*). Controls in which the reverse transcriptase was omitted from the RT reaction produced no amplification during the qPCR TaqMan reaction.

Microarray analyses. Microarray analysis was conducted using the R/MAANOVA open source software (Version 1.4) as part of the Bioconductor and R language open source software library (Version 2.4.0) (7). Robust Multiarray Averaging (RMA) is an analysis option within the R/MAANOVA package and a commonly applied technique for normalization of Affymetrix arrays. Statistical analysis was performed on RMA-normalized data using R/MAANOVA (8) with a fixed effect permutation ANOVA model consisting of the independent variable, smoking status (smoker *versus* nonsmoker), and the covariables gender of the offspring, and Hb content of the UC lysates (which controlled the analysis for the degree of UC contamination by blood). Using the magnitude of absorption at 418 nm for each sample as an indicator of its Hb (and by extension Hb mRNA) content, we trichotomized the samples into those with low absorption (OD_{418} <1.5), intermediate absorption (OD_{418} <2.5), and high blood content (OD_{418} ≥2.5).

To identify differentially regulated genes, we performed the F-tests F1, F2, F3, and Fs that are implemented in the R/MAANOVA software and assessed gene-centric (F1-test) and array centric (F3 test) variance through comparison of the respective lists of regulated genes with those provided by the F2 and Fs-tests, which interpolate between gene centric and array centric variance. R/MAANOVA uses the Benjamini Hochberg test to perform the false discovery rate, which was set to a p value of <0.005, and pooled across all gene lists to create an inclusive master list. We selected the subset of highly significantly differentially regulated genes that demonstrated a change in their expression level of at least 1.3-fold.

The differentially regulated genes were organized into clusters using the hierarchical clustering module of D-Chip software (revised 2006) (7) by both genes and samples with the clustering parameter set to (a) Euclidian distance and (b) a p value of 0.05. Differentially expressed genes were annotated using all publicly available databases including the ENTREZ, gene ontology, and IHOP databases.

Analysis of clinical variables. Analyses were conducted using SPSS software, version 15 (SPSS, Inc., Chicago, Illinois) and StatXact, version 4 (Cytel Software Corp., Cambridge, Massachusetts). Summary statistics (means, standard deviations, and percentages) were calculated to describe the characteristics of subjects in the study samples. For continuous variables, mean values were compared using the two-sample t test. For categorical variables, distributions of frequencies between samples were compared using the χ^2 test or the Fisher exact test.

Validation of microarray results using qPCR. For the *CSH1*, *CSH2*, and *CSHL1* genes, relationships between gene expression values as measured by qPCR and by microarray analysis were evaluated with correlation coefficients and their corresponding p values. In these analyses, gene expression values were normalized using expression levels of the *B2M* gene. The Spearman rank correlation coefficient was applied to account for skewness. Microarray expression values of different probes related to the same gene were averaged (after logarithmic transformation) to determine a single expression value before calculating a correlation with the qPCR value for the same gene.

RESULTS

Demographics of the subject population are shown in Table 1. Smokers were younger and they delivered infants at an earlier gestational age and at a lower birth weight than nonsmokers. Smokers were also more likely to report other drug use during pregnancy. Additionally, the proportion of male offspring born to nonsmoking mothers was higher. Other characteristics were similar between groups.

Six hundred seventy-eight probes corresponding to 545 genes were differentially expressed (*i.e.* an intensity ratio that exceeded ±1.3 and a corrected significance value $p < 0.005$) in tissue obtained from smokers *versus* nonsmokers. Of the 545 genes, 371 were upregulated and 174 were downregulated in smokers. Of the 545 differentially regulated genes, 458 genes are known. Only 87 probe identification numbers were not annotated as determined by the absence of a gene symbol.

The 25 genes with the largest increase or the largest decrease in mRNA abundance in the UC tissue of smokers compared with that of controls are shown in Tables 2 and 3. If more than one probe for the same gene was differentially expressed the mean value of the various probes is reported.

Hierarchical clustering based on similarity in gene expression using all differentially expressed genes (>1.3-fold increased or decreased RNA level) tied cases into two major groups (Fig. 1). One group consisted of nine nonsmokers and two

Table 1. *Characteristics of the study population (count, mean ± s.d., or percent, as indicated)*

	Non-smoking mothers	Smoking mothers	p
Number of subjects	15	15	
Maternal age (y)	29.8 ± 5.5	26.2 ± 6.5	0.12
Caucasian/white race	46.7%	60.0%	0.62
Hispanic ethnicity	26.7%	20.0%	0.68
Gravidity/total pregnancies			0.26
1	26.7%	20.0%	
2	53.3	20.0	
3	6.7	20.0	
≥4	13.3	33.3	
Unknown	0.0	6.7	
Parity/prior births			0.06
0	33.3%	40.0%	
1	60.0	20.0	
≥2	6.7	33.3	
Unknown	0.0	6.7	
Maternal body mass index	30.1 ± 5.4	32.4 ± 6.8	0.33
Evidence of alcohol or drugs at delivery	0.0%	33.3%	0.04
Infant sex			0.07
Male	60.0%	26.7%	
Female	40.0	73.3	
Gestational age (wk)	38.9 ± 1.5	36.8 ± 2.5	0.01
Infant weight (g)	3319 ± 425	2610 ± 911	0.01
Infant length (in)	19.9 ± 1.2	18.0 ± 1.8	0.004
Cigarettes/d			
Before pregnancy		20.6 ± 10.2	
In week before study entry		6.9 ± 8.7	
During 1st trimester		14.5 ± 8.6	
During 3rd trimester		10.5 ± 7.8	

Table 2. *Up-regulated genes in smokers (limited to 25)*

Entrez ID	Symbol	Mean ratio	P_IDs	Gene name	Gene function	Potential role in development
1442	*CSH1*	2.48	211739_×_at	Chorionic somatomammotropin hormone 1	Fetal growth	GS (15)
3050	*HBZ*	2.26	206647_at	Hemoglobin, zeta /// hemoglobin, zeta	O2 binding	HY (40)
4629	*MYH11*	2.23	1568760_at	Myosin, heavy polypeptide 11, smooth muscle	Hematopoieses	TM (28)
58155	*PTBP2*	2.17	1560271_at	Polypyrimidine tract binding protein 2	Cell cycle	CG (41)
1443	*CSH2*	2.16	203807_×_at	Chorionic somatomammotropin hormone 2	Collagen synthesis	GS (15)
3371	*TNC*	2.12	216005_at	Tenascin C (hexabrachion)	Tissue component	CG (24)
6453	*ITSN1*	2.07	215791_at	Intersectin 1 (SH3 domain protein)	Endocytosis	GS (21)
2120	*ETV6*	2.03	1561167_at	Ets variant gene 6 (TEL oncogene)	Fusion protein	AG (42)
4628	*MYH10*	2.03	237491_at	Myosin, heavy polypeptide 10, non-muscle	Cytokinesis	TM (27,43)
23240	*KIAA0922*	2.03	239946_at	KIAA0922		
51379	*CRLF3*	2.01	235803_at	Cytokine receptor-like factor 3		
81	*ACTN4*	2	241788_×_at	Actinin, alpha 4	Cell motility	TM (26)
10658	*CUGBP1*	2	242440_at	CUG triplet repeat, RNA binding protein 1	Translation p21	TM
6310	*ATXN1*	1.99	232744_×_at	Ataxin 1	Trinucletide repeats	
653471	*LOC653471*	1.98	217653_×_at	Ribosome biogenesis protein BMS1 homolog		
643314	*KIAA0754*	1.97	215268_at	Hypothetical LOC643314		
8847	*DLEAH2*	1.95	1556821_×_at	Deleted in lymphocytic leukemia, 2		
1444	*CSHL1*	1.94	205958_×_at	Chorionic somatomammotropin hormone-like 1	Fetal growth	GS (15)
1523	*CUTL1*	1.94	240798_at	Cut-like 1, CCAAT displacement protein	Gene expression	CG (44)
11030	*RBPMS*	1.94	241897_at	RNA binding protein with multiple splicing	Cell growth	CG (45)
11039	*SMA4*	1.94	215599_at	SMA4 /// similar to beta-glucuronidase precursor		
8662	*EIF3S9*	1.93	242550_at	Eukaryotic translation initiation factor 3, sub 9		CG (46)
51232	*CRIM1*	1.93	233073_at	Cysteine rich transmembrane BMP regulator 1	Capillary formation	AG (22)
23433	*RHOQ*	1.91	239258_at	Ras homolog gene family, member Q	Exocyst complex	CG (47)
4919	*ROR1*	1.89	1559394_a_at	Receptor tyrosine kinase-like orphan receptor 1	Neurite growth	TM

CG, cell and organ growth; AG, angiogenesis; TM, tissue and matrix remodeling; GS, growth-related signals; BI, barrier and immune function; HY, hypoxia related responses.

Table 3. *Down-regulated genes in smokers (limited to 25)*

Entrez ID	Symbol	Mean ratio	P_IDs	Gene name	Gene function	Potential role in development
200315	*APOBEC3A*	−2.78	210873_×_at	Apolipoprotein B mRNA editing enzyme	Cell regulation	BI (32)
4680	*CEACAM6*	−2.72	211657_at	Carcinoembryonic antigen-related CAM 6	Tissue architecture	CG (48)
2568	*GABRP*	−2.53	205044_at	γ-aminobutyric acid (GABA) A receptor, pi		
25984	*KRT23*	−2.48	218963_s_at	Keratin 23 (histone deacetylase inducible)	Structural protein	BI (49)
114569	*MAL2*	−2.45	224650_at	Mal, T-cell differentiation protein 2	Transcytosis	BI (34)
3854	*KRT6B*	−2.39	213680_at	Keratin 6B	Structural protein	BI (49)
5275	*SERPINB13*	−2.34	211361_s_at	Serpin peptidase inhibitor, clade B (ovalbumin)	Inhibit proteinases	AG (23)
220	*ALDH1A3*	−2.31	203180_at	Aldehyde dehydrogenase 1 family, member A3	Detoxification	
144568	*A2ML1*	−2.29	1564307_a_at	α-2-macroglobulin-like 1	Inhibit proteases	BI (35)
647456	*CD24*	−2.29	208650_s_at	CD24 molecule	Cell receptor	
5918	*RARRES1*	−2.28	221872_at	Retinoic acid receptor responder 1	Retinoic acid related	
8796	*SCEL*	−2.28	232056_at	Sciellin	Cell differentiation	BI (30)
6947	*TCN1*	−2.27	205513_at	Transcobalamin I (vitamin B12 binding protein)		
5055	*SERPINB2*	−2.25	204614_at	Serpin peptidase inhibitor, clade B (ovalbumin)	PAI-2 (proteinase inhibitor)	TM (25)
3934	*LCN2*	−2.18	212531_at	Lipocalin 2 (oncogene 24p3)	Granulocyte maturation	BI (36)
4118	*MAL*	−2.17	204777_s_at	Mal, T-cell differentiation protein	T-cell differentiation	BI (33)
8710	*SERPINB7*	−2.16	206421_s_at	Serpin peptidase inhibitor, clade B megsin	Proteinase inhibitor	AG (38)
3713	*IVL*	−2.11	214599_at	Involucrin	Epidermal differentiation	
26298	*EHF*	−2.11	225645_at	Ets homologous factor	Transcriptional repressor	BI (37)
9052	*GPRC5A*	−2.1	203108_at	G protein-coupled receptor, family C, group 5	Cell growth promotion	
6698	*SPRR1A*	−2.01	214549_×_at	Small proline-rich protein 1A		CG
3860	*KRT13*	−2	207935_s_at	Keratin 13	Structural protein	BI (49)
6699	*SPRR1B*	−2	205064_at	Small proline-rich protein 1B (cornifin)	Disrupts mitosis	CG
6707	*SPRR3*	−2	232082_×_at	Small proline-rich protein 3	Neoplastic marker	BI
1824	*DSC2*	−1.97	204751_×_at	Desmocollin 2		

smokers and the other group consisted of six nonsmokers and 13 smokers. The largest cluster of transcripts (cluster 1) consisted of 431 probes that predominantly were upregulated in smokers compared with nonsmokers. The second largest cluster (cluster 4) consisted of 197 probes that were primarily downregulated in smokers compared with nonsmokers. Further, small groups of genes (cluster 2 and 3 with 28 and 17 probes, respectively), did not show a consistent pattern of change as a function of smoking status. The genes in each cluster can be found in Supplementary Data Table 1 (available online at www.pedresearch.com). All genes that were examined in this microarray analysis can be found at GEO (http://www.ncbi.nlm.nih.gov/geo). Gene ontology analysis was performed with the DAVID ontology program V2.0 (9) using the lists of Affymetrix identification numbers for genes with either increased or decreased gene expression (>1.3-fold) in UC tissue from smokers. The upregulated and downregulated set of genes were each classified into five biologic functional families as shown in Table 4.

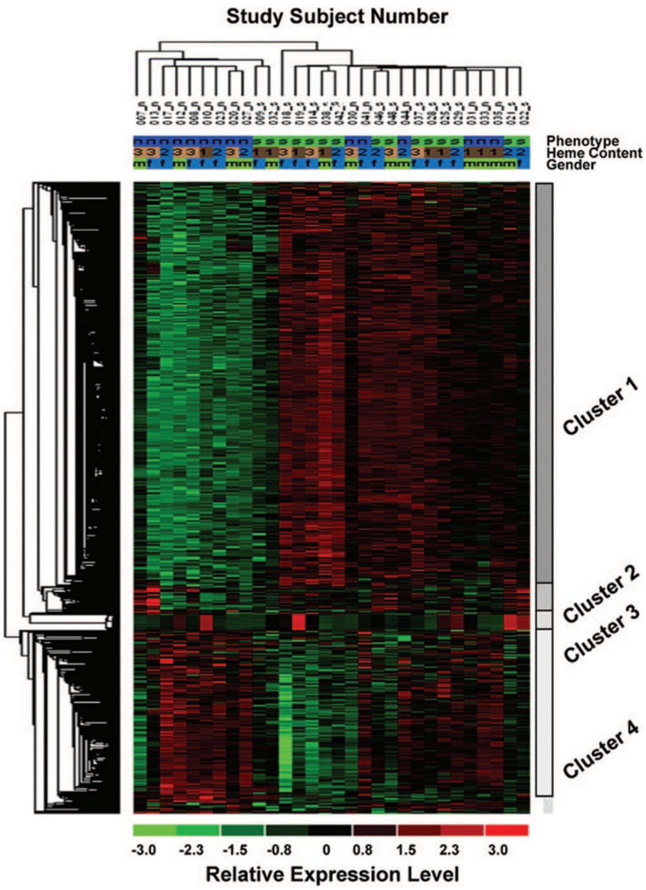

Figure 1. Hierarchical clustergram. Six hundred seventy-eight probes identified through ANOVA as being significantly regulated among the 30 subjects were clustered using the hierarchical clustering module in the D-Chip software using Euclidian distance and at a cluster significance value of 0.05. Two major subgroups of subjects (Phenotype: s = smokers; n = nonsmokers) and four major clusters of probes can be identified (discussed in the text). Heme content (1 = low, 2 = moderate, 3 = high) and sex of the baby (m = male, f = female) were used as covariates in ANOVA. The relative expression for each individual at a given probe is reflected by its color intensity (green = downregulated, red = upregulated).

The correlation coefficients between qPCR and microarray gene expression values for *CSH1*, *CSH2*, and *CSHL1* were +0.73, +0.68, and +0.69, respectively ($p < 0.0001$) as shown in Table 5.

DISCUSSION

To our knowledge, this is the first study to examine the effects of prenatal tobacco exposure on gene expression in the UC of infants born to smokers. As expected, we found that cigarette smoking was associated with reduced birth weight (1,10). Using microarray analysis of UC tissue, we identified 545 genes that were differentially expressed as a function of smoking status. Differentially expressed genes seem to cluster into a number of categories, including those involved in growth factor signaling or direct growth promotion, cellular growth, and differentiation, angiogenesis, extracellular matrix remodeling and connective tissue growth, and barrier and immune function. Correlation of the expression levels of three somatomammotropin genes with the qPCR results supported the validity of the microarray results.

Tobacco smoke is a complex mixture of toxicants, a number of which (*e.g.* carbon monoxide, hydrogen cyanide, nicotine, carcinogens) have been implicated in reducing fetal growth (4). Consistent with the theory that carcinogens may play a role in reduced birth weight, Wang *et al.* (11) found that pregnant smokers with the inducible *CYP1A1* genotype were more likely to deliver a low birth weight baby than smokers

Table 4. *Gene ontology classification using DAVID to group genes of similar functional families*

Group	No of genes	Enrichment score	Type of gene
Upregulated*			
1	71	6.37	Transcription regulatory/nuclear
2	14	5.8	RNA binding or RNA processing proteins
3	23	5.1	Primary metabolism/protein metabolism
4	24	4.27	Protein kinases
5	10	2.36	Vesicle associated intracellular transport
Downregulated†			
1	18	5.62	Transmembrane protein
2	19	4.47	Immunoglobulin domain containing transmembrane protein
3	7	4.4	Cytoskeleton/intermediate filament protein
4	4	3.16	Membrane protein
5	6	3.01	Epidermal morphogenesis

* There were 472 upregulated Affymetrix IDs with 384 matching DAVID dataset entries. Of these 142 were grouped.
† There were 201 downregulated Affymetrix IDs with 170 matching DAVID dataset entries. Of these 54 were grouped.

Table 5. *Spearman rank correlation coefficients between QPCR and microarray expression values for the same gene*

QPCR gene	Microarray probe	*B2M* normalization Corr.	*B2M* normalization p	*GAPDH* normalization Corr.	*GAPDH* normalization p
CSH1	CSH1–202493_×_at	+0.77	<0.0001	+0.76	<0.0001
	CSH1–206475_×_at	+0.63	0.0002	+0.62	0.0002
	CSH1–208356_×_at	+0.72	<0.0001	+0.74	<0.0001
	CSH1–208357_×_at	+0.74	<0.0001	+0.74	<0.0001
	CSH1–211739_×_at	+0.69	<0.0001	+0.70	<0.0001
	CSH1–208068_×_at	+0.63	0.0002	+0.65	0.0001
CSH2	CSH2–203807_×_at	+0.63	0.0002	+0.65	0.0001
	CSH2–207770_×_at	+0.58	0.0008	+0.56	0.001
	CSH2–207770_×_at1	+0.59	0.0006	+0.58	0.0007
	CSH2–208341_×_at	+0.72	<0.0001	+0.74	<0.0001
	CSH2–208342_×_at	+0.75	<0.0001	+0.77	<0.0001
CSHL1	CSHL1–205958_×_at	+0.64	0.0001	+0.64	0.0001
	CSHL1–207285_×_at	+0.63	0.0002	+0.62	0.0002
	CSHL1–208293_×_at	+0.61	0.0003	+0.63	0.0002
	CSHL1–208294_×_at	+0.67	<0.0001	+0.67	<0.0001
	CSHL1–208295_×_at	+0.79	<0.0001	+0.78	<0.0001

with the wild type variant. Further, one recent study examining the effects of tobacco exposure on gene expression in placental tissue (a composite of maternal and fetal tissue) found that phase 1 drug metabolism genes (particularly *CYP1A1*) were upregulated in smokers (12). Our study differs from that study in that we examined the effect of tobacco exposure on UC tissue (which is exclusively fetal tissue). The results that we obtained may reflect effects of prenatal tobacco exposure on fetal vascular gene expression. The difference in results from these two studies of gene expression may be explained by the fact that different areas in the placenta have differing patterns of gene expression, whereas the UC is not typically involved in drug metabolism.

Although, the exact mechanisms are unknown (13), changes in the pattern of gene expression in the UC tissue of smokers can be understood in the context of fetal adaptation to a nutrient-poor, or growth-limiting, environment. For example, studies of the effects of fetal malnutrition and anemia suggest that with fetal adaptation there are increases in growth-related genes, especially those encoding growth hormone (*GH1*, *GH2*) and chorionic somatomammotropin (*CSH-1*, *CSH-2* and *CSH-L* genes) (14). These genes are localized within a 66.5 kb cluster on human chromosome 17q23 (15). The proteins encoded by the genes in this cluster have a complex mechanism of action and regulation in both the pregnant mother and the fetus (15,16). Of note, fetal growth is mainly affected by human somatomammotropins via their effects on the GH receptor or the prolactin receptor. In addition to direct effects on fetal growth, these hormones have indirect effects that are mediated by insulin-like growth factors (15,16). Along with other growth factors, human somatomammotropin regulates insulin-like growth factors production and modulates intermediary metabolism, including the availability of glucose and amino acids to the fetus (15,16). Sheep and cattle adapt to poor fetal growth by increasing somatomammotropin activity in the fetal circulation (14,17). Our finding that the three *CSH* genes are the most highly upregulated among smokers may reflect a similar adaptive response. In addition to its association with lower birth weight, maternal smoking increases the risk of childhood obesity (18). In view of this, and the findings reported here, it is interesting that the growth hormone-lactogen gene cluster has been implicated in the association between low birth weight and the risk of metabolic syndrome later in life (19).

A number of growth factor signaling/direct growth promotion genes also showed differential expression in UC tissue obtained from smokers. Similarly, downregulation of the *CEACAM6* gene could lead to a delay in the termination of insulin's action, with growth promoting effects (20). The increased expression of the *ITSN1* (intersectin) gene may serve to increase epidermal growth factor-receptor turnover, another growth-enhancing adaptation (21). Increased expression of *CRIM1*, whose protein product promotes capillary tube formation (22) and decreased expression of *SERPINB13* [which encodes an inhibitor of angiogenesis (23)] may promote angiogenesis and fetal growth. Finally, upregulation of another gene, the tenascin gene [*TSN*; which promotes tissue healing and regeneration (24)], could contribute to the development of extracellular matrix (*i.e.* fibroblasts and smooth muscle cells) in the offspring of smokers.

Another potential interaction of genes that were differentially expressed involves the downregulation of *SERPINB2* (which encodes a plasminogen activator inhibitor-2, which may decrease tissue remodeling by inhibiting the plasminogen activators (25). The effects of decreased expression of *SERPINB2* may be augmented by increased expression of *ACTN4*, which encodes actinin alpha 4, a protein that interacts with *SERPINB2* and modulates its effects (26).

Two genes involved in the myosin chain showed increased expression in smokers. One of them, *MYH10*, is important in hypoxia-related myocardial adaptation (27) and the other, *MYH11*, is associated with arterial smooth muscle stiffening (28). It is noteworthy that stiffening of arteries is seen in infants with intra-uterine growth retardation (29). Further, one of the downregulated genes, *SCEL* (which encodes sciellin), is involved in stress-bearing blood vessel remodeling (30).

Some of the genes downregulated in cord tissue of smokers may provide immune and barrier protection (31). The most highly downregulated gene, *APOBEC3A*, is a protooncogene with presumed protective immune function through its action as a cytidine deaminase (32). Decreased expression of *MAL* (33), *MAL2* (34), and *KRT6B* may result in impaired epithelial barrier protection. Other downregulated genes, *A2ML1* (35), *LCN2* (36), and *EHF* (37), are associated with impaired immune cell function. Reduced expression of *SerpinB7*, and a lower concentration of its protein product megsin (which plays a role in megakaryocyte differentiation) may contribute to the development of thrombocytopenia that is seen in intrauterine growth retardation infants (38).

These findings provide insight into potential processes by which the fetus adapts to the adverse effects of maternal smoking. Strengths of this study include the well-defined study population, use of clinical variables to adjust for potential confounds in the analysis of the microarray results, and the validation of key findings using qPCR technology. One limitation of the study is that smoking could not be validated biochemically due to the inadequacy of infant hair samples for cotinine analysis. However, subjects were informed that their smoking status would be validated, which by itself can reduce reporting bias, particularly in relation to smoking. Further, the most common reporting bias in pregnant smokers is nondisclosure of smoking (39), which would have reduced the differences in gene expression seen between groups. Finally, although we adjusted for clinical variables that differed as a function of smoking status, it is possible that other variables that were not adjusted for will have influenced the results, including the clustering of groups based on smoking status.

In summary, we found that 545 genes were differentially expressed in the fetal tissue of offspring born to women who smoke. These findings require replication and subsequent studies should examine the potential mechanisms (*e.g.* epigenetic modification) by which maternal smoking exerts adverse effects on the fetus. Larger-scale studies are also needed to correlate gene expression results with longer-term clinical outcomes.

Acknowledgments. We thank Christine Abreu, Pamela Fall, John Glynn, Anupinder Kaur, Shama Praveen, Bruce Morris and Denise Ortiz in this work for their technical assistance. We also thank the other members of our tobacco research group (Marc Lalande, Henry Furneaux, Melinda Saunders, Anna Dongari-Batzoglou, John Greene, and Roxanne Stepnowski) who participated in valuable discussions during the conduct of the study and the analysis of study findings.

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