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IN UTERO TOBACCO EXPOSURE EPIGENETICALLY MODIFIES PLACENTAL CYP1A1 EXPRESSION

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Abstract

The metabolic pathways utilized by higher eukaryotic organisms to deal with potentially carcinogenic xenobiotic compounds from tobacco smoke have been well characterized. Carcinogenic compounds such as polycyclic aromatic hydrocarbons are metabolized sequentially in two-phases: in phase I *CYP1A1* catalyzes conversion into harmful hydrophilic DNA adducts, while in phase II *GSTT1* enables excretion via conjugation into polar electrophiles. In an effort to understand susceptibility to *in utero* tobacco exposure, we previously characterized known metabolic functional polymorphisms and demonstrated that while deletion of fetal *GSTT1* significantly modified birth weight in smokers, no polymorphism fully accounted for fetal growth restriction. Since smoking upregulates *CYP1A1* expression, we hypothesized that non-allelic (epigenetic) dysregulation of placental *CYP1A1* expression via alterations in DNA methylation (meCpG) may further modify fetal growth. In the present manuscript, we compared placental expression of multiple CYP family members among gravidae, and observed significantly increased *CYP1A1* expression among smokers relative to controls (4.4-fold, $p < 0.05$). To fully characterize *CYP1A1* meCpG status, bisulfite modification and sequencing of the entire proximal 1 kb promoter (containing 59 CpG sites) was performed. CpG sites immediately proximal to the 5'-XRE transcription factor binding element were significantly hypomethylated among smokers (55.6% vs 45.9% meCpG, $p = 0.027$), a finding which uniquely correlated with placental gene expression ($r = 0.737$, $p = 0.007$). Thus *in utero* tobacco exposure significantly increases placental *CYP1A1* expression in association with differential methylation at a critical XRE element.

INTRODUCTION

The metabolic pathways utilized by higher eukaryotic organisms to deal with potentially carcinogenic xenobiotic compounds from tobacco smoke have been well characterized (1–4). While differing susceptibilities to *in utero* tobacco exposure are not understood, genetic epidemiologic studies have linked cancer risk due to an altered ability to metabolize

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carcinogenic polycyclic aromatic hydrocarbons (PAH), or addictive (nicotine) compounds into less harmful intermediates along these characterized pathways (1–4).

Of the over 4000 substances in tobacco smoke, PAH compounds together with nitrosamines comprise the likely carcinogenic species (2–4). The majority of chemical carcinogens are metabolized in a sequential series of two-phase enzymatic metabolic reactions (Figure 1A). Phase I enzymes, such as the cytochrome P450 arylhydrocarbonhydroxylases (AHH), catalyze the conversion of PAH into reactive hydrophilic intermediates which have the potential to form harmful DNA adducts. Following high-affinity binding of PAH compounds to their intracellular aryl hydrocarbon (AH) ligands, the complex is translocated to the nucleus where it dissociates then heterodimerizes to form a DNA binding complex (AH:ARNT) to modulate chromatin disruption and regulate induction of *CYP1A1* expression (2–4). Induced *CYP1A1* thereby drives conversion of PAH into the hydrophilic intermediates to increased PAH-DNA adducts. In turn, these reactive electrophilic intermediates are made excretable by phase II enzymes, such as the *GSTT1* gene product, via conjugation into excreted polar electrophiles. Theoretically, any association of increased Phase I activity (*e.g.*, increased expression of *CYP1A1*) in combination with decreased Phase II activity (*e.g.*, decreased *GSTT1* expression) may yield increased susceptibility to tobacco-related adverse outcomes.

Maternal tobacco use has long-been identified as a major independent risk factor for intrauterine growth restriction (IUGR; 5–9). However, not all fetuses exposed to tobacco smoke experience growth restriction (5). This discrepancy cannot be accounted for by dose effect alone (10–12). Thus, current efforts aimed at understanding the potential genetic, epigenetic, and metabolic bases of this variable susceptibility to tobacco smoke exposure are of importance in perinatal medicine. Wang *et al* first reported in 2002 that an association between maternal cigarette smoking and infant birth weight differs by polymorphisms of two maternal metabolic genes: *CYP1A1* and *GSTT1* (13). These authors' analysis was limited to maternal genotype alone, and a test of interaction between maternal smoking and maternal *CYP1A1* and *GSTT1* genotypes on birth weight ratio was not statistically significant.

We have recently and significantly expanded the scope of these authors' original analysis with paired maternal *and* fetal samples from a large, prospective study conducted through the NICHD Maternal-Fetal Medicine Units Network (Aagaard-Tillery *et. al. in press*). Specifically, we performed blinded genotyping for known functional allelic variants of *CYP1A1* (Ile₄₆₂ValAA>AG/GG), *GSTT1* (deletion), and *CYP2A6* (Lys₁₆₀HisT>A) in smokers and their offspring alongside 1:1 matched controls. In our analysis, deletion of fetal *GSTT1* was singularly observed to significantly reduce the fetal birth weight ratio among smokers (*p* for interaction of 0.02). However, our study failed to fully account for susceptibility to fetal growth restriction *per se*. Thus, it remained a formal possibility that dysregulation in the expression of these integral genes (or their metabolic pathways) may play a significant role in modifying fetal growth in response to maternal tobacco use.

In considering potential candidates, two lines of evidence led us to focus on regulation of expression of the Phase I *CYP1A1* gene. First, aromatic hydrocarbon emissions are derived from both combustion of fossil fuels (coal, diesel, and gasoline) and environmental tobacco smoke. Multiple population-based analyses have demonstrated that the risk of fetal death, premature birth, and low birth weight is significantly higher for those with high prenatal ambient PAH exposure from all sources (14–20). Extension of these studies to include direct exposure measures (*i.e.*, quantitation of PAH level by personal air monitoring) reveal significant interactions between maternal *CYP1A1* haplotype and exposure to hydrocarbons on the detected level of PAH-DNA adducts present in cord blood (21). Second, in both human and animal models, environmental tobacco smoke induces aryl hydrocarbon hydroxylase activity and placental expression of *CYP1A1* (22,23).

Emerging evidence has shown that in addition to genomic base pair differences, gene expression can be silenced by non-allelic mechanisms including epigenetic influences such as covalent modifications of histones and DNA methylation (recently reviewed in 24,25). Other authors have previously observed that *CYP1A1* is inducible in its placental expression among smokers and that well-characterized xenobiotic response elements (XREs) in the proximal promoter are differentially methylated at CpG islands in lung tissue of smokers (26–29). Given these published observations of others alongside our prior observations we hypothesized that non-allelic modulation of *CYP1A1* expression may contribute to risk of adverse pregnancy outcomes (5,30,31). Since hypermethylation in key gene regulatory sequences at CpG islands is generally associated with gene silencing, we sought to compare placental gene expression of multiple CYP family members among gravidae, and determine methylation status of the proximal promoter region of the *CYP1A1* gene.

We report that placental *CYP1A1* expression was upregulated 4.4 fold ($p=0.003$) in smokers compared with non-smokers. Consistent with hypomethylation of crucial promoter regions increasing gene transcription, the CpG sites proximal to the XRE transcription factor binding element in Region I (*CYP1A1*-I) was specifically and significantly hypomethylated among smokers (55.6% vs 45.9% mCpG, $p=0.027$). Thus maternal smoking and aromatic hydrocarbon exposure leading to observed increases in DNA adduct formation in cord blood occurs via an epigenetic dysregulation of the *CYP1A1* promoter at a region surrounding a critical XRE element. We speculate that such hypomethylation at critical regulatory regions may serve as an epigenetic signature to leave a transcriptional memory of fetal exposure to maternal smoking, thereby potentially predisposing an individual to the further generation of reactive and potentially carcinogenic DNA adducts.

RESULTS

Study Subjects

Outcome data and placental samples for this study were obtained from a cohort of gravidae who had smoked during their pregnancy alongside non-smoking controls ($n=34$ total). This study was conducted for the purpose of evaluating differential gene expression and methylation in association with *in utero* tobacco exposure. The Institutional Review Board of Baylor College of Medicine and its affiliated institutions approved this study, and written informed consent was obtained from each participant at the time of enrollment. Inclusion criteria included singleton gestations at term (>35 weeks gestation) with self-admitted tobacco use or non-use controls (10,32–35). Exclusion criteria included multiple gestations, *a priori* known fetal anomalies or aneuploidy, chorioamnionitis, and maternal hepatic, hypertensive, or endocrine disorders. Data abstraction occurred at the time of enrollment, and included potential maternal comorbidities. Univariate analyses of our cohort failed to reveal significant differences among the groups by virtue of maternal age, BMI, gestational age, or fetal weight at delivery. As anticipated, fetal length was influenced by *in utero* tobacco use (Table I).

Placental *CYP1A1* expression is upregulated in smokers

We have previously demonstrated that among the known functional polymorphisms for *CYP1A1* and *GSTT1*, only deletion of fetal *GSTT1* significantly and specifically modified the effect of smoking on gestational age corrected birth weight. It remained a formal possibility that non-allelic regulation of *CYP1A1* expression could be further rendering adverse events in response to *in utero* tobacco exposure. We therefore analyzed placental expression of four CYP family genes (*CYP2A6*, *CYP2E1*, *CYP1B1* and *CYP1A1*) and observed that *CYP1A1* expression was specifically and significantly increased 4.4 fold among smokers compared with non-smokers ($p=0.003$; Figure 1B). In contrast, neither expression of the other CYP genes nor *GSTT1* differed by virtue of maternal smoking behavior (Figure 1B).

Regions of the CYP1A1 functional promoter which contain an XRE are differentially methylated in placental tissue of smokers compared with non-smokers

The methylation sites of the *CYP1A1* functional promoter in lung tissue from smokers and non-smokers have been previously characterized by other investigators (26). Based on relative proximity of CpG dinucleotides to critical transcriptional binding elements (*i.e.*, XRE sites), these authors designated 5 regions (Regions I-V) and subsequently determined the capacity for differential methylation among these regions with bisulfite modification and sequencing (26). Employing such an approach, these authors observed that the CpG dinucleotides proximal to XRE binding elements were differentially methylated in bronchial lavage samples between active smokers and long-term ex-smokers, while those distal from such elements did not demonstrate differential methylation (26). In order to characterize differential placental methylation in the *CYP1A1* promoter in response to maternal tobacco use, we employed two similar approaches.

As a first step in our analysis of the functional promoter, we bisulfite modified then amplified CpG dinucleotides in the *CYP1A1* promoter by *a priori* designated regions as previously described (26): Region I (−1411 to −1295), Region II (−1295 to −1006), Region III (−583 to −395) and Region IV (−395 to −228). In total, these regions contains a total of 59 CpG sites which have the potential to undergo differential methylation and include XRE transcriptional binding elements in Regions I and II (Figure 2A).. The total percent methylation for each region was calculated for both the smokers and non-smokers, and differences among regions were compared by the two-tailed Student t-test. As shown in Table II, the first core primed region (Region I) which contains an XRE transcriptional binding element, was unique in significant rate of methylation in smokers compared with non-smokers (55.6% vs 45.9% mCpG, $p=0.027$; Table II). In support of previously published data utilizing primary lung tissue (26) partial or no methylation was observed in placentas from smokers versus nonsmokers in primed regions II-IV (Figure 2B; Table II). Interestingly Region I contains a critical XRE which is known to regulate transcription of *CYP1A1* in lung (26).

As a second and alternate method of analyzing differential methylation across the *CYP1A1* promoter in relation to CpGs which surround an XRE, we alternately designated the CpGs within the functional promoter as belonging to one of 11 sequential (5' to 3') groups each containing 5 CpG dinucleotides. In such a manner, we were able to assess whether only those dinucleotides most proximal to and surrounding the XRE sites underwent differential methylation. As presented in Table III, among the 11 groups of 5 CpGs, only the two XRE-containing groups within these 55 CpGs demonstrated significant differential methylation (CpGs 1 to 5; $p = 0.027$ and CpGs 21 to 25; $p = 0.006$). Of note, the only group of dinucleotides with hypomethylation in association with smoking status was CpG 1 to 5, which was contained within Region I (Table 2 and 3).

Expression of CYP1A1 is inversely correlated with methylation status of Region I

Differential methylation of CpG dinucleotides in genomic DNA is generally considered to correlate with altered transcription (24,25). However, the direct evidence for true correlations in complex mammalian systems is limited. Given our differential methylation surrounding the XRE element in Region I, we therefore sought to better correlate placental *CYP1A1* expression with the level of site-specific methylation of Region I. To do so, we plotted the relative expression level of *CYP1A1* against the percent methylation for each region of the *CYP1A1* promoter in both smokers and non-smokers and interrogated the relationship with bivariate correlations (Pearson's correlation for variance) employing a two-tailed test for significance. As demonstrated in Figure 3, we found that the percent methylation of Region I inversely correlates with expression level ($r = -0.737$, $p=0.007$); this correlation held true regardless of maternal smoking behavior. In a linear regression model controlling for the potential covariates

of fetal gender and maternal comorbidities, percentage CpG methylation in Region I independently predicted *CYP1A1* expression (*d.n.s.*). Moreover, there was no correlation between methylation status of Regions II-IV and placental *CYP1A1* expression (*d.n.s.*).

DISCUSSION

The metabolic pathways utilized by higher eukaryotic organisms to deal with potentially carcinogenic xenobiotic compounds from tobacco smoke have been well characterized (1–4, 28). Several *CYP* family members are involved in the Phase I processing of these xenobiotics into reactive oxygenated intermediates (ROIs), which are further processed by the Phase II enzymes to form hydrophilic excreted conjugated compounds. The contribution of the *CYP* family members in the production of ROIs (which can not only form harmful DNA adducts but also set the “tumorigenesis machinery in motion”) has also been intensively studied (29). An increase in Phase I activity, which increases cellular levels of ROIs, without a subsequent increase in Phase II activity to rid the cell of the ROIs, leads to the accumulation of DNA adducts. With respect to fetal development and maternal tobacco use, it has been recently appreciated that aryl hydrocarbon activity and DNA adducts are concomitantly increased in the cord blood of smokers (15,21,23). The molecular mechanisms underlying such observations have been poorly understood.

Although there exists a number of acknowledged genetic and environmental factors which further influence birth weight, an established causal relationship with tobacco use and delivery of SGA infants exists (5–12). In 1957, Simpson and Linda reported their observations that infants born to mothers who smoked 10 cigarettes or more per day weighed an average 200 grams less than those delivered by reported non-smokers (36). In the interval since, multiple authors have repeatedly demonstrated the persistence of this association with Relative Risk estimates ranging from 1.5 to 2.9 (reviewed in 12). Moreover, causality has been implicated by repeated observations of a dose-response relationship as well as a positive effect of smoking cessation on fetal weight. Despite this causal relationship, an essential observation remains: not all infants exposed to tobacco are small for gestational age. It is therefore likely that the effect of smoking on fetal growth involves interactions between multiple epidemiologic, genetic or epigenetic, and sociodemographic factors.

Other investigators had previously demonstrated that *CYP1A1* expression increases in the lung tissue of smokers (reviewed in 30). We similarly find a significant increased *CYP1A1* expression in placental tissue from smokers (4.4 fold). This observed increase in *CYP1A1* expression was not accompanied by an increase in the Phase II enzyme, *GSTT1* nor other *CYP* enzymes. This is of probable true biologic relevance for a number of reasons.

First, we have previously demonstrated that *fetal* homozygous deletion of the singular phase II PAH gene integral to excretion of DNA adduct forming reactive intermediates (*GSTT1*) significantly and specifically modifies fetal growth patterns in response to maternal smoking (Aagaard-Tillery *et al.*, in press *Obstet Gynecol*). These findings persisted in multiple allelic interaction models to suggest an interaction between the fetal metabolic gene *GSTT1*, maternal smoking, and modification of birth weight. Of note, 18–22% of the population carries a homozygous deletion of *GSTT1*. As discussed, phase I gene-products, such as *CYP1A1*, are integral in metabolic activation of PAH compounds into oxidized derivatives, resulting in reactive oxygen intermediates capable of covalently binding DNA to form adducts; as a balance to such intermediary forming reactions, conjugation with endogenous species to form hydrophilic glutathione conjugates which are then readily excreted occurs. Our data support the notion that the discrepant variation in fetal susceptibility to smoking-related growth restriction may result from the diminished ability of the fetus to excrete these reactive intermediates (fetal phase II *GSTT1*(del)). However, the placenta is uniformly capable of

delivering the first “hit” to such fetuses by virtue of tobacco-induced increased *CYP1A1* expression.

Second, we have built on these observations and demonstrated that increased placental *CYP1A1* expression was specifically and significantly associated with hypomethylation of the *CYP1A1* promoter region in smokers compared with non-smokers (Figure 2 and Table II). Region I, which contains an XRE element that is involved in regulation of *CYP1A1* expression, was the only region which demonstrated significant differential methylation within the proximal promoter (Table II). This association held true within an individual, as there was a significant correlation between *CYP1A1* expression and Region I hypomethylation (Figure 3).

Thus, although our observations are unique in collective concept and findings, they are not without biologic plausibility and merit. First, mechanisms leading to IUGR following *in utero* tobacco exposure have generally often been attributed to chronic fetal hypoxia, yet, nicotine, cotinine and DNA adducts are known to cross or collect in the placenta of smokers. As discussed, phase I gene-products, such as *CYP1A1*, are integral in metabolic activation of PAH compounds into oxidized derivatives, resulting in reactive oxygen intermediates capable of covalently binding DNA to form adducts; as a balance to such intermediary forming reactions, conjugation with endogenous species to form hydrophilic glutathione conjugates which are then readily excreted occurs. Thus while it is possible that chronic hypoxia is a primary mediator of IUGR in response to *in utero* tobacco exposure, our data support the notion that the discrepant variation in fetal susceptibility to smoking-related growth restriction results from the diminished ability of the fetus to excrete these reactive intermediates (fetal phase II *GSTT1*(del)).

There are a limited number of methodological limitations which ought to be considered when broadly interpreting our findings. First, our samples were obtained from a relatively small cohort of smokers. Nevertheless, statistical significance was readily reached. Second, tobacco smoke contains multiple compounds, and while we have previously established functional polymorphisms along well-established metabolic pathways related to varying cancer susceptibility, the relative role of these genes in perinatal outcomes is unknown. Third, we cannot ascertain a dose-dependent effect nor have we directly assayed for serum or urine cotinine levels. However, multiple previous studies have validated maternal self-reporting of smoking behavior with 96% of “nonsmokers” having nondetectable serum cotinine levels at <10 ng/ml, in accordance with other authors findings (31–35).

Despite these limitations, the strengths of our study are several. First, we have provided an exhaustive characterization of the near entirety of the *CYP1A1* proximal promoter. This encompasses more than 1 kb, and 59 CpG sites. In total, we examined over 250 clones to encompass over 5000 CpG site reads to determine differential methylation. Second, we have attempted to directly correlate gene expression with site specific (rather than “global” or “net”) DNA methylation as a measure of causality. Third, we have performed these analyses in the most relevant tissue with relation to uteroplacental insufficiency.

Recent advances in human genome research, pharmacogenetics, medical genetics, and the evolving field of epigenetics have furthered our understanding of the interactions of the heritable genome and chromatin structure in the causal pathways employed in the development of human disease (24,25). The concept of epigenetic mechanisms providing a “memory” of previous transcriptional activation has also been proposed from yeast to man (37,38). The “developmental origins of adult disease” hypothesis builds on these observations to postulate that fetal exposures *in utero* can cause an epigenetic change within the fetus which can manifest in adulthood as an increased potential for disease (39,40).

Reports of the changes in DNA methylation patterns of specific genes attributed to gestational milieu are emerging in recent literature. In human population-based analyses, targeted genomic methylation profiling in known differentially methylated regions (DMRs) from peripheral mononuclear cells among adults which were exposed *in utero* to calorie restriction during the Dutch Hunger Winter demonstrated differential methylation at the imprinted *IGF2* region (39). In animal models, alterations in methylation patterns due to *in utero* exposure to maternal protein restriction can be seen even in the F2 generation (40).

Here we present the first evidence that maternal smoking alters DNA methylation levels *in utero* in human placenta. Because of the limitations of biologic material we did not assay for *CYP1A1* levels nor characterize its promoter directly in fetal blood nor hepatic tissue. Yet based on the reports of Jauniaux and Burton we would anticipate that *CYP1A1* levels in the fetus may similarly differ and would do so with a similar mechanism (41). If indeed this were observed to be true, then differential methylation of the *CYP1A1* promoter would serve as an epigenetic mechanism allowing for a molecular “memory” of fetal exposure to maternal tobacco smoke. In turn, based on the available evidence from both population based epigenetic studies (39) and animal models (40,42,43), this may function as a mechanism by which *in utero* tobacco exposure might potentially manifest in adulthood as an increased susceptibility to disease.

Along these lines, not only has a correlation between maternal smoking and a myriad of fetal morbidities has been established, but the susceptibility of the tobacco-exposed fetus to later childhood disorders has been well described. Maternal smoking has been linked not only to reduced birth weight, but a predisposition to asthma, reduced fecundity, and obesity in adulthood (44–46). Therefore, the potential exists that altered DNA methylation patterns established *in utero* can influence the etiology of adult diseases. Importantly, as established in animal models, in cases where an *in utero* exposure leads to hypomethylation of specific regions of the genome, supplementation of the diet with cofactors in the one carbon metabolism pathway alleviate the differential methylation (47–49). Such cofactors are found in standard prenatal vitamins (49).

Our collective data provide likely potential insights into such a mechanism. The implications of our findings are two-fold. First, our prior data illustrate that a fetal metabolic gene (*GSTT1*) which is integral in the excretion of reactive intermediates of aromatic hydrocarbons modifies fetal growth specifically in response to *in utero* tobacco exposure. These findings imply that tobacco metabolites may reach the fetus and thus modify fetal growth if not excreted. Second, our current data provide the epigenetic correlation for the ubiquitous increased expression of placental *CYP1A1* and subsequent DNA adduct accumulation which has been previously observed (14–17,32). Future studies aimed at illuminating the complex interplay of genomic-epigenomic-environmental interactions may help dissect multifactorial etiologies and identify at-risk populations for the common adverse pregnancy outcomes.

METHODS

Study Population

Outcome data and placental samples for this study were obtained from a cohort of gravidae who had smoked during their pregnancy alongside non smoking controls. Patients were admitted to either Ben Taub General Hospital or St. Luke’s Episcopal Hospital (Houston, TX). Inclusion criteria included singleton gestations at term (>35 weeks gestation) with self-admitted tobacco use or non-use controls (10,31–33). Exclusion criteria included multiple gestations, *a priori* known fetal anomalies or aneuploidy, chorioamnionitis, secondary substance exposure (*e.g.*, cocaine, heroine, marijuana, or others as detected in routine urine toxicology assessment), and maternal hepatic, hypertensive, or endocrine disorders. Data

abstraction occurred at the time of enrollment, and included potential maternal comorbidities. Toxicology screens were administered when clinically indicated.

Collection of placental samples

Within 30 minutes of delivery, equal full-thickness sections of placenta were uniformly collected and stored in 50mL conical tubes which were immediately placed on dry ice. Samples were stored at -80°C until use in accordance with IRB approved protocols.

Real Time PCR analysis

Approximately 50mg of tissue was lysed using a chaotropic buffer, and RNA was extracted from each placenta using the Machery Nagel NucleoSpin kit (Bethlehem, PA). RNA was quantitatively converted to cDNA from each sample using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA). Real-time quantitative RT-PCR (qRT-PCR) analyses were performed using 2 μL cDNA, and 2 μM final concentration of forward and reverse primers and TaqMan probes in a total reaction volume of 5 μL . We used the iQ5 Real-Time PCR Detection System from BioRad. Relative quantification of each gene was calculated after normalization to GAPDH by using the comparative Ct method (50).

Bisulfite Sequencing and Analysis

Sodium bisulfite treatment converts unmethylated cytidine into thymidine. Methylation of CpG sites can thus be detected by recognition of unmodified cytidine at CpG sites by sequencing. The EZ DNA methylation kit (Zymo Research, Orange, CA) was used to bisulfite modify and purify sample DNA. In a modification of previously published methods, four fragments of the *CYP1A1* promoter region 1400-bp upstream of the gene were amplified: Region I (−1411 to −1295), Region II (−1295 to −1006), Region III (−583 to −395) and Region IV (−395 to −228). Primer sequences are as follows:: Region I 5'-GTTAGTTGGGGTTAGGTTGAG-3' (sense) and 5'-CATAACCTAACTACCTACCTCC-3' (antisense); Region II 5'-GTTAGTTGGGGTTAGGTTGAG-3' (sense) and 5'-AAACCCCCACCCTACCCCC-3' (antisense); Region III 5'-GGGTTTTGGGGGATAGGTTT-3' (sense) and 5'-CG/ATACAAATACCTCCCCAAC-3' (antisense) Region IV 5'-GGAAGGAGGTTATTAA/TGGGG-3' (sense) and 5'-CACCTAAAAATCCCAATTCCAA-3' (antisense). PCR conditions were as follows: 5 μL template DNA, 5 μL 10X ABI (PE Applied Biosystems, Warrington, UK) Buffer, 4.5 μM MgCl_2 , 4 μL 2.5 μM dNTP, 5 μL 10 μM sense and antisense primers, and 0.5 μL TaqGold (ABI) Polymerase. Reactions were run under conditions of 95°C 15 min, with 40 cycles (95°C 30 sec, 56°C 30 sec, 72°C 1 min) with 10 min 72°C elongation for adenylation. The amplified PCR products were subsequently cloned, and a minimum of four clones from each reaction were sequenced. Samples were sequenced by Lone Star Labs (Houston, TX) and aligned using CLUSTAL W (Kyoto University). Figure 2A, which depicts the CpG sites, was created by the ABI Methyl Prime software.

Statistical Analysis

Univariate comparisons were performed using Chi-square or Fisher's exact test for discrete variables and the Wilcoxon test for the continuous variables. Real Time PCR was analyzed using the $2^{-\Delta\Delta\text{CT}}$ method as previously described (50). Results are displayed as fold change of smokers compared with non-smoking control samples. An independent samples students T-test was performed for each gene. An independent samples, one-tailed students T-test was performed to analyze the differential methylation between smokers and non-smokers given the a priori assumption of hypomethylation. For analysis of correlation, relative gene expression was calculated using the ΔCT from each subject and plotted against the percent methylation for that individual with analysis of variance by Pearson's coefficient. SPSS (SPSS, INC) was

uses for analyses and a nominal p-value <0.05 was considered significant. No adjustment was made for multiple comparisons.

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References

1. Smits KM, Benhamou S, Garte S, Weijenberg MP, Alamanos Y, Ambrosone C, Autrup H, Autrup JL, Baranova H, Bathum L, et al. Association of metabolic gene polymorphisms with tobacco consumption in healthy controls. *Int J Cancer* 2004;110:266–270. [PubMed: 15069692]
2. Raunio H, Rautio A, Gullsten H, Pelkonen O. Polymorphisms of CYP2A6 and its practical consequences. *Br J Clin Pharmacol* 2001;52:357–363. [PubMed: 11678779]
3. Larsen JE, Colosimo ML, Yang IA, Bowman R, Zimmerman PV, Fong KM. CYP1A1 Ile462Val and MPO G-463A interact to increase risk of adenocarcinoma but not squamous cell carcinoma of the lung. *Carcinogenesis* 2006;27:525–532. [PubMed: 16195240]
4. Hou L, Chatterjee N, Huang WY, Baccarelli A, Yadavalli S, Yeager M, Bresalier RS, Chanock SJ, Caporaso NE, Ji BT, et al. CYP1A1 Val462 and NQO1 Ser187 polymorphisms, cigarette use, and risk for colorectal adenoma. *Carcinogenesis* 2005;26:1122–1128. [PubMed: 15731166]
5. Aagaard-Tillery KM, Porter TF, Lane RH, Varner MW, Lacoursiere DY. In utero tobacco exposure is associated with modified effects of maternal factors on fetal growth. *Am J Obstet Gynecol* 2008;198:66, e61–66. [PubMed: 18166310]
6. Vogt Isaksen C. Maternal smoking, intrauterine growth restriction, and placental apoptosis. *Pediatr Dev Pathol* 2004;7:433–442. [PubMed: 15568214]
7. Wen SW, Goldenberg RL, Cutter GR, Hoffman HJ, Cliver SP, Davis RO, DuBard MB. Smoking, maternal age, fetal growth, and gestational age at delivery. *Am J Obstet Gynecol* 1990;162:53–58. [PubMed: 2301517]
8. Secker-Walker RH, Vacek PM. Relationships between cigarette smoking during pregnancy, gestational age, maternal weight gain, and infant birthweight. *Addict Behav* 2003;28:55–66. [PubMed: 12507527]
9. Cnattingius S, Haglund B. Decreasing smoking prevalence during pregnancy in Sweden: the effect on small-for-gestational-age births. *Am J Public Health* 1997;87:410–413. [PubMed: 9096542]
10. Peacock JL, Cook DG, Carey IM, Jarvis MJ, Bryant AE, Anderson HR, Bland JM. Maternal cotinine level during pregnancy and birthweight for gestational age. *Int J Epidemiol* 1998;27:647–656. [PubMed: 9758120]
11. Everson RB, Randerath E, Santella RM, Avitts TA, Weinstein IB, Randerath K. Quantitative associations between DNA damage in human placenta and maternal smoking and birth weight. *J Natl Cancer Inst* 1988;80:567–576. [PubMed: 3373547]
12. Cnattingius S. The epidemiology of smoking during pregnancy: smoking prevalence, maternal characteristics, and pregnancy outcomes. *Nicotine Tob Res* 2004;6(Suppl 2):S125–140. [PubMed: 15203816]
13. Wang X, Zuckerman B, Pearson C, Kaufman G, Chen C, Wang G, Niu T, Wise PH, Bauchner H, Xu X. Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight. *JAMA* 2002;287:195–202. [PubMed: 11779261]
14. Choi H, Jedrychowski W, Spengler J, Camann DE, Whyatt RM, Rauh V, Tsai WY, Perera FP. International studies of prenatal exposure to polycyclic aromatic hydrocarbons and fetal growth. *Environ Health Perspect* 2006;114:1744–1750. [PubMed: 17107862]
15. Whyatt RM, Perera FP, Jedrychowski W, Santella RM, Garte S, Bell DA. Association between polycyclic aromatic hydrocarbon-DNA adduct levels in maternal and newborn white blood cells and glutathione S-transferase P1 and CYP1A1 polymorphisms. *Cancer Epidemiol Biomarkers Prev* 2000;9:207–212. [PubMed: 10698483]
16. Whyatt RM, Jedrychowski W, Hemminki K, Santella RM, Tsai WY, Yang K, Perera FP. Biomarkers of polycyclic aromatic hydrocarbon-DNA damage and cigarette smoke exposures in paired maternal

- and newborn blood samples as a measure of differential susceptibility. *Cancer Epidemiol Biomarkers Prev* 2001;10:581–588. [PubMed: 11401906]
17. Daube H, Scherer G, Riedel K, Ruppert T, Tricker AR, Rosenbaum P, Adlkofer F. DNA adducts in human placenta in relation to tobacco smoke exposure and plasma antioxidant status. *J Cancer Res Clin Oncol* 1997;123:141–151. [PubMed: 9119879]
 18. Gladen BC, Zadorozhnaja TD, Chislovska N, Hryhorczuk DO, Kennicutt MC 2nd, Little RE. Polycyclic aromatic hydrocarbons in placenta. *Hum Exp Toxicol* 2000;19:597–603. [PubMed: 11211237]
 19. Perera FP, Jedrychowski W, Rauh V, Whyatt RM. Molecular epidemiologic research on the effects of environmental pollutants on the fetus. *Environ Health Perspect* 1999;107(Suppl 3):451–460. [PubMed: 10346993]
 20. Perera FP, Tang D, Rauh V, Tu YH, Tsai WY, Becker M, Stein JL, King J, Del Priore G, Lederman SA. Relationship between polycyclic aromatic hydrocarbon-DNA adducts, environmental tobacco smoke, and child development in the World Trade Center cohort. *Environ Health Perspect* 2007;115:1497–1502. [PubMed: 17938742]
 21. Wang S, Chanock S, Tang D, Li Z, Jedrychowski W, Perera FP. Assessment of interactions between PAH exposure and genetic polymorphisms on PAH-DNA adducts in African American, Dominican, and Caucasian mothers and newborns. *Cancer Epidemiol Biomarkers Prev* 2008;17:405–413. [PubMed: 18268125]
 22. Huel G, Godin J, Moreau T, Girard F, Sahuquillo J, Hellier G, Blot P. Aryl hydrocarbon hydroxylase activity in human placenta of passive smokers. *Environ Res* 1989;50:173–183. [PubMed: 2792058]
 23. Czekaj P, Wiaderkiewicz A, Florek E, Wiaderkiewicz R. Tobacco smoke-dependent changes in cytochrome P450 1A1, 1A2, and 2E1 protein expressions in fetuses, newborns, pregnant rats, and human placenta. *Arch Toxicol* 2005;79:13–24. [PubMed: 15448981]
 24. Zilberman D. The evolving functions of DNA methylation. *Curr Opin Plant Biol* 2008;11:554–559. [PubMed: 18774331]
 25. Liu L, Li Y, Tollefsbol TO. Gene-environment interactions and epigenetic basis of human diseases. *Curr Issues Mol Biol* 2008;10:25–36. [PubMed: 18525104]
 26. Anttila S, Hakkola J, Tuominen P, Elovaara E, Husgafvel-Pursiainen K, Karjalainen A, Hirvonen A, Nurminen T. Methylation of cytochrome P4501A1 promoter in the lung is associated with tobacco smoking. *Cancer Res* 2003;63:8623–8628. [PubMed: 14695173]
 27. Alexandrov K, Cascorbi I, Rojas M, Bouvier G, Kriek E, Bartsch H. CYP1A1 and GSTM1 genotypes affect benzo[a]pyrene DNA adducts in smokers' lung: comparison with aromatic/hydrophobic adduct formation. *Carcinogenesis* 2002;23:1969–1977. [PubMed: 12507920]
 28. Vineis P, Anttila S, Benhamou S, Spinola M, Hirvonen A, Kiyohara C, Garte SJ, Puntoni R, Rannug A, Strange RC, et al. Evidence of gene gene interactions in lung carcinogenesis in a large pooled analysis. *Carcinogenesis* 2007;28:1902–1905. [PubMed: 17307802]
 29. Nebert DW, Dalton TP. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer* 2006;6:947–960. [PubMed: 17128211]
 30. Raunio H, Hakkola J, Hukkanen J, Lassila A, Paivarinta K, Pelkonen O, Anttila S, Piipari R, Boobis A, Edwards RJ. Expression of xenobiotic-metabolizing CYPs in human pulmonary tissue. *Exp Toxicol Pathol* 1999;51:412–417. [PubMed: 10445407]
 31. Huuskonen P, Storvik M, Reinisalo M, Honkakoski P, Rysa J, Hakkola J, Pasanen M. Microarray analysis of the global alterations in the gene expression in the placentas from cigarette-smoking mothers. *Clin Pharmacol Ther* 2008;83:542–550. [PubMed: 17928820]
 32. England LJ, Grauman A, Qian C, Wilkins DG, Schisterman EF, Yu KF, Levine RJ. Misclassification of maternal smoking status and its effects on an epidemiologic study of pregnancy outcomes. *Nicotine Tob Res* 2007;9:1005–1013. [PubMed: 17852766]
 33. Klebanoff MA, Levine RJ, Morris CD, Hauth JC, Sibai BM, Ben Curet L, Catalano P, Wilkins DG. Accuracy of self-reported cigarette smoking among pregnant women in the 1990s. *Paediatr Perinat Epidemiol* 2001;15:140–143. [PubMed: 11383579]
 34. Silbergeld EK, Patrick TE. Environmental exposures, toxicologic mechanisms, and adverse pregnancy outcomes. *Am J Obstet Gynecol* 2005;192:S11–21. [PubMed: 15891707]

35. McDonald SD, Perkins SL, Walker MC. Correlation between self-reported smoking status and serum cotinine during pregnancy. *Addict Behav* 2005;30:853–857. [PubMed: 15833588]
36. Simpson WJ, Linda L. A preliminary report on cigarette smoking and the incidence of prematurity. *Am J Obstet Gynecol* 1957;73:808–815.
37. Ng RK, Gurdon JB. Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. *Nat Cell Biol* 2008;10:102–109. [PubMed: 18066050]
38. Zacharioudakis I, Gligoris T, Tzamarias D. A yeast catabolic enzyme controls transcriptional memory. *Curr Biol* 2007;17:2041–2046. [PubMed: 17997309]
39. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008;105:17046–17049. [PubMed: 18955703]
40. Burdge GC, Slater-Jefferies J, Torrens C, Phillips ES, Hanson MA, Lillycrop KA. Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *Br J Nutr* 2007;97:435–439. [PubMed: 17313703]
41. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev* 2007;83:699–706. [PubMed: 17900829]
42. Burdge GC, Lillycrop KA, Phillips ES, Slater-Jefferies JL, Jackson AA, Hanson MA. Folic acid supplementation during the juvenile-pubertal period in rats modifies the phenotype and epigenotype induced by prenatal nutrition. *J Nutr* 2009;139:1054–1060. [PubMed: 19339705]
43. Dolinoy DC, Huang D, Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci U S A* 2007;104:13056–13061. [PubMed: 17670942]
44. Jurisicova A, Taniuchi A, Li H, Shang Y, Antenos M, Detmar J, Xu J, Matikainen T, Benito Hernandez A, Nunez G, et al. Maternal exposure to polycyclic aromatic hydrocarbons diminishes murine ovarian reserve via induction of Harakiri. *J Clin Invest* 2007;117:3971–3978. [PubMed: 18037991]
45. Ino T. A Meta-Analysis of Association between Maternal Smoking during Pregnancy and Offspring Obesity. *Pediatr Int*. 2009
46. Keil T, Lau S, Roll S, Gruber C, Nickel R, Niggemann B, Wahn U, Willich SN, Kulig M. Maternal smoking increases risk of allergic sensitization and wheezing only in children with allergic predisposition: longitudinal analysis from birth to 10 years. *Allergy* 2009;64:445–451. [PubMed: 19170671]
47. Burdge GC, Lillycrop KA, Jackson AA, Gluckman PD, Hanson MA. The nature of the growth pattern and of the metabolic response to fasting in the rat are dependent upon the dietary protein and folic acid intakes of their pregnant dams and post-weaning fat consumption. *Br J Nutr* 2008;99:540–549. [PubMed: 17761015]
48. Waterland RA, Travisano M, Tahiliani KG, Rached MT, Mirza S. Methyl donor supplementation prevents transgenerational amplification of obesity. *Int J Obes (Lond)* 2008;32:1373–1379. [PubMed: 18626486]
49. Zeisel SH. Importance of methyl donors during reproduction. *Am J Clin Nutr* 2009;89:673S–677S. [PubMed: 19116320]
50. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. *Methods* 2001;25:402–408. [PubMed: 11846609]

Figure 1A. **Figure 1B.**

Figure 1. CYP1A1 is significantly upregulated in the placenta from smokers compared with non-smokers

Panel 1A. Schematic of the metabolism of inhaled polycyclic aromatic hydrocarbons from cigarette smoke. PAHs are metabolized into reactive intermediates by the Phase I enzymes, such as *CYP1A1*. The reactive intermediates are metabolized into excretable substances by the Phase II enzymes, such as *GSTT1*. Thus, any combinatorial association of increased Phase I activity (e.g., increased expression of *CYP1A1*) in combination with decreased Phase II activity (e.g., decreased *GSTT1* expression) may yield increased susceptibility to tobacco-related adverse outcomes. We have recently demonstrated that the relatively prevalent fetal *GSTT1* deletion in modulates birth weight in response to *in utero* tobacco exposure. **Panel 1B.**

CYP1A1 expression is significantly upregulated in the placenta from smokers compared with non-smokers ($p=0.003$). Placental expression of other CYP family members (*CYP2A6*, *CYP2E1*, and *CYP1B1*) as well as *GSTT1* did not differ significantly between the two groups.

Figure 2A.

Figure 2B.

Figure 2. The XRE containing Region I of the CYP1A1 promoter is hypomethylated in placenta from smokers compared with non-smokers
Panel A. The four analyzed regions of the CYP1A1 promoter are depicted. Magenta lines represent CpG dinucleotides, and the red stars designate localization of the XREs. In total, among all four regions the precise methylation status of 59 CpG dinucleotides was determined in bisulfite-treated placental DNA from 19 nonsmokers and 15 smokers employing sequencing.
Panel B. Methylation status of the 5 CpG sites in Region I, and 26 CpG sites in Region II. Two representative clones (from a minimum of four done in total) for each of 8 representative subjects are depicted. Open circles represent an unmethylated CpG, closed circles represent a methylated CpG site. Among smokers, 55.6% placental methylation within the XRE-containing Region I was observed, versus 45.9% among non-smokers ($p=0.027$). Region II is not differentially methylated between the two groups.

Figure 3. Plot of percent methylation of the promoter region in relation to relative expression of CYP1A1

Each point on the graph represents one subject, and includes both smokers and nonsmokers. The average methylation from a minimum of clones is plotted against the relative gene expression for each individual in order to provide direct estimates of the correlation between DNA methylation in a given region and expression of *CYP1A1*. Consistent with hypermethylation yielding gene silencing, increased methylation in the promoter of Region I, which contains a critical XRE, significantly and inversely correlates with gene expression ($r = -0.737$, $p = 0.007$). Consistent with our findings as presented in Tables II and III, there is no significant correlation between the degree of methylation at any CpG dinucleotide Regions II-IV and gene expression (data not shown).

Table I
Characteristics of the study population

We found that the two groups did not differ significantly by virtue of maternal comorbidities (preeclampsia, gestational diabetes etc), nor maternal age, BMI or gestational age at delivery. As anticipated, fetal length was influenced by smoking.

	Non-smokers	Smokers	<i>P</i> value
Maternal Age (yrs)	26.5 ± 1.4	28.6 ± 2.4	0.411
Maternal BMI	28.9 ± 1.7	31.9 ± 3.2	0.379
Gestational Age (wks)	38.4 ± 0.6	36.2 ± 1.3	0.145
Infant Weight (grams)	3393 ± 165	2982 ± 130	0.074
Infant Length(cm)	49.4 ± 0.5	44.3 ± 0.5	0.022

Region I of the CYP1A1 promoter is significantly hypomethylated (55.6% vs 45.9%, p=0.027) in placenta from smokers compared with non-smokers

Table II

Methylation within Regions II-IV does not significantly differ between the two groups.

CYP1A1 Promoter Region	Promoter Region Contains XRE	Smoker	Number of Clones Sequenced	CpG/Total (CG+TG)	% of methylated CpG
I	Yes	No Yes	87 44	242/435 101/220	55.6% 45.9% (p= 0.027)
II	Yes	No Yes	87 44	485/2262 231/1144	21.4% 20.2% (p= 0.765)
III	Yes	No Yes	39 15	5/585 5/225	0.9% 2.2% (p= 0.156)
IV	No	No Yes	42 19	1/546 3/247	0.2% 1.2% (p= 0.150)

Table III
Only CpGs proximally surrounding an XRE are differentially methylated

By dividing the CpG sites into sequential groups of 5 within the *CYP1A1* functional promoter, we find that only dinucleotide proximally surrounding an XRE element (1 to 5 and 21 to 25) are differentially methylated (CpGs 1 to 5; $p = 0.027$; CpGs 21 to 25; $p = 0.006$; bold type)

CpGs Within CYP1A1 Promoter	Contains XRE	Smoker	Number of Clones Sequenced	CpG/Total (CG + TG)	% of methylated CpG
1 to 5 Region I	Yes	No Yes	87 44	242/435 101/220	55.6% 45.9% ($p = 0.027$)
6 to 10 Region II	No	No Yes	87 44	288/435 125/220	66.2% 56.8% ($p = 0.138$)
11 to 15 Region II	No	No Yes	87 44	111/435 61/220	25.5% 27.7% ($p = 0.529$)
16 to 20 Region II	No	No Yes	87 44	53/435 23/220	12.2% 10.5% ($p = 0.526$)
21 to 25 Region II	Yes	No Yes	87 44	8/435 12/220	1.8% 5.5% ($p = 0.006$)
26 to 30 Region II	No	No Yes	87 44	22/435 10/220	5.1% 4.5% ($p = 0.759$)
31 to 35 Region III	No	No Yes	38 15	2/190 1/75	1.1% 1.3% ($p = 0.846$)
36 to 40 Region III	No	No Yes	38 15	0/190 1/75	0.0% 1.3% ($p = 0.112$)
41 to 45 Region III	No	No Yes	38 15	3/190 3/75	1.6% 4.0% ($p = 0.218$)
46 to 50 Region IV	No	No Yes	42 19	0/210 1/95	0.0% 1.1% ($p = 0.138$)
51 to 55 Region IV	No	No Yes	42 19	1/210 2/95	0.5% 2.1% ($p = 0.179$)