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In utero tobacco exposure epigenetically modifies placental *CYP1A1* expression

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Abstract

The metabolic pathways used 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 2 phases: in phase I, *CYP1A1* catalyzes conversion into harmful hydrophilic DNA adducts, whereas 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 although deletion of fetal *GSTT1* significantly modified birth weight in smokers, no polymorphism fully accounted for fetal growth restriction. Because smoking up-regulates *CYP1A1* expression, we hypothesized that nonallelic (epigenetic) dysregulation of placental *CYP1A1* expression via alterations in DNA methylation (meCpG) may further modify fetal growth. In the present article, 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, $P < .05$). To fully characterize *CYP1A1* meCpG status, bisulfite modification and sequencing of the entire proximal 1-kilobase promoter (containing 59 CpG sites) were performed. CpG sites immediately proximal to the 5′-xenobiotic response element transcription factor binding element were significantly hypomethylated among smokers (55.6% vs 45.9% meCpG, $P = .027$), a finding that uniquely correlated with placental gene expression ($r = 0.737$, $P = .007$). Thus, in utero tobacco exposure significantly increases placental *CYP1A1* expression in association with differential methylation at a critical xenobiotic response element.

1. Introduction

The metabolic pathways used by higher-eukaryotic organisms to deal with potentially carcinogenic xenobiotic compounds from tobacco smoke have been well characterized [1-4]. Although differing susceptibilities to in utero tobacco exposure are not understood, genetic epidemiologic studies have linked cancer risk due to an altered ability to metabolize carcinogenic polycyclic aromatic hydrocarbons (PAHs) or addictive (nicotine) compounds into less harmful intermediates along these characterized pathways [1-4].

Of the more than 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 2-phase enzymatic metabolic reactions (Fig. 1A). Phase I enzymes, such as the cytochrome P450 arylhydrocarbonhydroxylases, catalyze the conversion of PAHs into reactive hydrophilic intermediates that have the potential to form harmful DNA adducts. After 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

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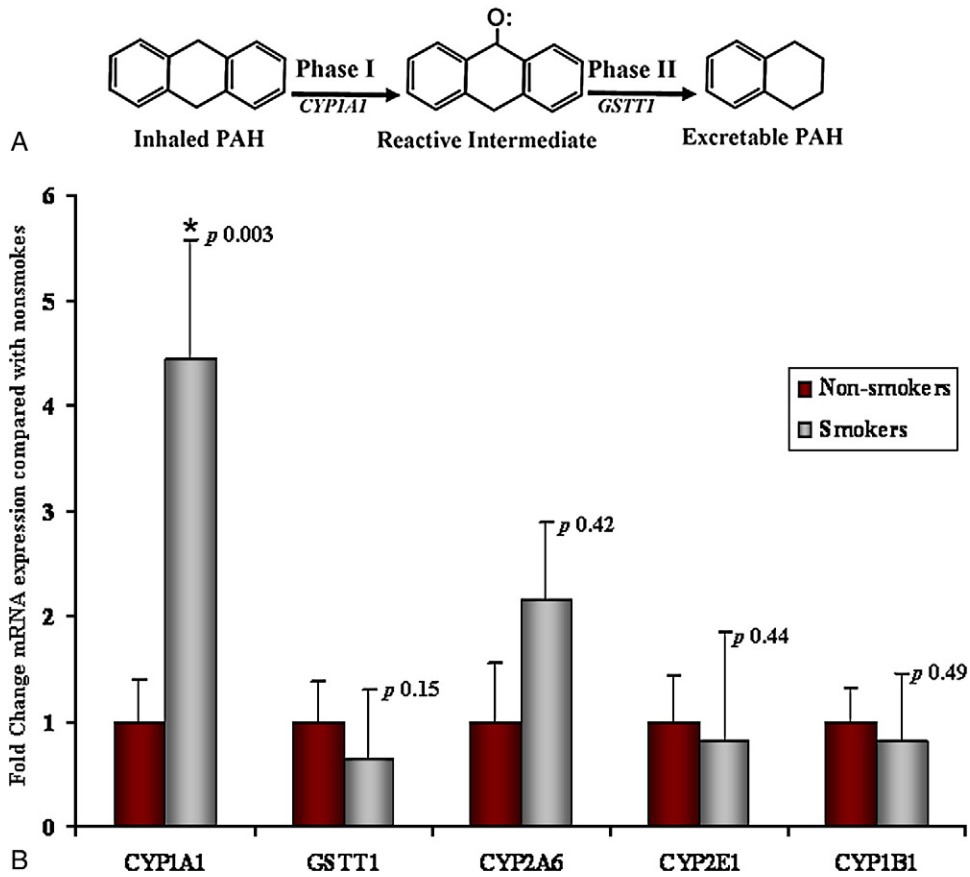

Fig. 1. *CYP1A1* is significantly up-regulated in the placenta from smokers compared with nonsmokers. A, Schematic of the metabolism of inhaled polycyclic aromatic hydrocarbons form cigarette smoke. Polycyclic aromatic hydrocarbons are metabolized into reactive intermediates by the phase I enzymes, such 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 (eg, increased expression of *CYP1A1*) in combination with decreased phase II activity (eg, decreased *GSTT1* expression) may yield increased susceptibility to tobacco-related adverse outcomes. We have recently demonstrated that the relatively prevalent fetal *GSTT1* deletion modulates birth weight in response to in utero tobacco exposure. B, *CYP1A1* expression is significantly up-regulated in the placenta from smokers compared with nonsmokers ($P = .003$). Placental expression of other CYP family members (*CYP2A6*, *CYP2E1*, and *CYP1B1*) as well as *GSTT1* did not differ significantly between the 2 groups.

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 (eg, increased expression of *CYP1A1*) in combination with decreased phase II activity (eg, 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 [13] first reported in 2002 that an association between maternal cigarette smoking and infant birth weight differs by polymorphisms of 2 maternal metabolic genes: *CYP1A1* and *GSTT1*. 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 National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network (Aagaard-Tillery et al, in press). Specifically, we performed blinded genotyping for known functional allelic variants of *CYP1A1* (Ile_{462}ValAA>AG/GG), *GSTT1* (deletion), and *CYP2A6* (Lys_{160}HisT>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 .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, 2 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 (ie, quantitation of PAH level by personal air monitoring) reveals significant interactions between maternal *CYP1A1* haploytpe 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 AH 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 nonallelic mechanisms including epigenetic influences such as covalent modifications of histones and DNA methylation (recently reviewed by Zilberman [24] and by Liu et al [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 nonallelic modulation of *CYP1A1* expression may contribute to risk of adverse pregnancy outcomes [5,30,31]. Because 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 up-regulated 4.4 fold (P = .003) in smokers compared with nonsmokers. 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% meCpG, P = .027). Thus, maternal smoking and aromatic hydrocarbon exposure leading to observed increases in DNA adduct formation in cord blood occur 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.

2. Results

2.1. Study subjects

Outcome data and placental samples for this study were obtained from a cohort of gravidae who had smoked during their pregnancy alongside nonsmoking 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 nonuse 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, body mass index (BMI), gestational age, or fetal weight at delivery. As anticipated, fetal length was influenced by in utero tobacco use (Table 1).

2.2. Placental CYP1A1 expression is up-regulated 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 nonallelic regulation of *CYP1A1* expression could be further rendering adverse events in response to in utero tobacco exposure. We therefore analyzed placental expression of 4 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 nonsmokers (P = .003, Fig. 1B). In contrast, neither expression of the other CYP genes nor *GSTT1* differed by virtue of maternal smoking behavior (Fig. 1B).

Table 1
Characteristics of the study population

	Nonsmokers	Smokers	P value
Maternal age (y)	26.5 ± 1.4	28.6 ± 2.4	.411
Maternal BMI	28.9 ± 1.7	31.9 ± 3.2	.379
Gestational age (wk)	38.4 ± 0.6	36.2 ± 1.3	.145
Infant weight (g)	3393 ± 165	2982 ± 130	.074
Infant length (cm)	49.4 ± 0.5	44.3 ± 0.5	.022

We found that the 2 groups did not differ significantly by virtue of maternal comorbidities (preeclampsia, gestational diabetes, etc), maternal age, BMI, or gestational age at delivery. As anticipated, fetal length was influenced by smoking.

2.3. Regions of the CYP1A1 functional promoter that contain an XRE are differentially methylated in placental tissue of smokers compared with nonsmokers

The methylation sites of the *CYP1A1* functional promoter in lung tissue from smokers and nonsmokers have been previously characterized by other investigators [26]. Based on relative proximity of CpG dinucleotides to critical transcriptional binding elements (ie, 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]. Using 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, whereas those distal from such elements did not demonstrate differential methylation [26]. To characterize differential placental methylation in the *CYP1A1* promoter in response to maternal tobacco use, we used 2 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 that have the potential to undergo differential methylation and include XRE transcriptional binding elements in regions I and II (Fig. 2A). The total percentage of methylation for each region was calculated for both the smokers and nonsmokers, and differences among regions were compared by the 2-tailed Student *t* test. As shown in Table 2, the first core primed region (region I) that contains an XRE transcriptional binding element was unique in significant rate of methylation in smokers compared with nonsmokers (55.6% vs 45.9% meCpG, $P = .027$, Table 2). In support of previously published data using primary lung tissue [26], partial or no methylation was observed in placentas from smokers vs nonsmokers in primed regions II to IV (Fig. 2B, Table 2). Interestingly, region I contains a critical XRE that 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 that surround an XRE, we alternately designated the CpGs within the functional promoter as belonging to 1 of 11 sequential (5′ to 3′) groups each containing 5 CpG dinucleotides. In such a manner, we were able to assess

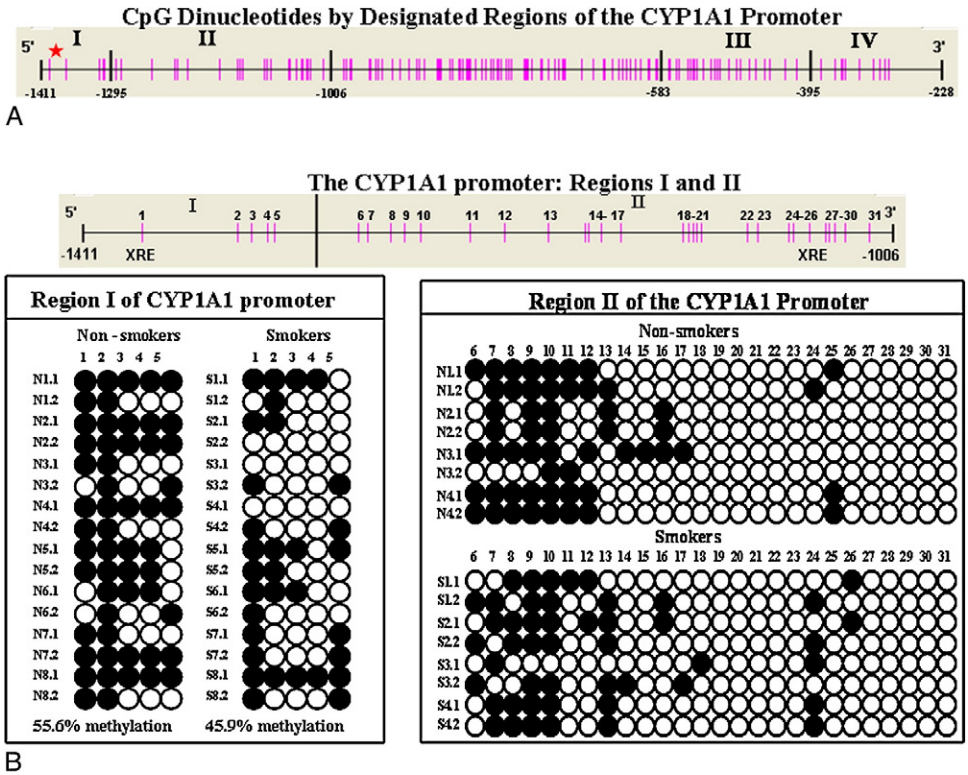

Fig. 2. The XRE-containing region I of the *CYP1A1* promoter is hypomethylated in placenta from smokers compared with nonsmokers. A, The 4 analyzed regions of the *CYP1A1* promoter are depicted. Magenta lines represent CpG dinucleotides, and the red stars designate localization of the XREs. In total, among all 4 regions, the precise methylation status of 59 CpG dinucleotides was determined in bisulfite-treated placental DNA from 19 nonsmokers and 15 smokers using sequencing. 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 4 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, vs 45.9% among nonsmokers ($P = .027$). Region II is not differentially methylated between the 2 groups.

Table 2
CYP1A1 promoter methylation

CYP1A1 promoter region	Promoter region contains XRE	Smoker	No. of clones sequenced	CpG/total (CG + TG)	% of methylated CpG
I	Yes	No	87	242/435	55.6%
		Yes	44	101/220	45.9% (P = .027)
II	Yes	No	87	485/2262	21.4%
		Yes	44	231/1144	20.2% (P = .765)
III	Yes	No	39	5/585	0.9%
		Yes	15	5/225	2.2% (P = .156)
IV	No	No	42	1/546	0.2%
		Yes	19	3/247	1.2% (P = .150)

Region I of the *CYP1A1* promoter is significantly hypomethylated (55.6% vs 45.9%, P = .027) in placenta from smokers compared with nonsmokers. Methylation within regions II to IV does not significantly differ between the 2 groups.

whether only those dinucleotides most proximal to and surrounding the XRE sites underwent differential methylation. As presented in Table 3, among the 11 groups of 5 CpGs, only the 2 XRE-containing groups within these 55 CpGs demonstrated significant differential methylation (CpGs 1-5, P = .027; CpGs 21-25, P = .006). Of note, the only group of dinucleotides with hypomethylation in association with smoking status was CpGs 1 to 5, which were contained within region I (Tables 2 and 3).

2.4. 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 percentage of methylation for each region of the *CYP1A1* promoter in both smokers and nonsmokers and interrogated the relationship with bivariate correlations (Pearson correlation for variance) using a 2-tailed test for significance. As demonstrated in Fig. 3, we found that the percentage of methylation of region I inversely correlates with expression level (r = −0.737, P = .007); this correlation held true regardless of maternal smoking behavior. In a linear regression model

Table 3
CYP1A1 promoter methylation status of CpGs flanking an XRE

CpGs within *CYP1A1* Promoter	Contains XRE	Smoker	No. of clones sequenced	CpG/total (CG + TG)	% of methylated CpG
1-5	**Yes**	**No**	**87**	**242/435**	**55.6%**
Region I		**Yes**	**44**	**101/220**	**45.9% (P = .027)**
6-10	No	No	87	288/435	66.2%
Region II		Yes	44	125/220	56.8% (P = .138)
11-15	No	No	87	111/435	25.5%
Region II		Yes	44	61/220	27.7% (P = .529)
16-20	No	No	87	53/435	12.2%
Region II		Yes	44	23/220	10.5% (P = .526)
21-25	**Yes**	**No**	**87**	**8/435**	**1.8%**
Region II		**Yes**	**44**	**12/220**	**5.5% (P = .006)**
26-30	No	No	87	22/435	5.1%
Region II		Yes	44	10/220	4.5% (P = .759)
31-35	No	No	38	2/190	1.1%
Region III		Yes	15	1/75	1.3% (P = .846)
36-40	No	No	38	0/190	0.0%
Region III		Yes	15	1/75	1.3% (P = .112)
41-45	No	No	38	3/190	1.6%
Region III		Yes	15	3/75	4.0% (P = .218)
46-50	No	No	42	0/210	0.0%
Region IV		Yes	19	1/95	1.1% (P = .138)
51-55	No	No	42	1/210	0.5%
Region IV		Yes	19	2/95	2.1% (P = .179)

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 dinucleotides proximally surrounding an XRE element (1-5 and 21-25) are differentially methylated (CpGs 1-5, P = .027; CpGs 21-25, P = .006; bold type).

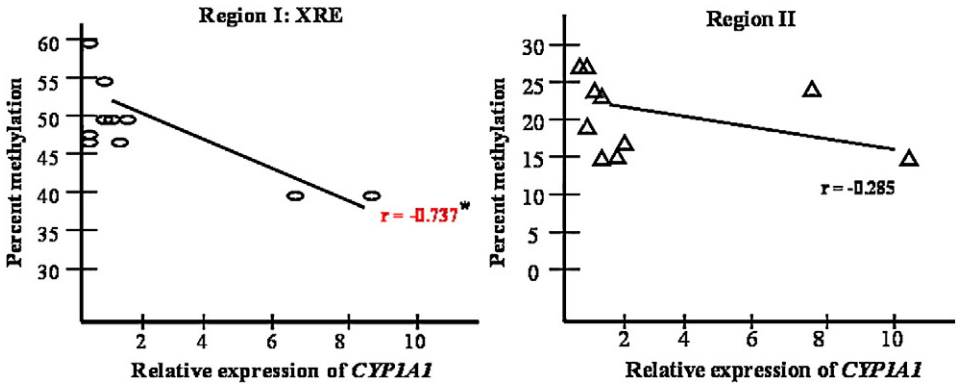

Fig. 3. Plot of percentage of methylation of the promoter region in relation to relative expression of *CYP1A1*. Each point on the graph represents one subject, and includes both smokers and nonsmokers. The average methylation from a minimum of clones is plotted against the relative gene expression for each individual 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 ($r = -0.737$, $P = .007$). Consistent with our findings as presented in Tables 2 and 3, there is no significant correlation between the degree of methylation at CpG dinucleotide regions II to IV and gene expression (data not shown).

controlling for the potential covariates of fetal sex and maternal comorbidities, percentage of CpG methylation in region I independently predicted *CYP1A1* expression (data not shown). Moreover, there was no correlation between methylation status of regions II to IV and placental *CYP1A1* expression (data not shown).

3. Discussion

The metabolic pathways used 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 AH 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 that further influence birth weight, an established causal relationship with tobacco use and delivery of small for gestational age infants exists [5-12]. In 1957, Simpson and Linda [36] reported their observations that infants born to mothers who smoked 10 cigarettes or more per day weighed an average 200 g less than those delivered by reported nonsmokers. 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 by Cnattingius [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 by Raunio et al [30]). We similarly find a significantly 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 probably 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% to 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 nonsmokers (Fig. 2 and Table 2). Region I, which contains an XRE element that is involved in regulation of *CYP1A1* expression, was the only region that demonstrated significant differential methylation within the proximal promoter (Table 2). This association held true within an individual, as there was a significant correlation between *CYP1A1* expression and region I hypomethylation (Fig. 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 after 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 that are then readily excreted occurs. Thus, although 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 that 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 although 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 less than 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 kilobase and 59 CpG sites. In total, we examined more than 250 clones to encompass more than 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 used 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 that 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 from peripheral mononuclear cells among adults who 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 or hepatic tissue. Yet based on the reports of Jauniaux and Burton [41], we would anticipate that *CYP1A1* levels in the fetus may similarly differ and would do so with a similar mechanism. 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 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 1-carbon metabolism pathway alleviates 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 2-fold. First, our prior data illustrate that a fetal metabolic gene (*GSTT1*) that 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 that 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.

4. Methods

4.1. Study population

Outcome data and placental samples for this study were obtained from a cohort of gravidae who had smoked during their pregnancy alongside nonsmoking 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 nonuse controls [10,31-33]. Exclusion criteria included multiple gestations; a priori known fetal anomalies or aneuploidy; chorioamnionitis; secondary substance exposure (eg, 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.

4.2. Collection of placental samples

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

4.3. Real-time polymerase chain reaction analysis

Approximately 50 mg 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 complementary DNA (cDNA) from each sample using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA). Real-time quantitative reverse transcriptase polymerase chain reaction (PCR) analyses were performed using 2 μL cDNA and 2 μmol/L 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 (Hercules, CA). Relative quantification of each gene was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase by using the comparative Ct method [50].

4.4. 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, 4 fragments of the *CYP1A1* promoter region 1400 base pairs 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′-AAACCCCCACCCTACCCCCC-3′ (antisense); region III, 5′-GGGTTTTGGGGGATAGGTTT-3′ (sense) and 5′-CG/ATACAAATACCTCCCCAAC-3′ (antisense); and region IV, 5′-GGAAGGAGGTTATTAA/TGGGG-3′ (sense) and 5′-CACCTAAAAATCCCAATTC-CAA-3′ (antisense). The PCR conditions were as follows: 5 μL template DNA, 5 μL 10× ABI (PE Applied Biosystems, Warrington, United Kingdom) buffer, 4.5 μmol/L MgCl2, 4 μL 2.5μmol/L dNTP, 5 μL 10 μmol/L sense and antisense primers, and 0.5 μL TaqGold (ABI) polymerase. Reactions were run under conditions of 95°C for 15 minutes, with 40 cycles (95°C 30 seconds, 56°C for 30 seconds, 72°C for 1 minute) and with 10-minute 72°C elongation for adenylation. The amplified PCR products were subsequently cloned, and a minimum of 4 clones from each reaction was sequenced. Samples were sequenced by Lone Star Labs (Houston, TX) and aligned using CLUSTAL W (Kyoto University). Fig. 2A, which depicts the CpG sites, was created by the ABI Methyl Prime software.

4.5. Statistical analysis

Univariate comparisons were performed using χ^2 or Fisher exact test for discrete variables and the Wilcoxon

test for the continuous variables. Real-time PCR was analyzed using the $2^{-\Delta\Delta CT}$ method as previously described [50]. Results are displayed as fold change of smokers compared with nonsmoking control samples. An independent-samples Student *t* test was performed for each gene. An independent-samples, 1-tailed Student *t* test was performed to analyze the differential methylation between smokers and nonsmokers 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 percentage of methylation for that individual with analysis of variance by Pearson coefficient. SPSS (SPSS Inc, Chicago, IL) was uses for analyses, and a nominal P value < .05 was considered significant. No adjustment was made for multiple comparisons.

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