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Placental DNA methylation alterations associated with maternal tobacco smoking at the *RUNX3* gene are also associated with gestational age

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Abstract

Aims—The developmental origins of health and disease hypothesis states that later-life disease may be influenced by the quality of the *in utero* environment. Environmental toxicants can have detrimental effects on fetal development, potentially through effects on placental development and function. Maternal smoking during pregnancy is associated with low birth weight, preterm birth and other complications, and exposure to cigarette smoke *in utero* has been linked to gross pathologic and molecular changes to the placenta, including differential DNA methylation in placental tissue. The aim of this study was to investigate the relationship between maternal smoking during pregnancy, methylation changes in the placenta and gestational age.

Materials & methods—We used Illumina[®]s (CA, USA) Human Methylation27 BeadChip technology platform to investigate the methylation status of 21,551 autosomal, non-SNP-associated CpG loci in DNA extracted from 206 human placentas and examined loci whose variation in methylation was associated with maternal smoking during pregnancy.

Results—We found that methylation patterns of a number of loci within the *RUNX3* gene were significantly associated with smoking during pregnancy, and one of these loci was associated with decreased gestational age ($p = 0.04$).

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Conclusion—Our findings, demonstrating maternal smoking-induced changes in DNA methylation at specific loci, suggest a mechanism by which *in utero* tobacco smoke exposure could exert its detrimental effects upon the health of the fetus.

Keywords

epigenetics; gestational age; *in utero*; methylation; placenta; pregnancy; *RUNX3*; smoking

It has long been suspected that environmental exposures *in utero* can increase susceptibility to adult disease. A growing body of literature suggests that the *in utero* environment may play a role in the development of cardiovascular disease, diabetes and certain cancers [1-5]. The Barker hypothesis posits that the *in utero* environment can affect offspring, altering risk for the development of disease throughout life [6-9]. Indeed, it has been suggested that even grandmaternal exposures can affect future disease susceptibility [10]. Fetal programming in response to the *in utero* environment is thought to be of epigenetic origin, where heritable changes to gene expression occur without direct changes to DNA sequence [11-13].

The placenta plays an important role in regulating fetal growth and development as it produces a number of growth factors and hormones. Additionally, the placenta exhibits a significant degree of metabolic activity, including the metabolism of potentially toxic compounds [14]. However, many toxicants are capable of crossing the placenta, acting directly or potentially by altering the metabolic function of the placenta. Environmental toxicants that cross the placenta may affect placental function by modifying the epigenetic state of the tissue, including altering DNA methylation [15,16]. Thus, epigenetic marks in the placenta can serve as a record of *in utero* exposure [14].

Maternal tobacco smoking during pregnancy is associated with significant morbidity and mortality, both perinatally and later in life. Several chemicals found in tobacco smoke, including nicotine, can cross the placenta and negatively impact upon the fetus [17]. Nicotine accumulates in fetal blood and amniotic fluid [18], and fetal nicotine levels have been shown to be 15% higher than maternal levels [19]. The detrimental effects of maternal tobacco smoking include premature birth [20,21], low birth weight [22,23], abnormal neurobehavioral outcomes [24], childhood obesity [25-27], respiratory tract diseases and sudden infant death syndrome [28]. Prenatal tobacco exposure can have damaging effects through both genetic and epigenetic mechanisms [14,29], and maternal tobacco smoking during pregnancy is associated with altered DNA methylation patterns in the placenta [30-32]. Furthermore, DNA methylation profiles associated with gestational age have been identified [33]. We hypothesized that maternal smoking during pregnancy is associated with changes to DNA methylation in the placenta and that smoking-associated DNA methylation alterations are, in turn, associated with altered gestational age, thereby providing a biological mechanism linking this exposure to important reproductive outcomes.

Materials & methods

Study design

A total of 206 placental samples were collected from infants delivered to the Women and Infants Hospital in Providence (RI, USA) between September 2008 and September 2009 [34], using institutional review board-approved protocols at all involved institutions. This cohort is oversampled for small-for-gestational-age (<10th percentile of birth weight) infants and clinical intrauterine growth restricted diagnoses. Placental samples were collected within 2 h of birth and full thickness sections were taken from the maternal side of the placenta, 2 cm from the umbilical cord insertion site. The samples were placed in RNAlater® (Applied Biosystems, Inc., CA, USA; AM7020) immediately after collection.

After being stored at 4°C for at least 72 h, the samples were blotted dry of RNAlater and then frozen at -80°C prior to processing. Smoking status at any time during pregnancy was recorded from patient charts and was analyzed as a dichotomous variable.

DNA extraction & bisulfite modification

As previously described [34], DNA extractions were performed using the QIAmp® DNA Mini Kit (Qiagen, Inc., Hilden, Germany; 51304) and quantified by NanoDrop™ ND-1000 spectrophotometer (NanoDrop, DE, USA). Bisulfite modification using the EZ DNA Methylation™ Kit (Zymo Research, CA, USA; D5008) was performed on 1 µg of extracted placental DNA. DNA from one peripheral blood sample from an adult not included in the study was also extracted and bisulfite-modified; the bisulfite-modified DNA from this blood sample was run on each BeadChip to control for interarray variability.

DNA methylation profiling

Bisulfite-modified DNA samples were arrayed using the Illumina® (CA, USA) Infinium® HumanMethylation27 BeadArray [101] at the University of California San Francisco Institute for Human Genetics Genomic Core Facility [34]. β -values representing the methylation status at each CpG locus were calculated from the intensity of the methylated (M) and unmethylated (U) alleles, where the ratio of fluorescent signals is:

$$\beta = \frac{Max(M, 0)}{(Max[M, 0] + Max[U, 0] + 100)}$$

and $0 < \beta < 1$.

β -values near 1 indicate complete methylation and values near 0 indicate absence of methylation. Array quality assurance was assessed according to the method described previously [34], and 21,551 autosomal, non-SNP-associated CpG loci were utilized in this analysis.

Statistical analysis

As the β -values of these loci were non-normally distributed, they were logit transformed [35]. To adjust the data for batch effect (BeadChip), the ComBat procedure was applied to the methylation array data [36]. These statistical methods are detailed in the Supplementary Material (see online at www.futuremedicine.com/doi/suppl/epi.13.63). After ComBat adjustment, associations between differential methylation at each of 21,551 CpG loci and maternal smoking during pregnancy were investigated using a locus-by-locus approach. This consisted of a series of independent linear regression models modeling logit-transformed, ComBat-adjusted methylation values as the dependent variable, and smoking status during pregnancy as the independent variable. False discovery rate estimation was used to control for the large number of tests performed [37]. RUNX3 CpG loci exhibiting associations with maternal smoking during pregnancy were subsequently analyzed for associations with gestational age (dichotomized, <37 weeks gestation versus \geq 37 weeks gestation, as this is considered the clinical threshold for preterm birth) using both two-tailed Student's t-tests and multivariable logistic regression models, controlled for potential confounders (maternal age, infant gender, birth weight and delivery method [vaginal vs cesarean section]).

Bisulfite pyrosequencing DNA methylation analysis

Pyrosequencing was performed to confirm array findings for specific CpG loci of interest. After bisulfite-modifying DNA from 22 placental samples (11 samples from smoking

mothers and 11 samples from nonsmoking mothers), pyrosequencing was performed on the PCR-amplified product. Pyrosequencing assays were designed using Pyromark™ Assay Design 2.0 software (Qiagen, Inc.; 9019077) and ordered from Invitrogen™ (Life Technologies, NY, USA). Pyrosequencing was performed on a Pyromark MD pyrosequencing instrument running Pyromark qCpG 1.1.11 software (Qiagen, Inc.). PCR was performed using HotStarTaq® DNA Polymerase (Qiagen, Inc.; 203205). cg06037693, cg00117172 and cg04757093 were assessed by pyrosequencing. Additional details can be found in the Supplementary Material.

Results

The demographics of the study population are described in Table 1. The population was over-sampled for small-for-gestational-age infants (28%) and the majority of the infants in this study were born at or near term, with a mean gestational age of 38.2 weeks. Approximately 11% of mothers reported smoking during pregnancy.

The DNA methylation status of the 206 placenta samples in this study was interrogated using the Illumina Infinium HumanMethylation27 BeadChip, which examines 27,578 loci [101]. Poor-performing loci, those associated with the sex chromosomes and those whose probe contained a SNP, were removed, leaving 21,551 loci in the 206 samples for study. Principal component analysis was performed to test for associations between the first three principal components with several variables including BeadChip. Since BeadChip was significantly associated with the top three principal components, which represent the maximal variation in methylation across the array, ComBat was used to normalize the array methylation data according to BeadChip [36]. These statistical methods are further detailed in the Supplementary Material and are described by Supplementary Figures 1–6 & Tables 1 & 2.

A locus-by-locus analysis assessed possible associations between smoking during pregnancy and differential methylation status at each of the 21,551 autosomal CpG loci. Analysis revealed that 1918 of these loci had differential methylation patterns associated with maternal smoking during pregnancy ($p < 0.05$; the top 50 CpG loci and p-values are given in Supplementary Table 3), although the lowest q-value observed was 0.3. A Manhattan plot describing the distribution of p-values derived from these associations by chromosomal location is shown in Figure 1. For the 1918 CpG loci significantly associated with smoking during pregnancy, the lowest $\delta\beta$ -difference (for median absolute β -values ranging from 0 to 1) is found at cg22863122, with a $\delta\beta$ -difference between smokers and nonsmokers of -0.0006. The highest $\delta\beta$ -difference is found at cg13315147, with a $\delta\beta$ -difference between smokers and nonsmokers of 0.1865. Among the top 50 CpG loci by p-value (Supplementary Table 3), the lowest methylation difference between smokers and nonsmokers is found at cg13269964, with a methylation difference of 0.0166. CpG loci of interest, described below, were pyrosequenced to confirm methylation differences in the placental tissue of smoking and nonsmoking mothers.

Multiple genes were found to have multiple CpG loci residing within them that were associated with smoking during pregnancy. We observed three genes (*APBA2*, *ATP10A* and *PTPRO*) with four CpG loci each that were associated with smoking during pregnancy, as well as six other genes (*CASP8*, *CHFR*, *KLK10*, *L3MBTL*, *MLH1* and *RBI*) with three CpG loci each that were associated with smoking during pregnancy. Some of these genes have previously been associated with prenatal smoke exposure (*PTPRO* [38] and *CASP8* [39]) and smoking-related cancers (*CHFR* [40]). Most notably, seven loci residing within the intronic and promoter regions of the *RUNX3* gene displayed differential methylation patterns that were significantly associated with maternal smoking during pregnancy (Table 2). The

RUNX3 gene has been associated with airway hyper-responsiveness and asthma and these conditions are also associated with maternal smoking [41-52], and as our analysis was focused on identifying CpG loci of potential biological relevance to maternal smoking during pregnancy, these loci within the *RUNX3* gene were chosen for further analysis, as well as follow-up bisulfite pyrosequencing.

These seven CpG loci within the *RUNX3* gene, the p-values for their associations with smoking during pregnancy and their median absolute methylation values in the placentas of smoking and nonsmoking mothers are given in Table 2. cg06037693 is situated immediately preceding the first promoter region, and cg14182690 in the first intronic region after the first promoter region; cg24019564, also situated in the first intronic region, precedes the second promoter region; and the remaining four loci associated with maternal smoking during pregnancy are located in intronic regions two (cg00117172), four (cg08705994 and cg00572797) and five (cg04757093) of this gene.

As maternal smoking during pregnancy is associated with decreased gestational age and preterm birth [21,53,54], we investigated the methylation status of each *RUNX3* CpG locus identified from the smoking analysis for associations with gestational age. Gestational age was modeled as a dichotomous variable consisting of infants of <37 weeks gestation and of 37 weeks gestation [55]. Univariate analysis revealed that two of the seven CpG loci within *RUNX3*, which were associated with smoking during pregnancy, cg04757093 and cg14182690, were significantly or near-significantly associated with gestational age ($p = 0.07$ and $p = 0.01$, respectively). Multivariable logistic regression models were used to test for associations between the methylation status of these two CpG loci with gestational age, controlling for maternal age, infant gender, birth weight and delivery method. One CpG locus, cg04757093, was significantly associated with gestational age in the model (Table 3). Along with cg06037693 and cg00117172, this locus exhibited significant hypermethylation in the placentas of smoking mothers ($n = 22$) compared with nonsmoking mothers ($n = 184$; for cg04757093, $p = 0.03$; Table 3 & Figure 2) and a 1-logit increase in methylation was associated with a tenfold increased risk for preterm birth (odds ratio: 10.2, 95% CI: 1.1–103.3; $p < 0.04$).

In order to confirm array results using an orthogonal technique, we performed bisulfite pyrosequencing of cg04757093, which was associated with gestational age, as well as two neighboring loci (cg06037693 and cg00117172), in a subset of placenta samples (total $n = 22$; Figure 3). Pyrosequencing confirmed that the placental tissue from infants exposed to maternal tobacco smoke had significantly higher DNA methylation at cg04757093 ($p < 0.02$) and showed the same trend as the array analysis at cg00117172 ($p < 0.09$), while the trend was not confirmed at cg06037693 by pyrosequencing.

Discussion

In a sample of 206 human placentas, we observed a significant association between maternal smoking during pregnancy and placental hypermethylation of cg04757093, a CpG locus within the body of the *RUNX3* gene. A growing body of literature suggests that *RUNX3* plays an important role in normal immune system development [56,57], susceptibility to early life disease as a result of *in utero* exposures [41], and many cancers [58-68]. *RUNX3* is important for normal cellular differentiation and development, including T-cell differentiation [56,57,66,69], macrophage differentiation [70], neuronal cell development [71] and cell-cycle progression [63], and is known to negatively regulate dendritic cell maturation [72]. *RUNX3* is a tumor suppressor gene [58,60,62,67,73,74] and it interacts with β -catenin [61]. When upregulated, *RUNX3* is known to inhibit cyclins D1 and E and increase *p27*, *Rb* and *TIMP-1* expression [59,60]. *RUNX3* expression changes are associated with

clear cell renal cell carcinoma [60] and hepatocellular carcinoma [68]. *RUNX3* hypermethylation is associated with cancers of the breast [58], stomach [63-65], prostate [75] and lung [76], as well as many others [77]. It has also been demonstrated that hypermethylation of *RUNX3* in smokers is associated with bladder cancer [78]. The precise function of *RUNX3* in the placenta has not been described.

Prenatal exposure to tobacco smoke is highly prevalent [28] and is associated with the development of childhood asthma [45-50,52], in part by increasing airway hyper-responsiveness [51]. In murine models, the *RUNX* family of transcription factors has been shown to play a role in the development of prenatal smoke exposure-induced airway hyper-responsiveness [41]. This is supported by research demonstrating that *RUNX3*-knockout mice spontaneously develop asthma-like disease [42]. Increased expression of *CCR7* in the absence of *RUNX3* expression has been shown to allow increased dendritic cell migration to draining lymph nodes, a feature associated with an asthma-like phenotype including airway hyper-responsiveness [42]. Further investigation is necessary to determine the precise nature of the influence of prenatal smoke exposure on placental *RUNX3* expression that may result from epigenetic changes, especially considering the limitations of this research, which has observed differential methylation at a single CpG locus within the gene body. More comprehensive coverage of *RUNX3* CpG loci, for example, via Illumina's now-available HumanMethylation450 BeadChip technology or through comprehensive next-generation sequencing, may reveal relevant methylation changes within *RUNX3* in more detail. To date, no studies looking at the association between maternal smoking and DNA methylation using the Illumina BeadChip array platforms have identified *RUNX3*. It is important to keep in mind that, although most of the other studies have focused on identifying alterations in infant cord blood related to maternal smoking [79,80], this study is examining alterations in the placenta. Owing to the highly tissue-specific nature of DNA methylation, it is not surprising that different loci are identified.

In addition to *RUNX3*, our initial genome-wide scan also identified a number of genes with multiple CpG loci, whose methylation was associated with maternal tobacco use during pregnancy. Three genes (*APBA2*, *ATP10A* and *PTPRO*) each had four CpG loci that were associated with smoking during pregnancy. *PTPRO* is a protein tyrosine phosphatase implicated as a tumor suppressor and involved in the development of a number of specific cellular lineages [81]. *APBA2* encodes a protein involved in synaptic vesicle exocytosis [82], although hypermethylation of this gene has been identified in a variety of tumor types including pancreatic cancer [83], hepatocellular carcinoma [84] and oral cancers [85]. *ATP10A* is a maternally expressed imprinted gene encoding an ATPase whose loss had been identified in children with Angelman syndrome [86]. Interestingly, both *APBA2* and *ATP10A* have been implicated in the development of psychiatric diseases including autism and schizophrenia [87-89].

A limitation of this study is its use of the HumanMethylation 27 BeadChip array platform, which does not provide as much coverage of the human methylome as the newer HumanMethylation 450 BeadChip array platform, and also does not interrogate as many non-promoter regions as the HumanMethylation 450 BeadChip array platform. The use of the HumanMethylation 450 BeadChip array platform in future studies would greatly help to elucidate the potential role of the CpG sites not interrogated by the HumanMethylation27 BeadChip array platform. In addition, a larger sample size would help to investigate the effects of smoking during pregnancy on placental *RUNX3* methylation, as a limitation of this research was the relatively small number of smoking mothers in this study population (n = 22). We are also limited in our examination of the dosage of exposure due to the use of self-reporting of tobacco smoking. Other more quantitative measures of tobacco smoking may provide a more accurate measure of the extent of tobacco smoking and, thus, additional

information on the mechanisms underlying these effects. Although, this research does point to a possible role for *RUNX3* in tobacco-associated adverse pregnancy outcome and, as *RUNX3* expression is controlled by a retinoic acid-sensitive signaling pathway in some cell types, this may suggest novel interventional approaches that could be utilized [77,78,90,91].

Conclusion

An extensive literature has reported associations between maternal tobacco smoking during pregnancy and the gestational age at birth [20,53,54,92,93], and a recent study by Joubert *et al.* linked maternal tobacco smoking during pregnancy to altered DNA methylation in infant cord blood, suggesting epigenetic mechanisms for tobacco smoke toxicity [80]. Although the methylation status of *RUNX3* was not specifically identified as being significantly associated with smoking during pregnancy, Joubert *et al.* reported that the methylation status of another member of the same gene family, *RUNX1*, was significantly associated with smoking during pregnancy [80]. Our data provide further evidence for epigenetic toxicity of tobacco smoke exposures *in utero*, and a potential role for this alteration in preterm birth, which is supported by other studies which have linked epigenetic alterations to this outcome [16]. Furthermore, this work suggests that altered placental DNA methylation of this locus, and potentially others, should be examined in studies of preterm birth and other adverse pregnancy outcomes, as this may provide novel avenues for intervention.

Additional research is warranted to elucidate the molecular pathways involved in altered gestational age resulting from maternal smoking during pregnancy, and the role that DNA methylation changes in the placenta may play in this process. Further investigation will lead to a greater understanding of the roles of placental DNA methylation in the context of the developmental origins of health and disease.

Future perspective

In the coming years, research emphasis will be placed on the developmental origins of diseases and disorders such as cardiovascular disease, cancer and neurobehavioral disorders. It will become increasingly crucial to elucidate the role that prenatal exposures and the quality of the *in utero* environment may play in modulating susceptibility to both early and later life diseases, particularly through epigenetic mechanisms such as altered DNA methylation patterns in the placenta, as well as other tissues. By investigating epigenetic alterations associated with both early-life exposures and later-life disease, a significantly greater understanding of disease risk will be gained with the goal of increasing the efficiency of clinical interventions for susceptible individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive summary

Background

- Smoking during pregnancy has been associated with preterm birth, low birth weight, abnormal neurobehavioral outcomes, childhood obesity, asthma, airway hyper-responsiveness and sudden infant death syndrome, as well as altered placental DNA methylation patterns. We hypothesized that associations exist between maternal smoking during pregnancy, placental methylation changes and preterm birth.

Results

- RUNX3* is a tumor suppressor gene with unknown function in the placenta that has been associated with asthma and airway hyper-responsiveness, as well as cancers of the breast, stomach, prostate, lung and bladder. A total of seven CpG loci in the *RUNX3* gene were observed to be associated with maternal smoking during pregnancy; one of these loci was also associated with preterm birth (<37 weeks gestation).
- We have observed an association between *RUNX3* methylation alterations, maternal smoking during pregnancy and preterm birth (<37 weeks gestation).

Discussion

- The precise signaling pathway mediating this effect remains to be elucidated. These results have facilitated a greater understanding of both the role of placental DNA methylation changes in smoking during pregnancy and preterm birth, as well as the greater developmental origins of health and disease.

Summary

- The methylation status of >27,000 CpG loci in 206 placental samples was investigated using the Illumina® (CA, USA) Infinium® HumanMethylation 27 Bead Array.
- Analysis was limited to autosomal and non-SNP-associated CpG loci only; these data were then logit-transformed and ComBat-adjusted, a previously published method of adjusting for batch effects within array data.
- A total of 1918 CpG loci were observed to have methylation patterns associated with maternal smoking during pregnancy.
- A total of seven of these CpG loci resided within the *RUNX3* gene.
- As the *RUNX3* gene has been associated in the literature with asthma and airway hyper-responsiveness, and these phenotypes have been associated in the literature with maternal smoking during pregnancy, we limited further investigation to these seven *RUNX3* CpG loci associated with maternal smoking during pregnancy.
- One of these CpG loci, cg04757093, was associated with both maternal smoking during pregnancy and preterm birth (<37 weeks gestation) after controlling for potential confounders.
- These placental methylation changes may represent a potential mechanism by which maternal smoking during pregnancy is associated with preterm birth.

- Future research is necessary to further elucidate the role that placental methylation alterations may play in the developmental origins of health and disease.

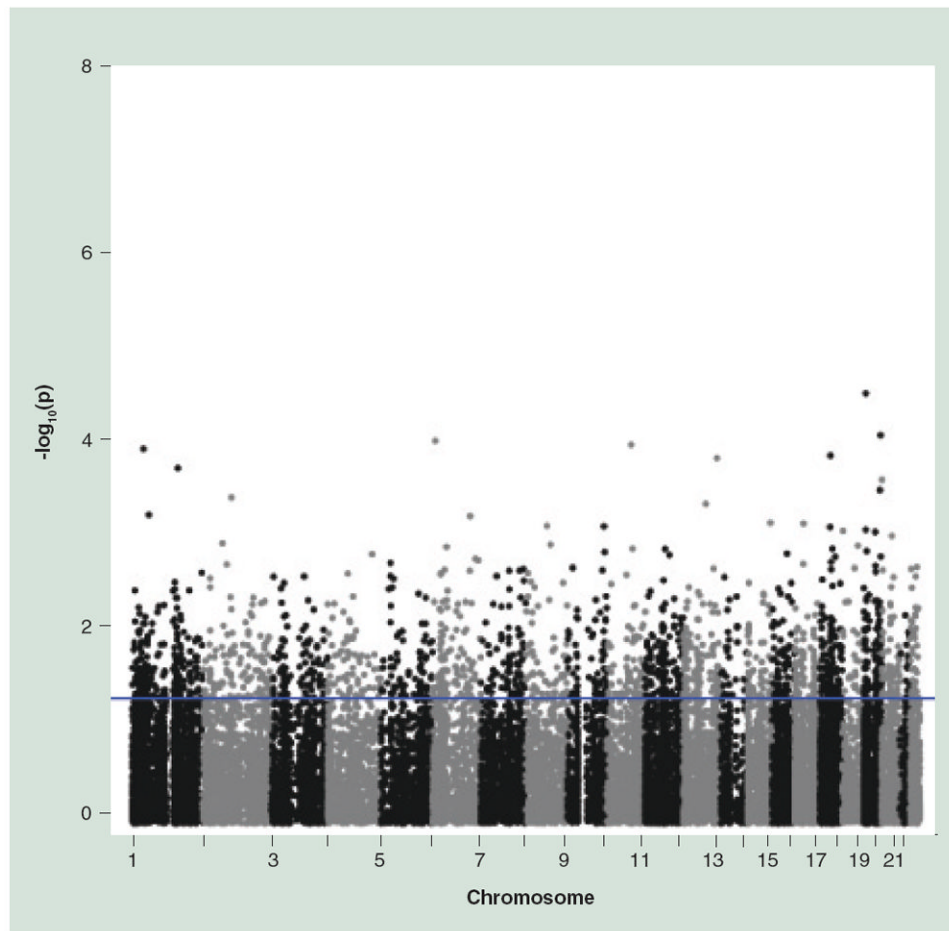


Figure 1. Manhattan plot of log p-values for locus-by-locus analysis of methylation and maternal smoking during pregnancy

Logit-transformed, ComBat-adjusted methylation status of 1918 CpG loci was significantly ($p < 0.05$) associated with maternal smoking during pregnancy, indicated by points above the solid line.

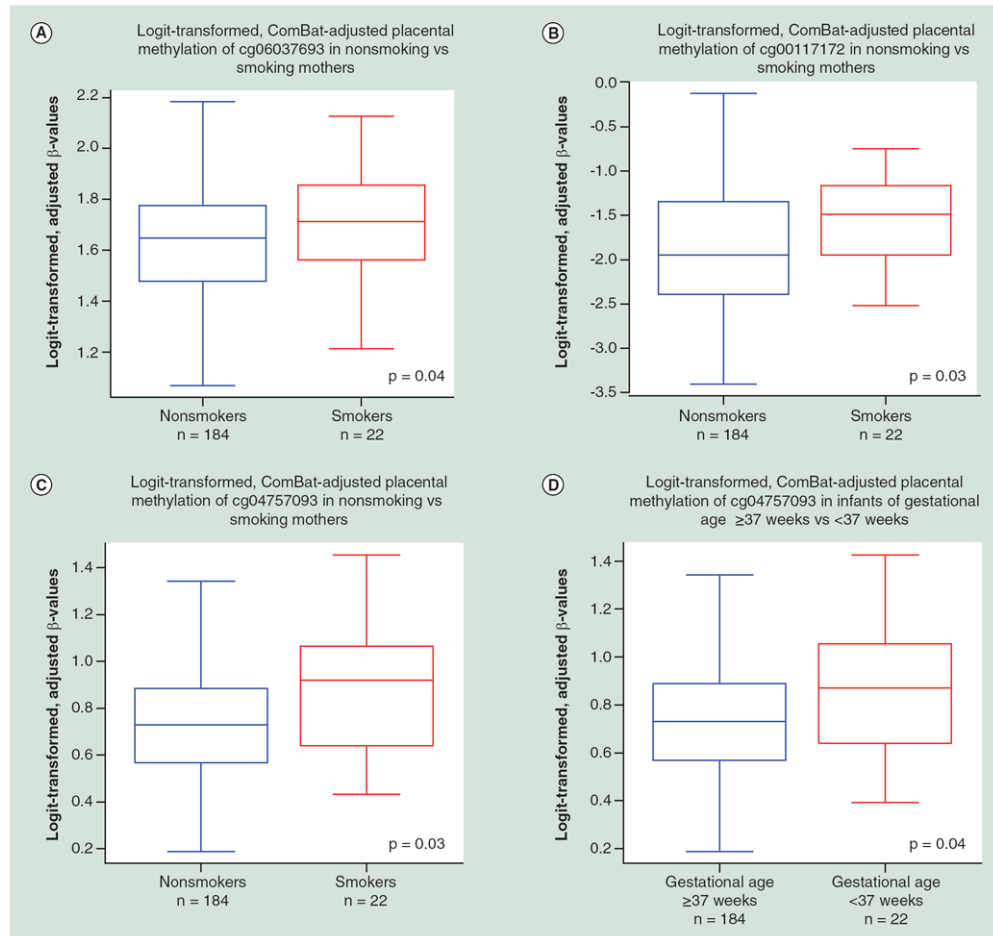


Figure 2. Boxplots of logit-transformed, ComBat-adjusted placental methylation in placentas of nonsmoking versus smoking mothers and infants of gestational age ≥ 37 weeks or < 37 weeks Smoking mothers exhibit significant placental hypermethylation at the (A) cg06037693, (B) cg00117172 and (C) cg04757093 loci within the *RUNX3* gene compared with nonsmoking mothers (Table 3). (D) There is also significant placental hypermethylation at the cg04757093 locus within the *RUNX3* gene in infants of gestational age < 37 weeks compared with infants of gestational age > 37 weeks (Table 2).

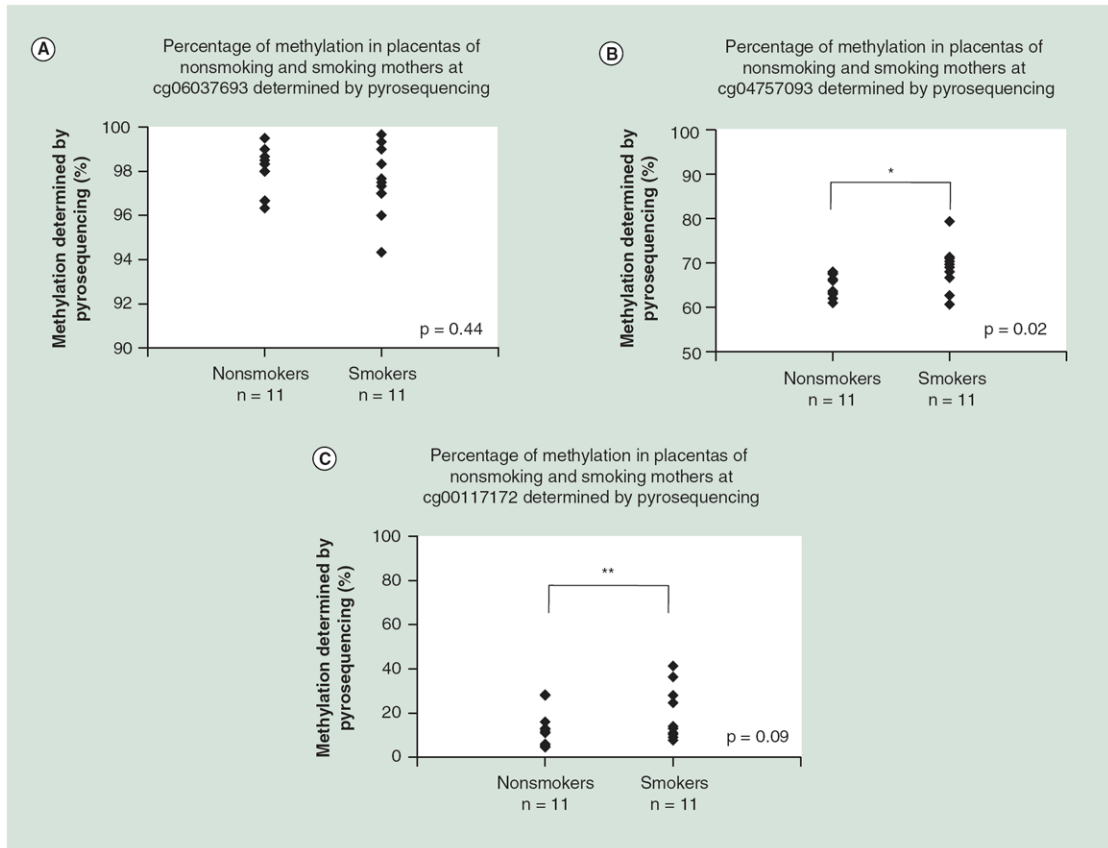


Figure 3. Percentage of methylation in placentas of nonsmoking and smoking mothers determined by pyrosequencing

(A) No significant differential methylation was observed at *RUNX3* locus cg06037693 between smoking and nonsmoking mothers in these 22 placental samples. (B) Pyrosequencing confirmed significant placental hypermethylation at *RUNX3* locus cg04757093 ($p < 0.02$) in smoking mothers compared with nonsmoking mothers. (C) Pyrosequencing confirmed near-significant placental hypermethylation at *RUNX3* locus cg00117172 ($p < 0.09$) in smoking mothers compared with nonsmoking mothers. Mean placental methylation for nonsmoking versus smoking mothers: cg06037693, 98 and 97.6, respectively (ranges of 96.3–99.5 and 94.3–99.7); cg04757093, 64.5 and 69.1, respectively (ranges of 61–68 and 60.7–79.3); and cg00117172, 12.8 and 21.1, respectively (ranges of 4.5–28.3 and 7.7–41.3). * $p < 0.05$; ** $p < 0.10$.

Table 1

Study population demographics[‡].

Demographic	Population (n = 206)	Small-for-gestational-age samples	Non-small-for-gestational-age samples	p-value
Infant gender				
Male, n (%)	99 (48.1)	31 (53.4)	68 (45.9)	0.42
Female, n (%)	107 (51.9)	27 (46.6)	80 (54.1)	
Maternal age (years)				
Mean (SD)	27.9 ± 5.9	27.8 ± 6.4	27.9 ± 5.7	0.93
Median (range)	27.5 (18–43)	27 (18–43)	28 (18–41)	
Tobacco use				
Smokers (lifetime), n (%)	25 (12.1)	14 (24.1)	11 (7.4)	0.002*
Smokers (during pregnancy), n (%)	22 (10.7)	12 (20.7)	10 (6.8)	0.008*
Birth weight status				
Appropriate for gestational age, n (%)	139 (67.5)	0 (0)	139 (93.9)	–
Small for gestational age, n (%)	58 (28.2)	58 (100)	0 (0)	
Large for gestational age, n (%)	9 (4.4)	0 (0)	9 (6.1)	
Gestational age (weeks)				
Mean ± SD	38.2 ± 2.0	38.1 ± 2.2	38.3 ± 1.9	0.65
Median (range)	39 (28.0–41.2)	38.7 (28.0–41.2)	39 (28.0–41.1)	
Birth weight (g)				
Mean ± SD	2937.4 ± 599.0	2392.3 ± 380.8	3151.0 ± 530.1	<2.2 × 10 ⁻¹⁶
Median (range)	2892.5 (890–4270)	2465 (890–3065)	3185 (1100–4270)	
Maternal race				
Caucasian, n (%)	106 (51.5)	25 (43.1)	81 (54.7)	0.70
Noncaucasian, n (%)	100 (48.5)	33 (56.9)	67 (45.3)	
Maternal insurance				
Private, n (%)	106 (51.5)	26 (44.8)	80 (54.1)	0.63
Other, n (%)	100 (48.5)	32 (55.1)	68 (45.9)	
Cesarean section[‡]				
Yes, n (%)	63 (30.6)	19 (32.8)	44 (29.7)	0.74

Demographic	Population (n = 206)	Small-for-gestational-age samples	Non-small-for-gestational-age samples	p-value
No, n (%)	140 (68.0)	39 (67.2)	101 (68.2)	
<i>Maternal recreational drug use[§]</i>				
Yes (lifetime), n (%)	11 (5.3)	4 (6.9)	7 (4.7)	0.57
Yes (during pregnancy), n (%)	6 (2.9)	2 (3.4)	4 (2.7)	0.79

[†] Continuous variables were analyzed using a Welch two-sample t-test; categorical variables were analyzed using a χ^2 test.

[‡] Three samples missing delivery method data.

[§] One sample missing recreational drug use during pregnancy data.

* $p < 0.05$.

SD: Standard deviation.

Table 2

Median placental absolute methylation value of seven *RUNX3* CpG loci associated with smoking during pregnancy, and p-values for the association with maternal smoking during pregnancy.

Illumina® (CA, USA) CpG designation	Median absolute methylation value in nonsmokers	Median absolute methylation value in smokers	p-value for the association with maternal smoking during pregnancy
cg06037693	0.83869186	0.84721389	0.044 *
cg14182690	0.49822096	0.55306253	0.035 *
cg24019564	0.04111224	0.03571554	0.040 *
cg00117172	0.12450197	0.18461105	0.030 *
cg08705994	0.81939773	0.89643210	0.015 *
cg00572797	0.87099528	0.92693759	0.046 *
cg04757093	0.67464321	0.71486633	0.032 *

* p < 0.05.

Table 3

Gestational age <37 weeks was significantly associated with logittransformed, ComBat-adjusted cg04757093 methylation status ($p = 0.04$), while controlling for potential confounders.

Covariate	Odds ratio (95% CI)	p-value
Logit-transformed, ComBat-adjusted cg04757093 methylation	10.15 (1.07–103.30)	0.04*
Maternal age (years)	1.09 (0.99–1.19)	0.08**
Infant gender		
Male	Ref	0.59
Female	1.36 (0.45–4.28)	
Birth weight (g)	0.997 (0.996–0.998)	3.19×10^{-6} *
Delivery method		
Vaginal	Ref	0.57
Cesarean section	0.70 (0.19–2.29)	–

* $p < 0.05$;

** $p < 0.10$.

Ref: Reference group.