Validation of differential DNA methylation in newborns exposed to tobacco smoke during gestation using bisulfite pyrosequencing

Christine Crute1,2, Yihan Liao3, Esther Son4, Carole Grenier2, Zhiqing Huang2, Cathrine Hoyo5 and Susan K. Murphy1,2§

1Integrated Toxicology and Environmental Health Program, Nicholas School of the Environment, Duke University, Durham, NC
2Division of Reproductive Sciences, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC
3Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC
4University of North Carolina at Chapel Hill, Chapel Hill, NC
5Department of Biology, North Carolina State University, Raleigh, NC

§To whom correspondence should be addressed: susan.murphy@duke.edu

Abstract

Maternal exposure to tobacco smoke during pregnancy has been associated with many negative child health outcomes. Tobacco smoke exposure alters DNA methylation in the developing embryo/fetus and may be a mechanism that increases risk of later life disease. Previous studies have identified CpG sites in umbilical cord blood that are associated with in utero tobacco smoke exposure. We sought to validate findings for CpG sites within several of the top hit genes, AHRR, CYP1A1, and GFI1, using targeted quantitative bisulfite pyrosequencing. Comparing results from cord blood specimens of tobacco smoke-exposed to unexposed newborns, we confirmed significance at all previously identified CpG sites tested, including one in AHRR (p=0.007), three in CYP1A1 (p<0.0001), and one in GFI1 (p=0.008). These assays also captured novel differentially methylated CpGs located near the identified sites that were not included in the prior array-based studies (p value range, 0.02 to <0.0001). These results validate the prior findings and provide a simplified and more economical approach to analysis of CpG sites for expanded use as biomarkers of in utero tobacco smoke exposure.
Figure 1: Schematics and pyrosequencing results for unexposed (U) and exposed (E) individuals for the (A) AHRR gene (RefSeq: NM_001242412), (B) CYP1A1 gene (RefSeq: NM_000499) and (C) GFI1 gene (RefSeq: NM_001127216). Illumina BeadChip probe positions are designated. Black arrows, transcription start sites; gray arrows, alternative start sites. Gray ovals, CpG islands; black rectangles, exons; shorter rectangles, UTRs. Whiskers, 5-95 percentiles. p≤0.05*, p≤0.01**, p≤0.001***, p≤0.0001****.

Description

The Developmental Origins of Health and Disease (DOHaD) hypothesis is based on anecdotal and empirical findings that early life exposures are associated with later life susceptibility to disorders and diseases (Barker 1990). Our susceptibility to in utero toxicant exposures is now a major focal point in public health research. Gestational exposure to tobacco smoke is associated with low birth weight (Sbrana et al. 2011), airway hyperreactivity associated with asthma (Lee et al. 2015), and neurodevelopmental effects such as attention deficit hyperactivity disorder (Nomura et al. 2010; Sciberras et al. 2011). These outcomes have been linked to epigenetic alterations, including changes in DNA methylation (Suter et al. 2013; Markunas et al. 2014; Richmond et al. 2015).

A previous study utilizing the Infinium HumanMethylation450 (450k) BeadChip reported differential methylation related to in utero tobacco smoke exposure (Joubert et al. 2012) and identified 26 significant CpG probes. A number of these are in coding regions of the growth factor independent 1 transcription repressor (GFI1) and the aryl-hydrocarbon receptor repressor (AHRR) genes, in addition to a region upstream of the cytochrome P450 gene, CYP1A1. Further studies have validated these results using the same or similar array-based methods (Richmond et al. 2015; Joubert et al. 2016; Rotroff et al. 2016; Rzehak et al. 2016). Here we sought to validate these findings using bisulfite pyrosequencing assays to measure DNA methylation in umbilical cord blood leukocytes from infants who were unexposed or exposed to tobacco smoke during pregnancy.

The sequence and positions of the specific CpGs analyzed are presented in Figure 1A-1C. The 146 bp PCR amplicon for AHRR contains three CpGs, one of which corresponds to probe cg05575921. The 226 bp CYP1A1 amplicon contains six CpG sites, including CpGs corresponding to probes cg22549041, cg11924019, and cg18092474. The 61 bp GFI1 amplicon is 61 bp long and contains six CpG sites, one of which corresponds to probe cg09935388.

Pyrosequencing assays were tested in duplicate or triplicate with mixtures of Qiagen’s Epitect fully methylated and unmethylated human DNAs containing 0%, 25%, 50%, 75% or 100% methylated DNA. Measured methylation was plotted against expected methylation levels. Goodness of fit assays for each CpG showed the assays measured incremental increases in DNA methylation as the amount of input methylated DNA increased across the range of possible values (AHRR: R²=0.97, p=0.0028; CYP1A1: R²=0.96, p=0.0029; GFI1: R²=0.92, p=0.003).

The assays were then used to measure methylation in human umbilical cord blood DNAs from the Newborn Epigenetics Study (NEST) cohort. Differential methylation was substantiated for the HumanMethylation450 probe CpGs for AHRR cg05575921 (p=0.0007) (Figure 1A), CYP1A1 cg11924019, cg22549041, and cg18092474 (p<0.0001) (Figure 1B), and GFI1 cg09935388 (p=0.008) (Figure 1C).

An advantage of targeted analyses by pyrosequencing is the ability to assess adjacent CpG sites that may not be represented on the 450k platform. For AHRR, sites p1 and p2 showed an average decrease in the exposed group of 3.9% (p=0.0009) and 4.9% (p<0.0001), respectively. For CYP1A1, sites p2, p3, and p4 showed an average increase in the exposed newborns of 8.4% (p=0.0001), 6.7% (p<0.0001), and 6.0% (p=0.0006), respectively. The GFI1 assay identified only one additional CpG site as significantly differentially methylated. Site p5 exhibited a 9.1% (p<0.02) decrease in the exposed group.

Using an independent quantitative method, we corroborated the findings from previously published studies of cord blood showing altered DNA methylation in association with in utero tobacco smoke exposure using array-based platforms (Joubert et al. 2012; Joehanes et al. 2016; Joubert et al. 2016; Tehranifar et al. 2018). We found significantly different levels of methylation between unexposed and exposed infant cord blood at identified sites for AHRR, CYP1A1, and GFI1. We also showed significant differences in methylation at adjacent CpG sites that were not represented on the arrays used in the prior studies. These CpGs may collectively be useful as biomarkers for in utero tobacco smoke exposure using a relatively cost-efficient and scalable platform.

AHRR and CYP1A1 are involved with the aryl-hydrocarbon receptor (AhR) pathway, which is known to facilitate changes in gene expression after toxicant exposure. Specifically, this pathway is known to mediate toxicity of polycyclic aromatic hydrocarbons (PAHs), several of which are found in cigarette smoke (Nguyen and Bradfield 2008). Although GFI1 has not been previously functionally associated with tobacco smoke exposure, it is involved with fundamental development processes such as hematopoiesis, inner ear and pulmonary neuroendocrine cell development, cellular proliferation, differentiation, and
apoptosis, and pre-mRNA splicing control as well as immune system response (Duan et al. 2005; Khandanpour et al. 2011; Moroy and Khandanpour 2011; Joubert et al. 2012; Rotroff et al. 2016).

A limitation of the study was that we used self-reported exposure rather than cotinine levels for some cases in which these data were missing. There is a tendency for participants to underestimate exposure, which is either intentional under-reporting due to societal expectations or a lack of awareness of secondhand exposure. The overall effect of under-reporting would diminish the differences between groups, as the group classified as unexposed may contain exposed individuals.

In conclusion, we have shown concordance between the previously reported 450k BeadChip findings and those from bisulfite pyrosequencing of an independent cohort. Our results provide the foundation for implementation of these relatively low-cost assays for defined analysis of these regions in other human cohort studies seeking to define exposure effects on these vulnerable regions of the genome.

**Methods**

*Request a detailed protocol*

**Samples.** This research was approved by the Duke Institutional Review Board (protocol Pro00043033). Cord blood specimens were selected based on self-reported smoking status during pregnancy (exposed, mother or other person living in the household smoked tobacco during her pregnancy; unexposed, mother did not smoke and was not exposed to tobacco smoke in her residence during pregnancy). These specimens were derived from the Durham, NC-based Newborn Epigenetics STudy (NEST), a mother-infant prospective cohort designed to determine the influence of early life exposures on the epigenome and health-related outcomes [see (Hoyo et al. 2014)].

Pregnant women were recruited from 2005 to 2011. Cord blood DNA was extracted from leukocytes separated by centrifugation from whole blood collected in vacutainer tubes containing EDTA. Samples were included in the analyses if we obtained clean PCR amplicons, the numbers of which were as follows: **AHRR**—42 unexposed, 58 exposed; **CYP1A1**—43 unexposed, 59 exposed; **GFI1**—41 unexposed, 50 exposed. Of these, maternal blood collected at enrollment for ~88% underwent measurement for cotinine, a major metabolic breakdown product of nicotine. For 12 women with missing cotinine values, self-report of tobacco exposure was used for exposure status classification. There was 87% congruence between self-reported exposure and exposure status from cotinine levels for the 820 women with plasma cotinine measurements in the parent study (Schechter et al. 2018).

**DNA Methylation Analysis.** Genomic DNA was purified using Puregene Reagents (Qiagen) and 800 ng was modified with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research). Pyrosequencing assays were designed to determine methylation levels at the previously identified significant CpG sites in **AHRR**, **CYP1A1**, and **GFI1**. All assay designs included adjacent CpGs not included on the array platform used in prior studies. Primers (Sigma) were designed using Qiagen Assay Design Software 1.0.6. **AHRR** PCR primers: F, 5'-TGG GGA TTG TTT ATT TTT GAG AG-3'; R, 5'-[biotin]AAA AAA CCC TAC CAA AAC CAC TC-3'. **CYP1A1** PCR primers: F, 5'-[biotin]ATG GGA GTG GAG GGG ATT-3'; R, 5'-CCC CAA TAC CAT TTA ACA TAA C-3'. **GFI1** PCR primers: F, 5'-AGG GAG TTA AGT GGT TAG ATA A-3'; R, 5'-[biotin]TAT ACC CAC AAC ACT CCA ATT C -3'. PCR was performed in 8-well strip tubes with total reaction volumes of 10 µL per well: 1 µL (~20 ng) BS-treated DNA and 9 µl master mix containing 5 µL PyroMark PCR buffer, 0.6 µL 25mM MgCl₂, 1 µL 10x Coral Load (Qiagen), 0.12 µL of both primers at 10 µM (Sigma), and 2.16 µL molecular grade water. PCR conditions were: **AHRR**, 95°C for 15 minutes; then 55 cycles of 94°C for 30s, 58°C for 30s and 72°C for 30s; final extension 72°C for 10 minutes; **CYP1A1**, 95°C for 15 minutes; then 55 cycles of 94°C for 30s, 55°C for 30s and 72°C for 30s; final extension of 72°C for 10 minutes; **GFI1**: 95°C for 15 minutes; then 5 cycles of 95°C for 30s, 66°C for 30s and 72°C for 30s; 6 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s; then 45 cycles for of 95°C for 30s, 57°C for 30s, and 72°C for 30s with final extension of 72°C for 10 minutes.

Sequencing primers were: **AHRR**, 5'-TGG TTT ATT TTT GAG AGG GTA-3'; **CYP1A1**, 5'-CCA AAA AAA AAA TTA TAT T-3'; and **GFI1**: 5'-TTA AGT GGT TAG ATA AGG AT-3'. Validation of pyrosequencing assays was performed using EpiTect DNAs (Qiagen). Pyrosequencing was performed on a Qiagen Pyromark Q96 MD instrument and percent methylation was determined using Qiagen PyroMark CpG software 1.0.11.

**Statistical analysis.** All statistical analysis was conducted using GraphPad Prism version 7.03. Data from the unexposed and exposed samples were tested for normal distribution using the D’Agostino and Pearson normality test, and statistical differences between the two groups were determined using the Welch’s t-test for normally distributed data or Mann-Whitney test for data with a non-normal distribution.

**Acknowledgments:** We thank the Newborn Epigenetics STudy participants without whom this research would not be possible.
References

Barker DJ. 1990. The fetal and infant origins of adult disease. BMJ 301: 1111. PMID: 2252919.

Duan Z, Zarebski A, Montoya-Durango D, Grimes HL, Horwitz M. 2005. Gfi1 coordinates epigenetic repression of p21Cip/WAF1 by recruitment of histone lysine methyltransferase G9a and histone deacetylase 1. Mol Cell Biol 25: 10338-51. PMID: 16287849.

Hoyo C, Dalveit AK, Iversen E, Benjamin-Neelon SE, Fuemmeler B, Schildkraut J, Murtha AP, Overcash F, Vidal AC, Wang F, Huang Z, Kurtzberg J, Seewaldt V, Forman M, Jirtle RL, Murphy SK. 2014. Erythrocyte folate concentrations, CpG methylation at genomically imprinted domains, and birth weight in a multiethnic newborn cohort. Epigenetics 9: 1120-30. PMID: 24874916.

Joehanes R, Just AC, Marioni RE, Pilling LC, Reynolds LM, Mandavila PR, Guan W, Xu T, Elks CE, Aslibekyan S, Moreno-Macias H, Smith JA, Brody JA, Dinghra R, Yousefi P, Pankow JS, Kunze S, Shah SH, McRae AF, Lohman K, Sha J, Absher DM, Ferrucci L, Zhao W, Demerath EW, Bressler J, Grove ML, Huan T, Liu C, Mendelson MM, Yao C, Kiel DP, Peters A, Wang-Sattler R, Vischer PM, Wray NR, Starr JM, Ding J, Rodriguez CJ, Wareham NJ, Irvin MR, Zhi D, Barrdahl M, Vineis P, Ambatipudi S, Uitterlinden AG, Hofman A, Schwartz J, Colicino E, Hou L, Vokonas PS, Hernandez DG, Singleton AB, Bandinelli S, Turner ST, Ware EB, Smith AK, Klengel T, Binder EB, Psaty BM, Taylor KD, Gharib SA, Swenson BR, Liang L, DeMeo DL, O'Connor GT, Herceg Z, Ressler KJ, Conneely KN, Sotoodehnia N, Kardia SL, Melzer D, Baccarelli AA, van Meurs JB, Romieu I, Arnett DK, Ong KK, Liu Y, Waldenberger M, Deary IJ, Fornage M, Levy D, London SJ. 2016. Epigenetic Signatures of Cigarette Smoking. Circ Cardiovasc Genet 9: 436-447. PMID: 27651444.

Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, Reese SE, Markunas CA, Richardson RC, Xu CJ, Küpers LK, Oh SS, Hoyo C, Gruzieva O, Söderhäll C, Salas LA, Baiz N, Zhang H, Lepeule J, Ruiz C, Ligthart S, Wang T, Taylor JA, Duijts L, Sharp L, Jankipersadssing SA, Nilsen RM, Vaez A, Fallin MD, Hu D, Litonjuua AA, Fuemmeler BF, Huen K, Kere J, Kull I, Munthe-Kaas MC, Gehring U, Bustamante M, Saurel-Couboizolles MJ, Quraishi BM, Ren J, Tost J, Gonzalez JR, Peters MJ, Häberg SE, Xu Z, van Meurs JB, Gaunt TR, Kerkhof M, Corpeleijn E, Feinberg AP, Eng C, Baccarelli AA, Benjamin Neelon SE, Bradman A, Merid SK, Bergström A, Herceg Z, Hernandez-Vargas H, Brunekeef B, Pinart M, Heude B, Ewart S, Yao J, Leomonnier N, Franco OH, Wu MC, Hofman A, McArindle W, Van der Vlies P, Falahi F, Gillman MW, Barcellos LF, Kumar A, Wickman M, Guerra S, Charles MA, Holloway J, Auffray C, Tiemeier HW, Smith GD, Postma D, Hivert MF, Eskenazi B, Vrijheid M, Arshad H, Antō JM, Dehghan A, Karlmaus W, Annesi-Maesano I, Sunyer J, Ghanous A, Pershagen G, Holland N, Murphy SK, DeMeo DL, Burchard EG, Ladd-Acosta C, Snieder H, Nystad W, Koppelman GH, Retlon CL, Jaddoe VV, Wilcox A, Melēn E, London SJ. 2016. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. Am J Hum Genet 98: 680-96. PMID: 27040690.

Joubert BR, Háberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, Huang Z, Hoyo C, Midttun Ø, Cupul-Uicab LA, Ueland PM, Wu MC, Nystad W, Bell DA, Peddada SD, London SJ. 2012. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect 120: 1425-31. PMID: 22851337.

Khandanpour C, Kosan C, Gaudreau MC, Dührsen U, Hébert J, Zeng H, Möröy T. 2011. Growth factor independence 1 protects hematopoietic stem cells against apoptosis but also prevents the development of a myeloproliferative-like disease. Stem Cells 29: 376-85. PMID: 21732494.

Lee JW, Jaffar Z, Pinkerton KE, Porter V, Postma B, Ferrini M, Holian A, Roberts K, Cho YH. 2015. Alterations in DNA methylation and airway hyperreactivity in response to in utero exposure to environmental tobacco smoke. Inhal Toxicol 27: 724-30. PMID: 26525176.

Markunas CA, Xu Z, Hartlid S, Wade PA, Lie RT, Taylor JA, Wilcox AJ. 2014. Identification of DNA methylation changes in newborns related to maternal smoking during pregnancy. Environ Health Perspect 122: 1147-53. PMID: 24906187.

Möröy T, Khandanpour C. 2011. Growth factor independence 1 (Gfi1) as a regulator of lymphocyte development and activation. Semin Immunol 23: 368-78. PMID: 21920773.

Nguyen LP, Bradfield CA. 2008. The search for endogenous activators of the aryl hydrocarbon receptor. Chem Res Toxicol 21: 102-16. PMID: 18076143.

Nomura Y, Marks DJ, Halperin JM. 2010. Prenatal exposure to maternal and paternal smoking on attention deficit hyperactivity disorders symptoms and diagnosis in offspring. J Nerv Ment Dis 198: 672-8. PMID: 20823730.

Richmond RC, Simpkin AJ, Woodward G, Gaunt TR, Lyttleton O, McArindle WL, Ring SM, Smith AD, Timpson NJ, Tilling K, Davey Smith G, Retlon CL. 2015. Prenatal exposure to maternal and offspring DNA methylation across the
lifecourse: findings from the Avon Longitudinal Study of Parents and Children (ALSPAC). Hum Mol Genet 24: 2201-17. PMID: 25552657.

Rotroff DM, Joubert BR, Marvel SW, Häberg SE, Wu MC, Nilsen RM, Ueland PM, Nystad W, London SJ, Motsinger-Reif A. 2016. Maternal smoking impacts key biological pathways in newborns through epigenetic modification in Utero. BMC Genomics 17: 976. PMID: 27887572.

Rzehak P, Saffery R, Reischl E, Covic M, Wahl S, Grote V, Xhonneux A, Langhendries JP, Ferre N, Closa-Monasterolo R, Verducci E, Riva E, Socha P, Gruszfeld D, Koletzko B, European Childhood Obesity Trial Study group,. 2016. Maternal Smoking during Pregnancy and DNA-Methylation in Children at Age 5.5 Years: Epigenome-Wide-Analysis in the European Childhood Obesity Project (CHOP)-Study. PLoS One 11: e0155554. PMID: 27171005.

Sbrana E, Suter MA, Abramovic AR, Hawkins HK, Moss JE, Patterson L, Shope C, Aagaard-Tillery K. 2011. Maternal tobacco use is associated with increased markers of oxidative stress in the placenta. Am J Obstet Gynecol 205: 246.e1-7. PMID: 21803321.

Schechter JC, Fuemmeler BF, Hoyo C, Murphy SK, Zhang JJ, Kollins SH. 2018. Impact of Smoking Ban on Passive Smoke Exposure in Pregnant Non-Smokers in the Southeastern United States. Int J Environ Res Public Health 15: 83. PMID: 29316617.

Sciberras E, Ukoumunne OC, Efron D. 2011. Predictors of parent-reported attention-deficit/hyperactivity disorder in children aged 6-7 years: a national longitudinal study. J Abnorm Child Psychol 39: 1025-34. PMID: 21468666.

Suter MA, Anders AM, Aagaard KM. 2013. Maternal smoking as a model for environmental epigenetic changes affecting birthweight and fetal programming. Mol Hum Reprod 19: 1-6. PMID: 23139402.

Tehranifar P, Wu HC, McDonald JA, Jasmine F, Santella RM, Gurvich I, Flom JD, Terry MB. 2018. Maternal cigarette smoking during pregnancy and offspring DNA methylation in midlife. Epigenetics 13: 129-134. PMID: 28494218.

Funding: This work was supported by NIEHS P01ES022831, USEPA RD-83543701, NCRR UL1TR001117 and NIEHS T32-ES021432.

Author Contributions: Christine Crute: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review and editing. Yihan Liao: Investigation, Formal analysis, Visualization. Esther Son: Investigation, Formal analysis, Visualization. Carole Grenier: Investigation, Methodology. Zhiqing Huang: Formal analysis, Investigation, Methodology. Cathrine Hoyo: Funding acquisition, Resources. Susan K. Murphy: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Project administration, Supervision, Writing - review and editing.

Reviewed By: Wilfried Karmaus

History: Received August 23, 2021 Revision received December 5, 2021 Accepted December 29, 2021 Published January 7, 2022

Copyright: © 2022 by the authors. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Crute, C; Liao, Y; Son, E; Grenier, C; Huang, Z; Hoyo, C; Murphy, SK (2022). Validation of differential DNA methylation in newborns exposed to tobacco smoke during gestation using bisulfite pyrosequencing. microPublication Biology. https://doi.org/10.17912/micropub.biology.000509