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Epigenetic Variation in the Mu-Opioid Receptor Gene in Infants with Neonatal Abstinence Syndrome

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Objective Neonatal abstinence syndrome (NAS) from in utero opioid exposure is highly variable with genetic factors appearing to play an important role. Epigenetic changes in cytosine:guanine (CpG) dinucleotide methylation can occur after drug exposure and may help to explain NAS variability. We correlated DNA methylation levels in the mu-opioid receptor (*OPRM1*) promoter in opioid-exposed infants with NAS outcomes.

Study design DNA samples from cord blood or saliva were analyzed for 86 infants who were being treated for NAS according to institutional protocol. Methylation levels at 16 *OPRM1* CpG sites were determined and correlated with NAS outcome measures, including need for treatment, treatment with ≥2 medications, and length of hospital stay. We adjusted for covariates and multiple genetic testing.

Results Sixty-five percent of infants required treatment for NAS, and 24% required ≥2 medications. Hypermethylation of the *OPRM1* promoter was measured at the −10 CpG in treated vs nontreated infants (adjusted difference δ = 3.2% [95% CI, 0.3-6.0%], *P* = .03; nonsignificant after multiple testing correction). There was hypermethylation at the −14 (δ = 4.9% [95% CI, 1.8%-8.1%], *P* = .003), −10 (δ = 5.0% [95% CI, 2.3-7.7%], *P* = .0005), and +84 (δ = 3.5% [95% CI, 0.6-6.4], *P* = .02) CpG sites in infants requiring ≥2 medications, which remained significant for −14 and −10 after multiple testing correction.

Conclusions Increased methylation within the *OPRM1* promoter is associated with worse NAS outcomes, consistent with gene silencing. *(J Pediatr 2014;165:472-8)*.

Neonatal abstinence syndrome (NAS), a constellation of signs and symptoms caused by withdrawal from in utero opioid exposure, is a growing problem, now affecting 5.6 per 1000 births.[1,2] The incidence of NAS has tripled in the past decade, affecting 60%-80% of infants born to mothers on methadone, buprenorphine, or other prescription narcotics.[1] NAS is associated with long hospitalizations, extensive pharmacologic therapy, and variable newborn recovery with increased health care costs.[3,4] Much of what influences the variability in the incidence and severity of NAS remains unknown, with genetic factors appearing to be important.[5-7]

Genetic factors contribute to an individual's risk for opiate addiction, with candidate genes identified as modulators of opioid therapy in dependent adults.[8,9] Specifically, the mu-opioid receptor gene (*OPRM1*) is the primary site of action of endogenous and exogenous opioids. A number of studies have associated single-nucleotide polymorphisms (SNPs) in this gene with an increased risk for substance abuse in adults.[9-12] Common variants such as the 118A>G rs1799971 SNP are known to have functional consequences.[11,12] In the first study examining genetic variants in infants with NAS, infants with the *OPRM1* rs1799971 AG or GG genotype had improved NAS outcomes compared with infants with the AA genotype.[6]

In addition to changes in the DNA sequence, changes in gene expression attributable to epigenetic modifications may influence NAS. Epigenetic changes are important in adults and are triggered by the use of an addictive drug, leading to drug cravings and a diminished response to pharmacotherapy.[13] Cytosine methylation of DNA is a common epigenetic mechanism that occurs through the addition of a methyl group to the cytosine residues of cytosine:guanine (CpG) dinucleotides. Chronic opioid exposure may lead to modifications of methylation levels at specific CpG sites within promoter regions of a gene, potentially leading to an increase or decrease in gene expression.[13,14] Previous studies

CpG	Cytosine:guanine
EMMC	Eastern Maine Medical Center
NAS	Neonatal abstinence syndrome
OPRM1	Mu-opioid receptor gene
PCR	Polymerase chain reaction
SNP	Single-nucleotide polymorphism
Sp1	Specificity protein 1

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Supported by the National Institutes of Health (DA024806-01A2 [to M.H.], R01DA032889-01A1 [to J.D.], DA018197-05 and MD Anderson's Cancer Center Support Grant DA026120 [both to D.N.]), Tufts Medical Center's Natalie Zucker and Susan Saltonstall Grants (to E.W.), National Center for Advancing Translational Sciences (UL1 TR000073 through the Tufts Clinical and Translational Science Institute [to N.T. and J.D.]), and the Toomim Family Fund (to D.N.). The authors declare no conflicts of interest.

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http://dx.doi.org/10.1016/j.jpeds.2014.05.040

of *OPRM1* have demonstrated that an increase in promoter methylation is associated with a decrease in protein expression of the *OPRM1*.[15] In addition, hypermethylation at selected CpG sites within *OPRM1* was present in opioid-dependent adults but not in control individuals.[16-18] These changes also have been identified in sperm of opioid dependent males, suggesting heritability.[16]

Epigenetic changes in *OPRM1* have not been examined in NAS. Variability in the severity of NAS may be dependent on different methylation patterns, thus influencing opioid receptor system responsiveness to opioids. The purpose of this study is to examine CpG methylation patterns within the *OPRM1* promoter region in infants chronically exposed to opioids in utero and to correlate these epigenetic changes with NAS outcome measures.

Methods

Eighty-six infants ≥36 weeks' gestational age were enrolled at Tufts Medical Center and affiliated nurseries (Brockton Hospital, Melrose Wakefield Hospital, and Lowell General Hospital) and Eastern Maine Medical Center (EMMC) between 2011 and 2012. This study had the same infant DNA samples and dataset from a previously published study examining SNP genotype in the *OPRM1* gene in infants with NAS.[6] Eligibility criteria included maternal prescribed methadone or buprenorphine exposure in utero for at least 30 days before delivery, singleton pregnancies, and infants who were medically stable after delivery without other significant complications. The study was approved by the institutional review boards of all sites with written informed consent.

DNA was sampled from either cord blood (PAXgene Blood DNA tube; Qiagen, Venlo, The Netherlands) or saliva (Oragene OG-250 DNA collection kit with CS-1 sponges; DNA Genotek, Kanata, Ontario, Canada).[19,20] If a cord blood sample was not available at the time of delivery, a saliva sample was collected at any point during the infant's hospitalization. We reviewed the infant and maternal charts for demographic information, medical diagnoses, and details of maternal substance abuse treatment and NAS outcome measures. Infants were assessed and treated according to comparable institutional NAS treatment protocols. All infants were scored using a modified Finnegan scale.[21] Infants with 3 consecutive scores ≥8 or 2 consecutive scores ≥10 were started on first-line opioid replacement therapy, which was neonatal morphine solution (0.5-1.0 mg/kg/day) at the Tufts institutions and methadone (0.5-1.0 mg/kg/day) at EMMC. Second-line therapy was of phenobarbital (Tufts) or clonazepam (EMMC). Infants were weaned from morphine, methadone, and clonazepam as inpatients and monitored for 48 hours before discharge home. Phenobarbital weaning was completed as an outpatient.

Laboratory Methods

DNA Isolation. Blood and saliva samples were sent to the Tufts Medical Center Clinical and Translational Research Center Core Laboratory for DNA isolation. Blood samples collected in PaxGene DNA tubes (QIAGEN, Valencia, California) were frozen within 14 days at −70°C until DNA isolation was performed. Salivary specimens were stored at room temperature before DNA extraction using the prepIT-L2P kit (DNA Genotek, Ottawa, Ontario, Canada). DNA was genotyped for the *OPRM1* 118A>G (rs1799971, dbSNP database) SNP using established TaqMan technology (assay C_8950074_1; Life Technologies, Grand Island, New York).

Bisulfite DNA Conversion. Genomic DNA (300 ng) was treated with sodium bisulfite using the EZ DNA Methylation Gold Kit #D5005 (Zymo Research, Orange, California). The bisulfite-treated DNA was eluted in 20 μL of M-Elution Buffer. The Human Methylated & Non-Methylated DNA Control Set (Zymo Research; cat. no. D5014) was mixed to create DNA with various percentages of methylation (0%, 25%, 50%, 75%, 100%) to monitor the efficiency of the bisulfite treatment.

***OPRM1* CpG Methylation Analysis.** Cord blood and saliva methylation levels were used as biomarkers for methylation levels occurring in the central nervous system. *OPRM1* methylation analysis was conducted according to methods published by Nielsen et al[17] with minor modifications. Two CpG islands were located from 400 nucleotides upstream to 1000 nucleotides downstream of the transcription start site in the *OPRM1* promoter (Figure 1). The first CpG island is

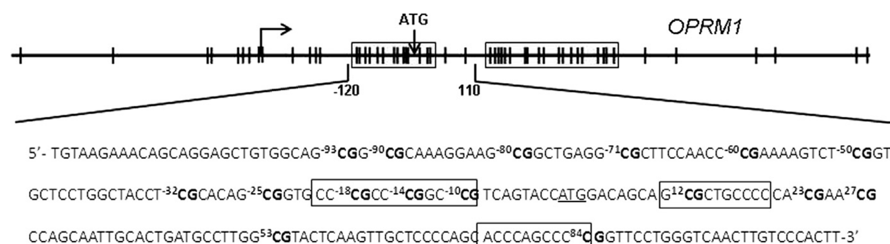

Figure 1. *OPRM1* promoter region. The *OPRM1* gene promoter region is shown with the 2 CpG islands *boxed* in and CpG dinucleotides indicated as |. The major transcription start site is indicated by the *arrow*, located −253 upstream of the ATG translation start site. The sequence of the amplified CpG island is also shown with the 16 CpG sites analyzed for cytosine methylation (*bold*) and their position relative to the ATG translation start site (*underlined*) indicated. Three putative Sp1 transcription factor binding sites are *boxed*.

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