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 may 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.3 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
of OPRM1 have demonstrated that an increase in promoter methylation is associated with a decrease in protein expression of the OPRM1. In addition, hypermethylation at selected CpG sites within OPRM1 was present in opioid-dependent adults but not in control individuals. These changes also have been identified in sperm of opioid dependent males, suggesting heritability.

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. 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). DNA was genotyped for the OPRM1 gene promoter region. The first CpG island is 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 (boxed) and their position relative to the ATG translation start site (underlined) indicated. Three putative Sp1 transcription factor binding sites are indicated. 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 (boxed) and their position relative to the ATG translation start site (underlined) indicated. Three putative Sp1 transcription factor binding sites are boxed.

**Cord Blood and Saliva 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 al17 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

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**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.
located from −97 to +27, labeled relative to the A of the ATG translation start site. We examined 16 CpG dinucleotides located at nucleotide −93, −90, −80, −71, −60, −50, −32, −25, −18, −14, −10, +12, +23, +27, +53, and +84. The −18, −14, −10, +12, and +84 CpG sites are located at potential specificity protein 1 (Sp1) transcription factor binding sites.

Primers for amplifying the upstream OPRM1 CpG island were as follows: (1) primer A: 5′-TTTTTTTTTTTGGTTATTT AGG-3′; (2) primer B: 5′-CAAATCCATCTAATAAAA-3′; (3) primer C: 5′-TGTAAGAAATAGGAGTGTGTG AG-3′; and (4) primer D: 5′-AATAAAACATATTAACCC AAAACC-3′. The first amplification was performed with 1 μL of bisulfite-treated DNA; 1 μM each of primers A and B; 250 μM each of dATP, dCTP, dGTP, and TTP; 4 mM MgCl2; 0.625 units of HotStarTaq Plus DNA Polymerase (Qiagen), and QIAGEN PCR Buffer in a final volume of 50 μL. Amplification consisted of 5 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 15 seconds at 52°C, and 30 seconds at 72°C, followed by a final elongation step at 72°C for 7 minutes.

A nested polymerase chain reaction (PCR) was performed using the same conditions as above with 1 μL of the initial PCR product and primers C and D. The nested amplification consisted of 5 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 15 seconds at 58°C, and 30 seconds at 72°C, followed by a final elongation step at 72°C for 7 minutes.

Direct Sequencing of OPRM1 CpG. Preparation for sequencing was according to Nielsen et al27 with minor modifications. To summarize, unincorporated nucleotides and primers were treated by mixing 5 μL of the nested PCR mixture with 2 μL of ExoSAP-IT (USB Corp, Cleveland, Ohio) followed by incubation at 37°C for 15 minutes and 80°C for 15 minutes. For an appropriate concentration for sequencing (5-10 ng/μL), 1 μL of the Exo-SAP-IT–treated DNA was diluted 1:50 with water. For sequencing, 2 μL of the diluted Exo-SAP-IT–treated DNA was added to 5 μM primer C or D in a final volume of 7.6 μL. Sequencing was performed on an ABI 3130 XL sequencer (Applied Biosystems, Foster City, California).

Determination of Percent DNA Methylation. Trace files (.abi) were analyzed using the ESME version 3.2.1 software from Epigenomics AG (Berlin, Germany). The percent methylation calls by the ESME were visually inspected using the associated electropherograms generated by the ESME software. Electropherograms were reviewed twice for accuracy. The promoter region was analyzed for predicted transcription factor binding sites using TESS: Transcription Element Search System (www.cbi.usenov.edu/downloads),22 and Patch 1.0 (www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) and AliBaba 2.1 (www.gene-regulation.com/pub/programs.html#alibaba2).23

Statistical Analyses
Our primary outcome measure for NAS severity was need for any NAS pharmacologic treatment, with secondary outcome measures of treatment with ≥2 medications (yes/no) and length of stay. Because infants treated with ≥2 medications represent the most severe phenotype of NAS, this factor was selected as a key outcome measure. Total opioid treatment days correlated strongly with length of stay ($r = 0.92$; $P < .001$); thus, opioid treatment days are not reported separately. We selected candidate variables for adjusted analyses by comparing treated vs nontreated infants in bivariate analysis using the $\chi^2$ (test of independence) and independent sample $t$ tests. Breastfeeding (yes/no) was defined as any amount of mother’s milk consumed during the hospitalization as documented in the infant’s medical chart. Then we averaged methylation levels across 16 CpG sites for each subject and tested the association of the subject level average (mean) with potential covariates using independent sample $t$ tests.

We tested the association of NAS outcome measures with level of methylation for each of the 16 CpG sites within the OPRM1 promoter region. Methylation levels also were compared among those infants who were treated with 0, 1, or 2 medications at each CpG site using ANOVA. Linear regression models were then created to adjust for covariates associated with methylation levels or NAS outcomes with $P < .05$ in bivariate analysis. Lastly, we applied the Benjamini and Hochberg24 method to account for the testing of 16 CpG sites in OPRM1 for each of the NAS outcomes.

Genotype frequencies were assessed for differences from the HapMap CEU database using the $\chi^2$ test (goodness of fit) and for Hardy-Weinberg equilibrium.25 Lastly, we used an independent sample $t$ test to compare methylation levels based on genotype in the OPRM1 118A>G SNP using a dominant genetic model (AA genotype vs AG/GG genotypes). Statistical analyses were performed with R programming (2010; The R Project for Statistical Computing, www.r-project.org).

Results

Of the 86 infants, 84 (98%) were white and 70 (81%) were ≥38 weeks’ gestational age. Fifty-five (64%) of the infants were exposed to maternal methadone (mean dose at delivery 106 mg [95% CI, 81-124]), and 31 (36%) were exposed to buprenorphine (mean dose at delivery 16 mg [95% CI, 13-19]). Sixty-seven (78%) of the infants also had concurrent in utero nicotine exposure; 10 (12%) benzodiazepines, and 4 (5%) selective serotonin reuptake inhibitors. Average length of stay for all infants was 22 days (95% CI, 19-26 days); and for treated infants, 32 days (95% CI, 28-35 days). Fifty-six (65%) of all infants were treated for NAS, with 38% of these infants also treated adjunctively with phenobarbital (n = 16) or clonazepam (n = 5).

Demographic variables were compared between treated and nontreated infants as shown in the Table. Medical comorbidities and maternal medical factors did not differ between the infants. Breastfed infants had decreased length of stay (16 vs 27 days; $P < .001$) and decreased need for any medical treatment for NAS (63% of nontreated vs 34% of treated infants were breastfed, $P = .009$). There were no
significant differences when we compared Tufts and the affiliated hospitals with EMMC for NAS outcome measures. Maternal treatment and doses of these medications at delivery did not correlate with any NAS outcome measure.

DNA samples including 24 from cord blood and 62 from saliva. Methylation levels across the 16 CpG sites did differ between infant DNA sources with a higher mean level of methylation from cord blood compared with saliva samples (10.0% [95% CI, 8.1-11.9] vs 6.7% [95% CI, 5.5-7.9], P = .003). However, mean methylation levels did not differ based on DNA source at the −14, −10, and +84 CpG sites.

DNA Methylation Levels and Genotype
For the OPRM1 118A>G SNP (rs1799971), the genotype frequencies were: AA 0.71, AG 0.28, and GG 0.01, with corresponding allele frequencies of A = 0.85 and G = 0.15. Hardy-Weinberg equilibrium was not violated (P = .42) and there were no differences from the frequencies observed with the HapMap CEU population (P > .05). There were no differences in methylation levels between genotypes at each of the 16 CpG sites.

DNA Methylation Levels and NAS Outcomes
Results for our primary outcome measure of need for any treatment for NAS by level of DNA methylation at each CpG site are shown in Figure 2. Methylation was increased at the −10 CpG in those infants receiving any treatment for NAS compared with nontreated infants in a model that adjusted for infant DNA source and breastfeeding (unadjusted difference 2.8% [3.0% vs 5.8%]; adjusted difference δ = 3.2% [95% CI, 0.3%-6.0%), P = .03). After adjustment for multiple testing, results were no longer significant. No additional CpG sites were found to be associated with need for treatment of NAS.

The need for treatment with ≥2 medications for NAS by level of DNA methylation at each of the 16 CpG sites is shown in Figure 3. Methylation was increased at the −14 (unadjusted difference 5% [5.2 vs 10.2%]; adjusted δ = 4.9% [95% CI, 1.8-8.1%], P = .003), −10 (unadjusted difference 5.0% [3.6 vs 8.6%]; adjusted δ = 5.0% [95% CI, 2.3-7.7, P = .0005]), and +84 (unadjusted difference 3.3% [1.7 vs 5.0%]; adjusted δ = 3.5% [95% CI, 0.6%-6.4%], P = .02) CpG sites in those infants requiring ≥2 medications compared with those treated with 0-1 medication. Linear regression models were adjusted for infant DNA source and breastfeeding. Results for the −10 and −14 CpG sites remained significant after correction for multiple testing. These 3 CpG sites are located at putative Sp1 transcription factor binding sites (Figure 1).

Although the results were not significant after adjustment for multiple testing, a positive correlation was found between level of methylation and length of stay (r = 0.27, P = .03) at the −10 CpG site. In a model that adjusted for DNA source and breastfeeding, each 1% increase in methylation level corresponded to a 0.8 day increase in length of stay (95% CI 0.1-1.5 days, P = .02). No other correlations were found with length of stay for the other CpG sites. Methylation levels also increased with the number of medications required to treat NAS (3.0% vs 4.6% vs 6.9% for 0, 1, and 2 medications, respectively; P = .05) in unadjusted analysis for the −10 CpG. The mean level of methylation for each subject averaging all 16 CpG sites did not correspond with any NAS outcome measures.

**Discussion**

Our results are consistent with the previous studies evaluating the importance of the OPRM1 gene in opioid addiction and NAS. The common 118A>G rs1799971 SNP causes to an amino acid change resulting in a 3-fold increase in the binding affinity of the receptor with β-endorphin, altering the function of the hypothalamic-pituitary-adrenal axis, and vulnerability to addiction.\(^{26,27}\) Data from animal models and in vivo studies indicate that the G allele is associated with a decrease in protein expression and gene expression.\(^{28,29}\) We found that infants with NAS who carried at least one copy of this minor G allele had a shorter length of stay and were less likely to receive any treatment for NAS compared with infants who were homozygotes for the A allele, suggesting improved tolerance to the process of opioid withdrawal.\(^{6}\)

In the present study, opioid exposed infants with a more severe phenotype of NAS had an increase in DNA methylation of 3 CpG sites in the OPRM1 promoter region. An increase in DNA methylation levels at the −14, −10, and +84 CpG sites were found in infants who required 2 or more medications to control withdrawal symptoms. The −14, −10, and +84 CpG sites are located at putative Sp1 transcription factor binding sites.
With an increased level of DNA methylation, Sp1 sites have a decreased binding affinity for their transcription factors which may lead to a decrease in gene expression.30,31 In this case, infants with hypermethylation at these specific CpG sites may have down-regulated OPRM1 gene expression, leading to reduced levels of the OPRM1. This in turn may lead to an increased need for opioid medications to control NAS symptoms. Our results do not show that the opioids caused the hypermethylation at 3 CpG sites, but that the hypermethylation of the OPRM1 promoter region may influence NAS outcome measures. The impact of changes in methylation should be evaluated in future studies for correlations with the level of the OPRM1, as well as methylation levels in the mothers. The changes in methylation levels before and after treatment should also be evaluated to determine whether opioid replacement alters any epigenetic changes in newborns with NAS.

Previous studies also have demonstrated that an increase in OPRM1 promoter methylation is associated with a decrease in protein expression of the OPRM1.13 Nielsen et al17 found increased levels of methylation at the −18 and +84 CpG sites in the OPRM1 promoter, sites of putative

**Figure 2.** Percent methylation of 16 CpG sites within the OPRM1 promoter in opioid-exposed infants who were treated (n = 65) vs nontreated (n = 21) for NAS. CpG sites in Sp1 transcription factor binding sites are indicated. *P < .05 in bivariable analyses.

**Figure 3.** Percent methylation of 16 CpG sites within the OPRM1 promoter in opioid-exposed infants treated with ≥2 medications (n = 21) vs <2 medications (n = 65) for NAS. CpG sites in Sp1 transcription factor binding sites are indicated. *P < .05 in bivariable analyses.
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Sp1 transcription factor binding, in lymphocytes of prior heroin addicts compared with controls. They also found that methylation levels differed depending on ethnicity in former heroin addicts, with an increased level of methylation at the −14 site noted in Hispanic subjects.16 Chorbov et al16 found hypermethylation at seven OPRM1 CpG sites in leukocyte DNA of male opioid addicts compared with controls. Similar changes at the −10 site were measured in European Americans with alcohol dependence compared with controls.3,4 Transgenerational effects of chronic opioid exposure have been demonstrated in animal models, suggesting that epigenetic changes in the mother caused by opioid addiction may be passed on to her infant altering sensitivity to narcotics and the process of withdrawal.33,34 The heritability of these changes is also supported by a study that evaluated sperm-derived DNA from opioid addicts, finding that methylation was increased at one CpG site within OPRM1.16

This study has a number of limitations. First, although the same NAS scoring system was used at all study centers, an intraobserver reliability program was not established between sites. Although the Finnegan scoring system is the gold standard, it remains subjective with the possibility for differences between institutions and individual providers. This study is also limited by a small sample size with the need for correction for multiple statistical comparisons, limiting our statistical power. Our sample size likely accounts for why more significant differences were not seen between treated and untreated infants. However, greater methylation levels were seen in those infants requiring ≥2 medications, representing the most severe NAS phenotype. The generalizability of our results is also limited by lack of ethnic variability as methylation levels vary depending on ethnic background.19 However, our results are consistent with previous studies in opioid-dependent white adults.17

The timing of the DNA samples varied with some samples collected prior to initiation and some during treatment with opioid medications for NAS, which may have influenced methylation levels. The source of DNA accounted for some of the variation in methylation, with differences seen in mean methylation levels between cord blood and saliva samples. Cord blood and saliva methylation levels can be considered as biomarkers for OPRM1 methylation in the central nervous system.15 Although DNA quantity and quality typically are greater from cord blood compared with saliva or buccal cells, it is often difficult to obtain cord blood at the time of delivery from all subjects.16 An increasing number of epigenetic studies have utilized saliva as a DNA source as it is readily available, can be followed serially, and is noninvasive.3,37 DNA source was adjusted for in all of our multivariate models and results remained unchanged when DNA source was included in the models.

The identification of key genetic factors through early noninvasive testing that correlate with NAS severity can lead to future individualized treatment regimens and improved care for these infants. Infants with high-risk genetic profiles could be treated more aggressively from birth, and those with low-risk profiles could potentially be discharged home from the hospital earlier. Identifying similar genetic markers in the mothers and placentas also represent an opportunity for earlier identification of high-risk infants.

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