Pharmacogenomics and Personalized Medicine

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ORIGINAL RESEARCH

DNA methylation at the mu-1 opioid receptor gene (*OPRM1*) promoter predicts preoperative, acute, and chronic postsurgical pain after spine fusion

This article was published in the following Dove Press journal:
Pharmacogenomics and Personalized Medicine
9 May 2017
Number of times this article has been viewed

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Introduction: The perioperative pain experience shows great interindividual variability and is difficult to predict. The mu-1 opioid receptor gene (*OPRM1*) is known to play an important role in opioid-pain pathways. Since deoxyribonucleic acid (DNA) methylation is a potent repressor of gene expression, DNA methylation was evaluated at the *OPRM1* promoter, as a predictor of preoperative, acute, and chronic postsurgical pain (CPSP).

Methods: A prospective observational cohort study was conducted in 133 adolescents with idiopathic scoliosis undergoing spine fusion under standard protocols. Data regarding pain, opioid consumption, anxiety, and catastrophizing (using validated questionnaires) were collected before and 2–3 months postsurgery. Outcomes evaluated were preoperative pain, acute postoperative pain (area under curve [AUC] for pain scores over 48 hours), and CPSP (numerical rating scale >3/10 at 2–3 months postsurgery). Blood samples collected preoperatively were analyzed for DNA methylation by pyrosequencing of 22 CpG sites at the *OPRM1* gene promoter. The association of each pain outcome with the methylation percentage of each CpG site was assessed using multivariable regression, adjusting for significant ($P<0.05$) nongenetic variables.

Results: Majority (83%) of the patients reported no pain preoperatively, while CPSP occurred in 36% of the subjects (44/121). Regression on dichotomized preoperative pain outcome showed association with methylation at six CpG sites (1, 3, 4, 9, 11, and 17) ($P<0.05$). Methylation at CpG sites 4, 17, and 18 was associated with higher AUC after adjusting for opioid consumption and preoperative pain score ($P<0.05$). After adjusting for postoperative opioid consumption and preoperative pain score, methylation at CpG sites 13 and 22 was associated with CPSP ($P<0.05$).

Discussion: Novel CPSP biomarkers were identified in an active regulatory region of the *OPRM1* gene that binds multiple transcription factors. Inhibition of binding by DNA methylation potentially decreases the *OPRM1* gene expression, leading to a decreased response to endogenous and exogenous opioids, and an increased pain experience.

Keywords: *OPRM1*, epigenetics, pain, chronic postsurgical pain, DNA methylation

Introduction

Inadequately controlled pain remains a significant problem after surgery, as it negatively affects quality of life and function and increases the risk of persistent postsurgical pain.[1] Approximately 50%–75% of patients undergoing surgery experience moderate or severe pain, and this is true for the 6 million children who undergo surgery every year in the US.[2,3] Chronic postsurgical pain (CPSP) is the pain that lasts beyond 2 months postsurgery.[4] In children, the incidence of CPSP ranges from 13% to 68.8%.[5,6] This implies that of the 1.5 million children who undergo major surgery every year in the US,

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http://dx.doi.org/10.2147/PGPM.S132691

~225,000–1,000,000 children develop CPSP.[7] Interindividual variability in pain sensitivity,[8] acute postsurgical pain,[9–11] and CPSP have been partly explained by genetic markers.[12] The mu-1 opioid receptor gene (*OPRM1*) that codes for the mu opioid receptor (MOR) plays an important role in opioid-pain pathways.[13] The *OPRM1* variant that has been most commonly studied is the A118G variant; however, the results of association studies with pain and β-endorphin-binding activity between this variant and the wild-type receptors are controversial.[14–17]

Beyond genetics, chronic pain is a classic example of gene–environment interaction,[18] and hence recently interest has been directed toward the role of epigenetics in pain. Epigenetics is the study of changes in chromosomes that do not alter the sequence of deoxyribonucleic acid (DNA),[19] but may still lead to alterations in gene expression. Genetics and epigenetics together are important factors in the transition of acute postsurgical pain to CPSP.[20,21] DNA methylation is a common epigenetic mechanism, which involves the addition of a methyl group to the 5′ position of a cytosine residue followed by a guanine residue (a CpG dinucleotide), which are often clustered (CpG islands) in the promoter regions of genes.[22] DNA methylation at the *OPRM1* promoter region regulates DNA binding of transcription factors and is a potent epigenetic repressor of gene transcription.[23] An increase in the *OPRM1* promoter methylation has been found to be associated with a decrease in protein expression of OPRM1.[24] Because DNA methylation is reversible, there is much interest in understanding its association with pain, as a potential target for intervention. The *OPRM1* DNA methylation levels have been found to be elevated in opioid and heroin addicts.[25,26] However, this has not been studied in relation to perioperative pain and CPSP. It has been hypothesized that DNA methylation at the promoter region of *OPRM1* will be associated with pain before and after surgery. Understanding the contribution of OPRM1 genetic–epigenetic interactions to pain outcomes will allow prediction of susceptibility to poor pain control and CPSP and will enable target identification for modification of risk studies in the future.

Methods

A prospective observational cohort study was conducted in 133 adolescents with idiopathic scoliosis undergoing posterior spine fusion under standard intraoperative anesthesia (propofol–remifentanil total intravenous anesthesia, guided by electroencephalography monitoring for depth of anesthesia) and postoperative analgesia with morphine patient-controlled analgesia along with adjuvants (scheduled intravenous acetaminophen, ketorolac, and diazepam as needed and methocarbamol) managed by perioperative pain team. The study was approved by the Cincinnati Children's Hospital Institutional Review Board. This study is registered with ClinicalTrials.gov identifiers NCT01839461 and NCT01731873. Written informed consent was obtained from parents, and assent was obtained from children before enrollment.

Participants

Healthy nonobese subjects with an American Society of Anesthesiologists (ASA) physical status ≤2 (mild systemic disease), aged 10–18 years, with a diagnosis of idiopathic scoliosis and/or kyphosis, and undergoing elective spinal fusion were recruited. The exclusion criteria included pregnant or breastfeeding females, presence of chronic pain defined as use of opioids in the past 6 months, liver or renal diseases, and developmental delays.

Data collection

Preoperatively, data regarding demographic factors (sex, age, and race), weight, pain scores (numerical rating scale [NRS]/0–10)[27] on the day of surgery (P0), and pain medications used were obtained. Anxiety scores for both child and a parent were assessed using the 0–10 visual analog scale (VAS), a simple validated scale that has been used previously in children.[28] Questionnaires were administered as described in the following sections. The intraoperative data collected included propofol and remifentanil doses, duration of surgery, and number of vertebral levels fused. In the immediate postoperative period (postoperative days [PODs] 1 and 2), pain scores (every 4 hours) and morphine and diazepam doses administered were noted. After hospital discharge, the questionnaires were administered per schedule presented in Table 1 to obtain psychosocial and pain measures in a standard fashion.

Outcomes

Pain outcomes evaluated were 1) preoperative pain, 2) acute postoperative pain (defined as area under curve [AUC] for pain scores over time on POD 1 and 2 [AUC_{POD1-2}] calculated using trapezoidal rule), and 3) chronic pain/CPSP (NRS >3/10 at 2–3 months postsurgery) as defined by the International Association for the Study of Pain.[29] These cut offs for NRS were used because NRS pain scores >3 (moderate/severe pain) at 3 months have been described as a predictor for persistence of pain and associated with functional disability.[30]

Measurement of DNA methylation

Blood was drawn upon intravenous line placement before surgery, from which the DNA was isolated on the same day and frozen at –20°C. To study DNA methylation, the focus was on a 251 bp region, including 22 CpG sites reaching from position –93 to position +159, whose methylation levels were previously shown to be associated with the *OPRM1* expression[24] and opioid/heroin addiction (Figure 1).[25,26] The CpG sites are numbered according to those studies[25,26] to allow for an easy comparison. This included a CpG site at +117, reported by Oertel et al.[24] The pyrosequencing assays utilize 50–500 ng of genomic DNA of acceptable quality (measured by Thermo Scientific NanoDrop spectrophotometer [Waltham, MA, USA] and with a 260/280 ratio ranging from 1.6 to 2.0). The extracted DNA was treated with bisulfite using Zymo EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions. Two sets of primers, long and nested, were designed using ZymoTaq Premix (Zymo Research) for two rounds of polymerase chain reaction (PCR). Methylation of the PCR products was quantified using PyroMark MD 1.0 software (Qiagen, Valencia, CA, USA). Primers used in the assays are listed in Table 2. Samples were repeated if the pyrosequencing runs did not pass quality checks of the pyrosequencer or if the internal bisulfite conversion controls failed. The sample runs were monitored through methylated and nonmethylated DNA as well as template-free negative controls.

Data analysis

Prior to the analyses, the data quality was assessed. Demographics and patients' clinical characteristics were summarized as mean (standard deviation [SD]), median, and frequency (percentage) according to the distribution of the data. Prior to evaluation of the association between pain outcome and DNA methylation, the effects of covariables were tested, which included age, sex, race, morphine dose in mg/kg on POD 1 and 2, preoperative anxiety score (VAS), preoperative pain score, duration of surgery, vertebral levels fused, propofol and remifentanil doses used during surgery (per kg), use of intravenous acetaminophen/ketorolac (yes/no), diazepam doses (mg/kg), and pain catastrophizing scale (parent version) and parent pain history scores and sequential scores for Childhood Anxiety Sensitivity Index and pain catastrophizing scale (child version).[31] Three pain outcomes were examined. Preoperative pain was dichotomized as yes versus no, because, although it was measured using NRS as a 0–10 scale, 83% of the patients reported no preoperative pain. It was then associated with covariables using logistic regression. Similarly, acute postoperative pain was analyzed using simple linear regression models, and CPSP using logistic regression models. Covariables associated at $P<0.10$ were entered into multivariable models, and stepwise selection was used to derive a final nongenetic model for each outcome where only variables with $P<0.05$ were retained.[32] DNA methylation levels were then added to the final nongenetic model to assess their association with the pain outcomes. Statistical analyses

Table 1 Data collection schema

Data variables	Preoperative	Intraoperative	Over 48 hours after surgery	2–3 months
Demographics Anxiety score	x			
Surgical duration Vertebral levels fused Propofol dose Remifentanil dose		x		
Pain scores Opioid consumption Diazepam use Analgesic adjuncts	x		x	x
Child questionnaires CASI PCS-C FDI Pain assessment	x			x
Parent questionnaires PPH PCS-P	x			

Notes: Time calculated from end of surgery. x indicates the phase in which the data is collected.
Abbreviations: CASI, Childhood Anxiety Sensitivity Index; FDI, Functional Disability Index; PCS-C, pain catastrophizing scale (child version); PCS-P, pain catastrophizing scale (parent version); PPH, parent pain history.

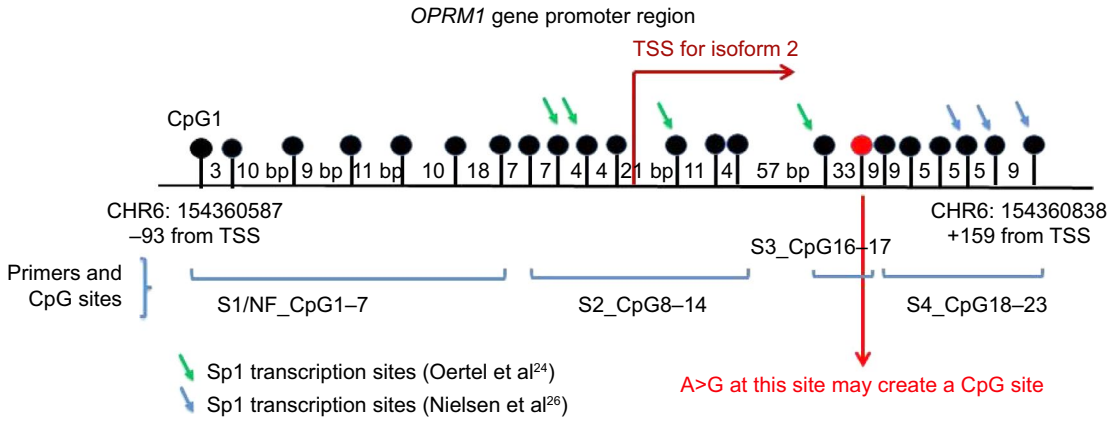

Figure 1 Depiction of the *OPRM1* promoter region (HG19; Chr 6: 154360587 to 154360838) and the location of the CpG sites. The knobs represent each CpG site, and the primers are indicated in brackets below. The red-colored knob at +117 indicates the CpG site (CpG17) associated with the variant A118G. The arrows indicate sites that have been described as Sp1 transcription factor-binding sites in previous studies (CpG sites 9, 10, 12, 16, 21, and 23 at –18, –14, 12, +84, +145, +150, and +159 from ATG site).
Abbreviations: *OPRM1*, mu-1 opioid receptor gene; TSS, transcription start site.

Table 2 Primers used in the pyrosequencing assay

Primer	Forward	CpG sites
OPRM1_NF	5′- TAAGAAATAGTAGGAGTTGTGGTAG -3′	
OPRM1_NR	5′-Biotin-AAAAACACAAACTATCTCTCCC -3′	
OPRM1_LF	5′- TGTAAGAAATAGTAGGAGTTGTGGTAG -3′	
OPRM1_LR	5′- AAATAAAACAAATTAACCCAAAAAC -3′	
OPRM1_S1/NF	5′-TAAGAAATAGTAGGAGTTGTGGTAG-3′	CpG1–7
OPRM1_S2	5′-GGTGTTTTTGGTTATTTGGTATAG-3′	CpG8–14
OPRM1_S3	5′-GTATTTAAGTTGTTTTTTAGTATTTAG-3′	CpG 16 and 17 (SNP-CpG)
OPRM1_S4	5′-GGGTTAATTTGTTTTATTTAGATGGT-3′	CpG18–22

Note: LF and LR: forward and reverse primers used in the first round, long PCR; NF and NR: forward and reverse primers used in the second round, nested PCR.
Abbreviations: *OPRM1*, mu-1 opioid receptor gene; PCR, polymerase chain reaction.

were performed using Statistical Analysis System, version 9.3 (SAS Institute Inc., Cary, NC, USA). $P \leq 0.05$ was used as the threshold for statistical significance. No multiple testing correction was done, thus it is possible that some findings occurred by chance.

Functional genomics analysis

To identify potential regulatory mechanisms in the *OPRM1* promoter, a large collection of functional genomics data sets from various sources, including ENCODE,[33] Roadmap Epigenomics,[34] Cistrome,[35] and ReMap-ChIP, was compiled.[36] The genomic coordinates of the *OPRM1* promoter were intersected against the genomic coordinates contained in each data set. In total, this database contains 4,953 data sets performed in 1,706 different cell types and conditions; 1,911 data sets monitor binding interactions of transcription factors with the human genome using ChIP-seq; 1,214 measure the presence of a particular histone mark using ChIP-seq; 226 measure open chromatin through DNase-seq; 57 measure expression quantitative trait loci (eQTLs); and 558 predict "ActiveChromatin" states using combinations of histone marks.[37] Collectively, 240 of these experiments were performed in brain-related cell lines and cell types.

Results

The final cohort comprised 133 participants; the mean age was 14.49 years (SD 1.91), and they were mostly white (83%) and female (74%) (Table 3). The recruitment timeline is described in Figure 2. Descriptions of variables that were evaluated for inclusion in the multiple regression model are presented in Table 3.

Pain descriptives

Acute and chronic postoperative pain data were collected for 128 and 121 patients, respectively. The overall incidence of CPSP was found to be 36.4% (44/121). The preoperative pain score was 0 in 83% (106/127) of the cohort.

DNA methylation and pain

Three pain outcomes were examined. For the association with dichotomized preoperative pain scores, logistic regression was used. After adjusting for age and sex, the methylation level of six CpG sites (sites 1, 3, 4, 9, 11, and 17, Table 4) was significantly associated with whether a patient reported preoperative pain. For all the six CpG sites, higher methylation was associated with higher odds of reporting preoperative pain, except for site 17. No impact of the methylation on the actual pain scores was detected (data not shown). For acute postoperative pain ($AUC_{POD1–2}$), significant impact of methylation was detected in CpG sites 4, 17, and 18 (Table 4) when preoperative pain and morphine consumption were adjusted. For all three CpG sites, higher methylation was associated with higher preoperative pain. For chronic postoperative pain, significant association was detected for the nonpromoter site, CpG13 and 22 (Table 4), with preoperative pain and morphine consumption being controlled. For all the CpG sites, higher methylation was associated with higher odds of having chronic pain. Methylation at two CpG sites was associated with both preoperative pain and $AUC_{POD1–2}$ (CpG 4 and 17); methylation at these sites also showed a trend toward CPSP risk, although not statistically significant. Figure 3 depicts estimated probabilities of developing CPSP using median preoperative pain score (0), median morphine doses (1.7 mg/kg), and 2.5th, 25th, 50th, 75th and 97.5th percentiles of the methylation data of each of the two sites CpG13 and CpG22.

Functional genomics analysis

Query of the region evaluated using a large collection of ChIP-seq, DNase-seq, and eQTL data, as described in the "Methods" section, showed that this region is located in open chromatin and is marked by H3K27ac, H3K4me1, and H3K4me3 (indicative of active regulatory regions)[38–40] in noncancer brain cells from the caudate–putamen, temporal, frontal lobes, and angular gyri. Moreover, this region contains ChIP-seq peaks for binding of multiple transcription factors, including REST, RAD21, SP1, YY1, and ZNF263 in various tissues. In particular, REST and RAD21 bind the *OPRM1* promoter region in three cell lines (SK-N-SH, PFSK-1, and SK-N-SH) derived from brain tissue where the opioid receptors responsible for analgesia are found (Table 5). Collectively, these results suggest that the differential DNA methylation patterns observed in the *OPRM1* promoter might functionally act by modulating the expression of *OPRM1* via alteration of the binding of REST and other neuron-expressed transcription factors.

Discussion

It has been previously shown that psychological and clinical variables contribute to CPSP.[31] This study finds associations between epigenetics and CPSP in children for the first time and adds to the emerging evidence linking epigenetic mechanisms to the development of chronic pain states.[41] Specifically, it was found that novel biomarkers (DNA methylation of certain CpG sites in the *OPRM1* promoter region) associated

Table 3 Demographics of the cohorts and description of the covariates used in the regression model

Variable	Acute postoperative pain (N=128)		CPSP		
		P-value[d]	No (N=77)	Yes (N=44)	*P*-value
Age[a] (years)	14.49 ± 1.91	0.15	14.20 ± 1.87	14.78 ± 1.67	0.10
Sex[b]		0.23			0.54
Male	35 (26%)		20 (26%)	9 (21%)	
Race[b]		0.21			0.13
White	111 (83%)		66 (86%)	32 (74%)	
Weight[c] (kg)	54.00 (48.00–61.90)	0.83	54.20 (48.00–61.9)	54.00 (50.00–61.00)	0.90
VAS anxiety[c] (child)	4.30 (2.50–6.80)	0.24	4.40 (2.60–6.80)	3.60 (1.80–5.20)	0.39
VAS anxiety[c] (parent)	5.50 (4.36–8.00)	0.24	5.40 (4.60–8.00)	5.90 (4.40–8.10)	0.94
Preoperative pain score[c]	0.00 (0.00–0.00)	<0.001	0.00 (0.00–0.00)	0.00 (0.00–2.00)	0.015
Number of vertebral levels fused[c]	12.00 (11.00–12.00)	0.58	12.00 (11.00–12.00)	12.00 (10.00–12.00)	0.91
Surgical duration (hours)[a]	4.91 ± 1.27	0.21	4.71 ± 1.07	5.09 ± 1.45	0.14
Pain $AUC_{POD1–2}$[a]	198.58 ± 73.78	–	189.04 ± 67.61	222.64 ± 80.44	0.018
Morphine dose POD1 & 2[c] (mg/kg)	1.60 (1.19–2.17)	0.15	1.59 (1.08–1.93)	1.89 (1.50–2.47)	0.003
CASI[a]	28.21 ± 5.87	0.18	27.86 ± 5.99	28.38 ± 5.80	0.71

Notes: [a]Data exhibited normal distribution, shown as mean ± SD and compared using *t*-tests for CPSP. [b]Shown as frequency (proportion) and compared using chi-squared tests for CPSP. [c]Data did not exhibit a normal distribution, shown as median (IQR) and compared using Wilcoxon rank-sum tests for CPSP. [d]Assessed using Spearman's rank correlation.

Abbreviations: AUC, area under curve; CASI, Child Anxiety Sensitivity Index; CPSP, chronic postsurgical pain; IQR, interquartile range; POD, postoperative day; SD, standard deviation; VAS, visual analog scale.

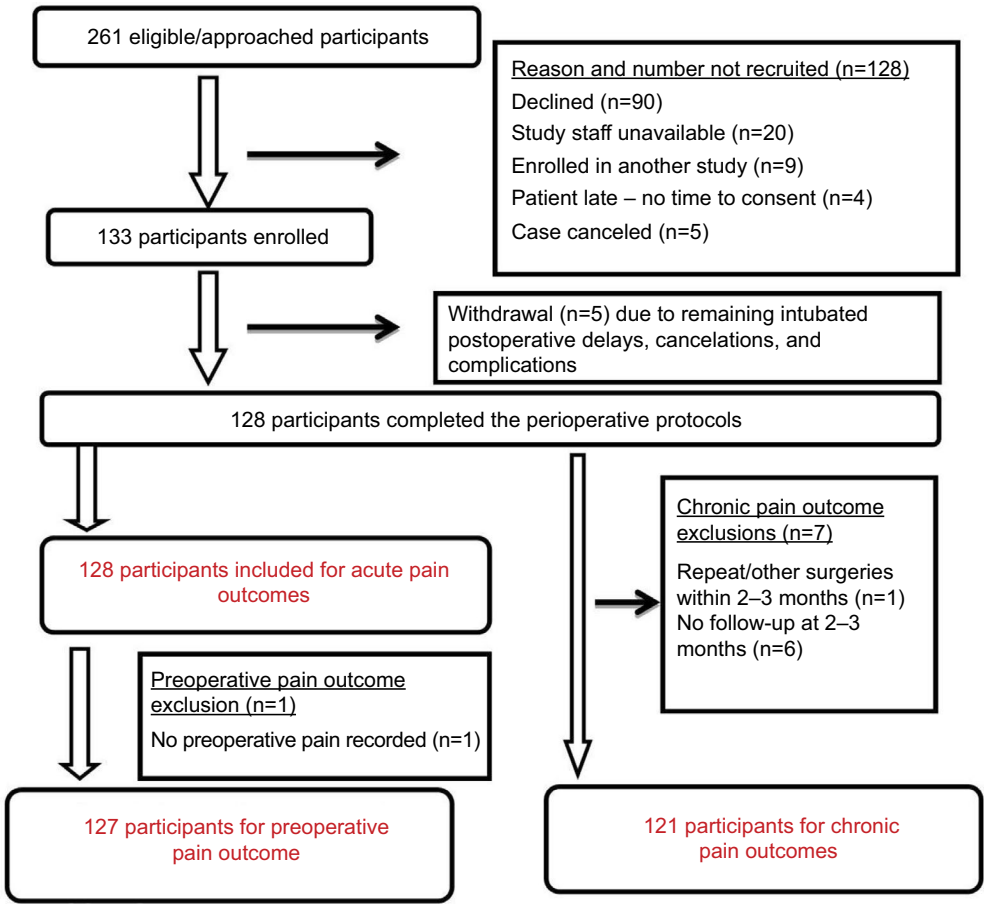

Figure 2 Recruitment timeline for the spine surgery study cohort is delineated. Of the 261 eligible patients who satisfied the inclusion/exclusion criteria, reasons for not enrolling and derivation of final cohorts included in the study with preoperative, acute, and chronic pain outcomes are described.

with preoperative pain, acute postoperative pain, and CPSP following posterior spine fusion in healthy adolescents. DNA methylation was also associated with pain before surgical stimulus and exposure to opioids. These findings allow for prediction of risk for the pain response to surgery and possible novel mechanisms that could be targeted for prevention and treatment of CPSP.

Endogenous opioid peptides and exogenously administered opioid analgesics bind to MOR to regulate pain responses. The MOR activity is regulated at different levels, including epigenetic mechanisms.[23] Many of the CpG sites found to be associated with pain outcomes (1, 3, 4, 9, 11, 13, 17, 18, and 22) have been previously described as putative Sp1 transcription factor-binding sites. These include CpG sites 9, 10, 12, 16, 21, and 23 at –18, –14, 12, +84, +145, and +159 from ATG site, respectively.[26] Methylation levels at some of these sites were previously found to be associated with opioid addiction/dependence. Nielsen et al found that CpG sites at –18 and +84 (9 and 16) were more strongly methylated in heroin addicts than controls. Similarly, seven CpG sites showed significant hypermethylation of blood DNA taken from male opioid addicts when compared to blood DNA from controls (CpGs 5, 9, 10, 11, 18, and 23).[26] Increased methylation within the *OPRM1* promoter (at –14, –10 [sites 10, 11]) was also found to be associated with worse neonatal abstinence syndrome outcomes in infants exposed to opioids in utero.[42] It has been shown in mouse brain tissues that DNA methylation of the *OPRM1* promoter decreases expression of the gene; through interaction with chromatin-remodeling factors, remodeling occurs, thus allowing access for Sp1 binding,[43] which results in the MOR upregulation. Thus, methylation at these sites can prevent the *OPRM1* activation, leading to decreased endogenous and exogenous opioid effects, manifested in the experience of increased perioperative pain. In addition, Chorbov et al also observed increased DNA methylation in the sperm of addicts, which may suggest a means of epigenetic heritability of opioid phenotypes.[25]

Animal studies have shown that *OPRM1* promoter methylation reduced *OPRM1* expression.[23] Knothe et al confirmed methylation effects on *OPRM1* expression through experiments in human cell lines (neuronal SHSY5Y and Kelly, and nonneuronal HEK-293) with and without the demethylating

Table 4 Association of DNA methylation of CpG sites at the *OPRM1* promoter with pain outcomes

CpG site*	Location	Genomic location	Preoperative pain score of 1[a]		Acute pain[b]		CPSP[c]		
			P-value	Regression coefficient	*P*-value	Regression coefficient	*P*-value	Regression coefficient	OR (95% CI)
1	–93	154360587	**0.023**	0.051	0.290	0.620	0.189	–0.028	0.972 (0.932–1.015)
2	–90	154360590	0.256	0.023	0.464	0.396	0.452	0.014	1.014 (0.978–1.053)
3	–80	154360600	**0.035**	0.041	0.153	0.772	0.368	–0.017	0.983 (0.946–1.021)
4	–71	154360609	**0.026**	0.054	**0.003**	1.864	0.995	0.000	1.000 (0.957–1.046)
5	–60	154360620	0.916	–0.003	0.495	0.412	0.411	0.017	1.017 (0.977–1.060)
6	–50	154360630	0.589	0.009	0.100	0.836	0.731	0.006	1.006 (0.973–1.040)
7	–32	154360648	0.145	0.019	0.221	0.466	0.567	0.007	1.007 (0.983–1.033)
8	–25	154360655	0.198	0.021	0.875	–0.070	0.227	0.019	1.019 (0.988–1.050)
9	–18	154360662	**0.020**	0.038	0.925	0.044	0.548	0.010	1.010 (0.978–1.043)
10	–14	154360666	0.979	0.001	0.886	–0.097	0.893	0.003	1.003 (0.958–1.051)
11	–10	154360670	**0.008**	0.049	0.443	0.404	0.147	0.029	1.029 (0.989–1.071)
12	12	154360691	0.716	0.013	0.500	0.625	0.117	0.051	1.052 (0.985–1.124)
13	23	154360702	0.305	0.018	0.460	0.356	**0.002**	0.067	1.069 (1.022–1.119)
14	27	154360706	0.810	–0.006	0.444	0.441	0.793	0.006	1.006 (0.964–1.049)
16	84	154360763	0.221	0.069	0.730	0.512	0.150	0.073	1.075 (0.973–1.188)
17	118	154360796	**0.029**	–0.997	**0.034**	17.736	0.114	0.516	1.675 (0.885–3.171)
18	126	154360805	0.205	0.019	**0.031**	0.921	0.804	0.004	1.004 (0.975–1.033)
19	135	154360814	0.334	0.019	0.415	0.418	0.856	–0.003	0.997 (0.962–1.033)
20	140	154360819	0.151	0.025	0.826	0.108	0.977	–0.001	1.000 (0.966–1.034)
21	145	154360824	0.103	0.029	0.914	0.053	0.861	0.003	1.003 (0.970–1.038)
22	150	154360829	0.446	0.014	0.314	0.497	**0.046**	0.036	1.037 (1.000–1.075)
23	159	154360838	0.491	0.023	0.385	0.750	0.480	0.022	1.022 (0.964–1.083)

Notes: *CpG sites are numbered the same as in other studies for ease of comparison. [a]Modeled using logistic regression on the probability of preoperative pain =1. Age and sex were controlled. Results shown represent the change of log OR with 1% increase in DNA methylation. [b]Modeled using linear regression adjusted for preoperative pain score and morphine consumption over postoperative days 1 and 2. [c]Modeled using logistic regression adjusted for preoperative pain score and morphine consumption over postoperative days 1 and 2. OR represents the odds of CPSP with 1% increase in the DNA methylation level. $P<0.05$ are presented in bold.
Abbreviations: CI, confidence interval; CPSP, chronic postsurgical pain; *OPRM1*, mu-1 opioid receptor gene; OR, odds ratio.

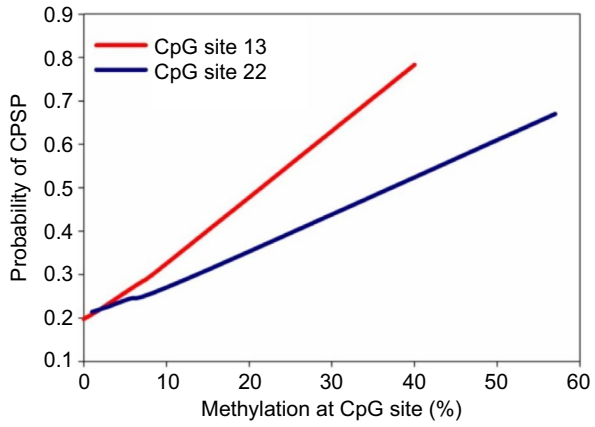

Figure 3 The probability of developing CPSP based on DNA methylation at CpG 13 and 22, derived from the regression model, is depicted. The probabilities were estimated using median preoperative pain scores (0), median morphine consumption (1.7 mg/kg), and 2.5%, 25%, 50%, 75%, and 97.5% of the methylation data of each of the two sites. The 97.5% values for DNA methylation in the data are 40% for CpG13 and 57% for CpG22. The nongenetic covariates are already adjusted for in the regression model. Hence, the probability of CPSP holding other variables constant increases with increased methylation at these sites.
Abbreviation: CPSP, chronic postsurgical pain.

agent 5′-aza-2′-deoxycytidine.[44] But they did not find that methylation profiles across the *OPRM1* gene from CpG position –93 to +159 in brain tissues collected postmortem from heroin addicts correlated with differences in *OPRM1* mRNA expression in the corresponding brain regions.[44] Opioid exposure in all their subjects may have played a role in minimizing methylation differences between the specimens and the individuals. Hence, the lack of correlation in their study does not rule out the differences that may exist between opioid-exposed and nonopioid-exposed (or pain vs no pain) subjects.

The findings of this study corroborate a growing body of evidence that *OPRM1* DNA methylation affects pain behaviors and contributes to the limited efficacy of opiates in certain cases,[45] which is improved upon blockade of methylation.[46] In a neuropathic animal model, it was shown that increased methylation of the MOR gene proximal promoter in dorsal root ganglion decreased morphine analgesia.[47] Administration of demethylating agents reversed

Table 5 Findings from evaluation of *OPRM1* promoter region using functional genomics datasets in neuronal cell-type

Data set name	Type	Cell-type label	Cell-type group	Chromosome 6	
				Start	End
ENCODE_ChIP-seq	REST	PFSK-1	Neuron	154360476	154360892
ENCODE_ChIP-seq	REST	SK-N-SH	Neuron	154360476	154360892
ENCODE_ChIP-seq	REST	U87	Glial_cell	154360476	154360892
ENCODE_ChIP-seq	RAD21	SK-N-SH_RA	Neuron	154360485	154360774
ENCODE_DNase-seq	DNase	Cerebellum_OC	Cerebellum	154360055	154361686
ENCODE_DNase-seq	DNase	SK-N-SH	Neuron	154360205	154361641
ENCODE_DNase-seq	DNase	Medullo	Neuron	154360485	154360635
ENCODE_DNase-seq	DNase	Medullo_D341	Neuron	154360500	154360704
ENCODE_DNase-seq	DNase	BE2_C	Neuroblast	154360520	154360670
ENCODE_DNase-seq	DNase	SK-N-MC	Neuron	154360560	154360710
ENCODE_DNase-seq	DNase	HA-h	Glial_cell	154360580	154360730
ENCODE_DNase-seq	DNase	HAc	Glial_cell	154360620	154360770
ENCODE_DNase-seq	DNase	SK-N-SH_RA	Neuron	154360660	154360810
Roadmapepigenomics_ActiveChromatin	10_TssBiv	Brain_Germinal_Matrix	Germinal_matrix	154360200	154361000
Roadmapepigenomics_ActiveChromatin	10_TssBiv	Brain_Inferior_Temporal_Lobe	Temporal_lobe	154360200	154361000
Roadmapepigenomics_ActiveChromatin	2_TssAFlnk	Neurosphere_Ganglionic_Eminence_Derived	Neurosphere	154360200	154360600
Roadmapepigenomics_ActiveChromatin	10_TssBiv	Brain_Angular_Gyrus	Angular_gyrus	154360400	154361200
Roadmapepigenomics_ActiveChromatin	10_TssBiv	Brain_Anterior_Caudate	Caudate–putamen	154360400	154361200
Roadmapepigenomics_ActiveChromatin	10_TssBiv	Brain_Cingulate_Gyrus	Cingulate_gyrus	154360400	154361800
Roadmapepigenomics_ActiveChromatin	10_TssBiv	Brain_Dorsolateral_Prefrontal_Cortex	Prefrontal_cortex	154360400	154361800
Roadmapepigenomics_ActiveChromatin	2_TssAFlnk	Neurosphere_Cortex_Derived	Neurosphere	154360400	154361000
Roadmapepigenomics_ActiveChromatin	1_TssA	Neurosphere_Ganglionic_Eminence_Derived	Neurosphere	154360600	154360800
Roadmapepigenomics_ActiveChromatin	2_TssAFlnk	Neurosphere_Ganglionic_Eminence_Derived	Neurosphere	154360800	154361000
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Germinal_Matrix	Germinal_matrix	154359832	154361779
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Cingulate_Gyrus	Cingulate_gyrus	154359959	154360615
Roadmapepigenomics_HistoneMarks	H3K4me3	Brain_Inferior_Temporal_Lobe	Temporal_lobe	154360134	154361957
Roadmapepigenomics_HistoneMarks	H3K4me3	Neurosphere_Cultured_Cells_Ganglionic_Eminence_Derived	Neurosphere	154360141	154361032
Roadmapepigenomics_HistoneMarks	H3K4me3	Brain_Anterior_Caudate	Caudate–putamen	154360158	154361200
Roadmapepigenomics_HistoneMarks	H3K27ac	Brain_Anterior_Caudate	Caudate–putamen	154360161	154361089
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Hippocampus_Middle	Hippocampus	154360219	154361820
Roadmapepigenomics_HistoneMarks	H3K9ac	Brain_Anterior_Caudate	Caudate–putamen	154360231	154360919
Roadmapepigenomics_HistoneMarks	H3K4me3	Neurosphere_Cultured_Cells_Cortex_Derived	Neurosphere	154360232	154360613
Roadmapepigenomics_HistoneMarks	H3K4me3	Brain_Germinal_Matrix	Germinal_matrix	154360236	154361028
Roadmapepigenomics_HistoneMarks	H3K4me3	Brain_Angular_Gyrus	Angular_gyrus	154360273	154361216
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Angular_Gyrus	Angular_gyrus	154360276	154361392
Roadmapepigenomics_HistoneMarks	H3K27ac	Brain_Mid_Frontal_Lobe	Frontal_lobe	154360280	154360645
Roadmapepigenomics_HistoneMarks	H3K4me3	Brain_Cingulate_Gyrus	Cingulate_gyrus	154360289	154361329
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Mid_Frontal_Lobe	Frontal_lobe	154360308	154360649
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Anterior_Caudate	Caudate–putamen	154360363	154361164
Roadmapepigenomics_HistoneMarks	H3K9ac	Brain_Mid_Frontal_Lobe	Frontal_lobe	154360541	154360711
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Substantia_Nigra	Substantia_nigra	154360548	154361656
Roadmapepigenomics_HistoneMarks	H3K27ac	Brain_Inferior_Temporal_Lobe	Temporal_lobe	154360556	154361011
Roadmapepigenomics_HistoneMarks	H3K4me3	Brain_Mid_Frontal_Lobe	Frontal_lobe	154360586	154361813
Roadmapepigenomics_HistoneMarks	H3K27me3	Brain_Inferior_Temporal_Lobe	Temporal_lobe	154360592	154360975
Roadmapepigenomics_HistoneMarks	H3K9ac	Brain_Cingulate_Gyrus	Cingulate_gyrus	154360645	154360850
Roadmapepigenomics_HistoneMarks	H3K4me1	Brain_Angular_Gyrus	Angular_gyrus	154360648	154360925
Roadmapepigenomics_HistoneMarks	H3K9ac	Brain_Angular_Gyrus	Angular_gyrus	154360654	154360843
Roadmapepigenomics_HistoneMarks	H3K4me1	Neurosphere_Cultured_Cells_Cortex_Derived	Neurosphere	154360679	154361066
Roadmapepigenomics_HistoneMarks	H3K9ac	Brain_Inferior_Temporal_Lobe	Temporal_lobe	154360694	154360980

(*Continued*)

Table 5 (*Continued*)

Data set name	Type	Cell-type label	Cell-type group	Chromosome 6	
				Start	End
Roadmapepigenomics_HistoneMarks	H3K4me3	Neurosphere_Cultured_Cells_Cortex_Derived	Neurosphere	154360717	154360925
Roadmapepigenomics_HistoneMarks	H3K4me1	Neurosphere_Cultured_Cells_Ganglionic_Eminence_Derived	Neurosphere	154360784	154361032
Roadmapepigenomics_HistoneMarks	H3K27ac	Brain_Mid_Frontal_Lobe	Frontal_lobe	154360822	154361016
UMMSBrain_H3K4me3	H3K4me3	Brain_prefrontal_cortex	Prefrontal_cortex	154360073	154362028

Notes: Chromatin-state learning markers based on a Core 15-state model (ChromHMM), which captures key interactions between the core set of five chromatin marks assayed in all epigenomes (H3K4me3, H3K4me1, H3K36me3, H3K27me3, and H3K9me3). H3K4me3, H3K27ac, H3K4me1, and H3K9ac are histone modifications characteristic of actively transcribed promoter regions, while H3K27me3 is involved in repression of transcription.
Abbreviations: TSS, transcription start site; 1TssA, active TSS; 2TssAFlnk, flanking active TSS; 10TssBiv, bivalent, poised TSS; 11BivFlnk, flanking bivalent TSS enhancer.

the hypermethylation of the *OPRM1* gene and improved the analgesic effect of morphine in mice pain models.[47,48] It is not clear if pain is the trigger or the consequence of increased methylation of the *OPRM1* promoter. In fact, chronic opioid use in methadone-substituted former opiate addicts was found to be associated with increased DNA methylation at the *OPRM1* position +126 (CpG 18), correlating with increased pain, compared to controls with pain not treated with opioids.[49] The authors note that causal relationships with opioid use could not be established, as there was no opioid dose dependency. In their discussion, they mentioned that the trigger for increased methylation may actually be the baseline increased pain.[50] This might partly explain the association of *OPRM1* methylation with baseline pain identified in this study, as MOR function is essential for endogenous opioid action even in the absence of opioids. Useful information would be gained by evaluating DNA methylation changes over time in those who do and do not develop CPSP, in the presence/absence of opioids.

Although the exact mechanisms by which DNA methylation of *OPRM1* promoter region contributes to pain are not known, the computational analysis revealed a DNA region bound by the repressor element 1 silencing transcription factor (REST) in multiple neuronal cell lines at the *OPRM1* promoter region (Table 5). Intriguingly, REST has previously been implicated in OPRM1 gene silencing via epigenetic modifications.[51] REST, a member of the zinc finger transcription factor family, represses transcription of neural genes[52] in nonneuronal cells by binding a DNA sequence element called the neuron-restrictive silencer element[53] and recruiting several chromatin-modifying enzymes.[54] There is also evidence for the involvement of REST in chronic neuropathy.[52] Nerve injury results in a long-lasting increase in REST expression in mouse dorsal root ganglia.[55] Hence, it is hypothesized that, in patients with already suppressed *OPRM1* expression due to DNA methylation of the *OPRM1* promoter, increased REST expression after injury possibly leads to further *OPRM1* gene silencing and worsens the pain experience. If true, this mechanism could potentially create new avenues for pain therapy. Besides the sequence-specific REST, RAD21 was another transcription factor that was found to be relevant in neuronal cells for the *OPRM1* promoter region. REST is involved in three-dimensional DNA organization. Recent research, using ChIP-seq and RNA-seq data from matching cell types from the human ENCODE resource, showed that RAD21 mostly functions as an activator of transcription.[56] Although not known to play a role in *OPRM1* regulation or pain, DNA methylation preventing binding of an activating transcription factor may also be responsible for decreased *OPRM1* function and hence pain in these patients.

This study has a few limitations, one of which is the use of blood samples for DNA methylation, instead of target tissue like brain, which are understandably inaccessible in clinical human studies. However, this approach has been employed successfully before.[25,26,42] Fan and Zhang compared methylation profiles of human chromosome 6 (*OPRM1* gene location), derived from 12 tissues, and reported that CpG island methylation profiles were highly correlated between somatic tissues.[57] Davies et al found that some interindividual variation in DNA methylation was reflected across brain and blood, indicating that peripheral tissues may have utility in studies of complex neurobiological phenotypes.[58]

In summary, we report novel associations of DNA methylation in the *OPRM1* promoter with preoperative, acute, and CPSP in children undergoing spine surgery. Since DNA methylation is influenced by multiple modifiable factors (diet, exercise, parental upbringing, and stress), understanding the role of epigenetic regulation of *OPRM1* in pain opens new avenues of pain research. Understanding susceptibility may act as a guide for targeted use of aggressive multimodal analgesia, use of calcium-channel modulators (like gabapentin) and preemptive analgesics like *N*-methyl-D-aspartate receptor antagonists (like ketamine), regional anesthesia, and behavioral therapies in patients with higher

risk.[59] Addressing this higher risk using alternative strategies may be especially important as the MOR protein encoded by *OPRM1* plays a key role in mediating not only pain responses but also the development of tolerance and physical dependence.[60,61] Future studies need to map methylation changes in the *OPRM1* promoter over the time course of CPSP, and the effect of preventive and therapeutic strategies on transition of acute pain to CPSP, and development of tolerance/opioid hyperalgesia.

Summary

Surgery is a stressful and painful experience; however, some individuals continue to experience pain, even months or years after surgery. This is because pain experience is unique. It has been shown that psychological factors play a role in shaping pain responses after surgery. Genes also play an important role in determining pain. One such gene is the mu-1 opioid receptor gene (*OPRM1*) that codes for the mu opioid receptor (MOR), where potent pain medications (opioids) bind. In this study, we evaluated whether nonstructural changes in the deoxyribonucleic acid (DNA) (DNA methylation) of the regulatory region of the *OPRM1* gene could predict the individual pain experience. In 133 adolescents undergoing spine fusion, after controlling for important nongenetic covariates identified from a previous analysis, it was found that methylation at several regulatory sites was associated with preoperative, immediate postoperative, and chronic postoperative pain. These sites were identified as binding regions for important transcription factors; methylation likely affects factor binding and decreases the *OPRM1* expression, leading to increased pain sensitivity. These serve as novel biomarkers for pain and, since DNA methylation is modifiable, might provide a basis for future preventive and therapeutic strategies.

Acknowledgments

The authors would like to acknowledge Ashley Ulm and Veda Yadagiri (Pyrosequencing Core, Cincinnati Children's Hospital Medical Center [CCHMC]) and Diane Kissell for their role in analyzing the DNA extraction and pyrosequencing, under the supervision of Hong Ji (Director, Pyrosequencing Core) and Kejian Zhang (Director of Molecular Genetics Lab, CCHMC). They would also like to acknowledge Kayla Stallworth and Hope Esslinger, CCRC IV, previous research coordinators for the Department of Anesthesia, CCHMC, for their help with patient recruitment in the earlier stages of the study. This study was supported by 5K23HD082782 through the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health (PI: Chidambaran), Center for Pediatric Genomics, and Shared Facility Discovery Award from CCHMC (PI: Chidambaran). The authors are solely responsible for the content, and it does not necessarily represent the official views of the National Institutes of Health.

Disclosure

The authors report no conflicts of interest in this work.

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