



Research Paper: Comparing *Oprm1* Gene Promoter Methylation in the Lymphocytes of Male Rats Addicted to Nicotine, Morphine, Methadone, and Buprenorphine



Maedeh Mohammad Alizadeh¹, Seyed Reza Kazemi Nezhad¹, Parvin Babaei², Parvaneh Keshavarz^{3*}

1. Department of Genetics, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran

2. School of Medicine, Neuroscience Research Center, Guilan University of Medical Sciences, Rasht, Iran

3. School of Medicine, Cellular and Molecular Research Center, Guilan University of Medical Sciences, Rasht, Iran



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ABSTRACT

Background: Addiction is a polygenic disorder caused by genetic and environmental factors. The opioid material can act as an epigenetic element, like DNA methylation.

Objectives: The present study aimed to examine the effect of epigenetic drugs such as nicotine, morphine, methadone, and buprenorphine on the methylation of two CpG sites in promoter of *Oprm1* gene in male Wistar rats.

Materials & Methods: In this case-control study, 48 male Wistar rats with Mean±SD (200±30) g were divided into 6 groups: These are five groups only The control (intact) group, Nicotine (0.4 mg/kg, SC injection for 5 days), morphine (10 mg/kg on days 1-3 and 20 mg/kg on days 4-6 and 40 mg/kg, IP injection on days 7-9), methadone (0.5 mg/kg, IP injection for 15 days), buprenorphine (0.05 mg/kg, SC injection for 6 days) and finally saline for each respected group. After the treatment, genomic DNA was extracted from the whole blood of the rats. Then, the extracted DNA was treated with sodium bisulfate. To identify the selected methylated areas of *Oprm1* promoter sites (CpG-107 and CpG+33), we used Methylation-Specific PCR (MSP).

Results: Our results showed no methylation in the two CpG sites of *Oprm1* promoter in all of the treated groups.

Conclusion: Addiction with nicotine, morphine, methadone, and buprenorphine in doses and duration used in this study was not associated with the methylation of the *Oprm1* promoter sites in the male Wistar rats.

Keywords: Nicotine; Morphine; Methadone; Buprenorphine; DNA methylation

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* Corresponding Author:

Parvaneh Keshavarz

Address: School of Medicine, Cellular and Molecular Research Center, Guilan University of Medical Sciences, Rasht, Iran

Tel: +98 (911) 2381567, **Fax:** +98 (13) 33754468

E-mail: keshavarz@gums.ac.ir

Highlights

- There is no association between *Oprm1* gene promoter methylation and addiction
- Nicotine, morphine, methadone, and buprenorphine do not effect on the methylation of *Oprm1* gene

Introduction

Drug addiction is one of the main concerns of health care organizations. According to the literature, there are around 29.5 million people (different reports from 15.3 to 43.1 million) in the world suffering from drug abuse. Biological, social, environmental, and genetic factors can influence the vulnerability to addiction [1, 2]. Studies on families, twins, and adopted children have shown that the odds of inheriting drug abuse disorders is moderate to high (0.30 to 0.70) [3]. However, genetic or environmental factors alone do not increase the tendency to addiction [4].

In drug addiction, compulsive drug-seeking and drug-taking behavior persist despite serious negative consequences. Addictive substances induce adaptive changes in the central nervous system and lead to tolerance, physical dependence, sensitization, craving, and relapse [5]. Morphine is one of the opioids which is widely used as a painkiller and an addictive drug [5-7]. Nicotine is the main ingredient of tobacco that causes addiction to smoke [7, 8]. Tobacco also is one of the leading causes of many diseases, mortality, and morbidity [9, 10]. Other opioids such as methadone and buprenorphine as an agonist of Mu-Opioid Receptor (MOR) are widely used for the treatment of dependence on other opioid drugs such as morphine [8].

Epigenetics refers to any change in the gene expression that does not alter the DNA sequence. DNA methylation and histone modification (methylation, Acetylation, phosphorylation, ubiquitylation, and sumoylation) are the most frequent epigenetic changes that regulate gene expression [11, 12]. Previous studies reported the involvement of DNA methylation and histone modifications in drug addiction [13-16]. Based on the reports, cytosine methylation induces epigenetic changes and transcriptional regulation of the mammalian genome. Cytosine methylation occurs on C5' of the cytosine ring by DNA-methyltransferase in the C-G dinucleotides [17]. There are approximately 45000 CpG islands in the human genome and 19568 in the rat genome [18, 19].

Generally, the DNA methylation in promoter sites is related to reduction of gene expression [20, 21].

Opioid receptors are G protein-linked receptors activated through the interaction of downstream signaling with heterotrimeric G-protein [8]. Mu (μ), kappa (κ), and delta (δ) opioid receptors are respectively coded by three genes: *OPRM1*, *OPRK1*, and *OPRD1* [22]. *OPRM1* is a likely candidate gene for smoking and the main target in most drug therapies [1, 23]. Various studies have reported the higher level of DNA methylation in specific CpG sites in the *Oprm1* promoter in addicted individuals to opioids compared to non-addicts [13, 22, 24, 25]. The μ opioid receptor is expressed in different cells, such as lymphocytes, macrophages, and neurons [26-28]. Hypermethylation of *Oprm1* gene is followed by lower transcription of *Oprm1* [29, 30]. *Oprm1* gene has a binding site for several important translation factors such as Elongation Factor (EF2), CREB (cAMP response element-binding protein), and AP-1 (activator protein 1) [31].

Human studies on the *OPRM1* gene of addicted people revealed more methylation rate of the promoter of this gene in the whole blood in addicted male compared with the control counterparts [22]. In Nielsen et al. studies on Caucasian and African-American populations, similar results were found in peripheral blood lymphocytes of the heroin addicts under Methadone Maintenance Treatment (MMT) [13, 24]. Because addicted people usually use more than one drug, it is difficult to distinguish the exact effect of each drug on methylation in human, therefore this study was designed to evaluate the impact of four most commonly-used drugs (nicotine, morphine, methadone, and buprenorphine) on the methylation level of two CpG sites at the *Oprm1* promoter in male Wistar rats separately. To the best of our knowledge, this is the first study to aim this critical goal.

Materials and Methods

Subjects

In this case-control study, 48 male Wistar rats with Mean \pm SD (200 \pm 30) g and aged two months were used. The

Table 1. The sequences of primers for methylated or unmethylated promoters

Name	Sequence
Methyl rat <i>Oprm1</i> - forward primer	5'- TGGTTTATGTAAGAATTTGAC <u>GGA</u> -3'
Methyl rat <i>Oprm1</i> - reverse primer	5'- ACTAAAAATCTAAACAATC <u>GCTAA</u> -3'
Unmethyl rat <i>Oprm1</i> - forward primer	5'- TGGTTTATGTAAGAATTTGA <u>IGGA</u> -3'
Unmethyl rat <i>Oprm1</i> - reverse primer	5'- ACTAAAAATCTAAACAATC <u>ACTAA</u> -3'



Underlined nucleotides show the primer changes for methylated versus unmethylated sequences

animals were housed with free access to food and water in a 12 h light/dark cycle and at constant temperature of $22\pm 2^\circ\text{C}$. They were allowed to get habituated to the examiner and laboratory conditions (hence were handled at least three days before the experiments). All procedures concerning animal care were following the International Ethical Guidelines, the Declaration of Helsinki, and the Ethics Committee of Guilan University of Medical Sciences.

Groups

The studied animals were classified into 6 groups with 8 rats in each group: 1. The control (intact) group; 2. The group receiving normal saline as a solvent for drug; 3. The group receiving nicotine: 0.4 mg/kg, SC injection for 5 days (N5260, Sigma-Aldrich, USA) [32]; 4. The group receiving incremental doses of morphine: 10 mg/kg on days 1-3 and 20 mg/kg on days 4-6 and 40 mg/kg, IP injection on days 7-9 (Daroupakhsh, Iran) [33]; 5. The group receiving methadone: 0.5 mg/kg, IP injection for 15 days (Daroupakhsh, Iran) [34]; and 6. The group receiving buprenorphine: 0.05 mg/kg, SC injection for 6 days (Faran, Iran) [35].

12 hours after the last injection, the male rats were anesthetized with ether. Their blood samples were taken from their venae cavae. To prevent the blood samples from clotting, we kept them in Ethylene Diamine Tetra acetic Acid (EDTA) tubes as an anticoagulant at -20°C until DNA extraction.

Genomic DNA extraction

DNA was extracted from blood lymphocytes of all rats using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's protocol. Briefly, cells were lysed by optimized buffers and enzymes of this kit. DNA was adsorbed to the QIAamp membrane. Alcohol was added, and lysates loaded onto the QIAamp spin column. Wash

buffers removed impurities, and pure, ready-to-use DNA was then eluted in water or low-salt buffer.

DNA methylation

The nucleotide sequence of the rat *Oprm1* promoter region (chr1: 37533440-37536440, Rat.rn4) and CpG island sequence located at the promoter region of *Oprm1*, were downloaded from the UCSC Genome Browser website (genome.ucsc.edu). It shows that the *Oprm1* gene is located at position 1p11; the 18 CpG sites are located in position 37535123-37535517. The primers were designed by the MethPrimer Web software (<http://www.urogene.org/methprimer/>) (Table 1). In the present research, 2 out of the 18 CpG sites (+33 CpG and -107 CpG) were investigated. As mentioned, the position of +33 CpG is an E2F transcription factor binding site. Also, the status of -107 CpG is binding sites of 2 transcription factors AP-1 and CREB [31].

To apply the MSP method, DNA is treated with sodium bisulfite, in which process, all unmethylated cytosine is converted to uracil [36, 37]. During the sulfonation reaction, however, methylated cytosine cannot be converted [38]. Therefore, the differences created by the reaction between the methylated and unmethylated cytosine can be distinguished by MSP. To characterize methylation percentage, genomic DNA (>300 ng) was treated with sodium bisulfite using the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's protocol. The final bisulfite-treated DNA was eluted in 20 μL elution buffer.

Methylation specific PCR

MSP is a PCR-based method for the analysis of methylation templates in CpG islands [36]. In the MSP method, 2 sets of methylated and unmethylated cytosine-specific primers of targeted sequences are used, and the PCR reaction is separately carried out. DNA



Figure 1. Location of MSP primers located on *Oprm1* gene promoter in the male rat

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amplification with primers was employed to amplify the *Oprm1* promoter sites (+33 CpG and -107 CpG) (Figure 1). The final product size of PCR was 180 base pairs. Amplification was performed with 1.5 μ L of bisulfite-treated DNA, 0.5 μ L of each primers, 2.5 μ L PCR buffer, 0.5 μ L dNTPs, 0.75 μ L $MgCl_2$, 0.13 μ L *Taq* DNA polymerase in 25 μ L total reaction volume. The amplification was done over 5 min at 94°C; then 30 cycles, including 30 s at 94°C, 30 s at 59°C, 30 min at 72°C and a final 5-min elongation step at 72°C.

To evaluate the accuracy and quality of the desired fragments, we loaded them onto the 2% agarose gel electrophoresis. The place and status of PCR products

in agarose gel were detected by staining with the fluorescent dye ethidium bromide under direct ultraviolet light. The results of electrophoresis in agarose gel were analyzed by observing the ultraviolet ray using gel documentation (Figure 1).

Results

Gel electrophoresis of amplified DNA showed DNA bands in the row of PCR created by unmethylated primers. Figure 2 shows the results of the PCR process in which all samples have a positive response amplified by the unmethylated primer. Comparing the two CpG sites in the *Oprm1* promoter in 48 rats in 6 groups, no

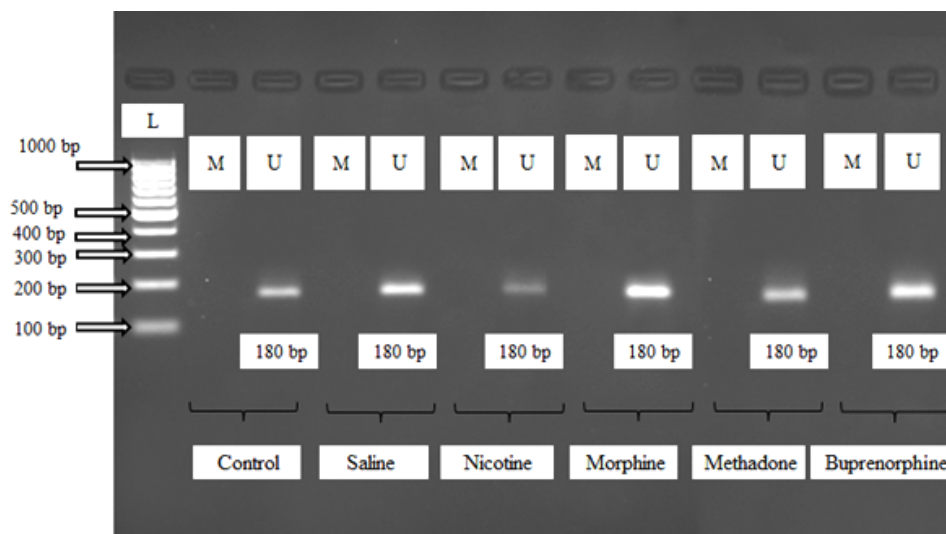


Figure 2. The PCR results of 180 bp fragment on the 2% agarose gel

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L: Ladder; M: Methylated; U: Unmethylated

Table 2. Frequency of methylation status of the study groups

Methylation	Groups						Total
	Control	Saline	Nicotine	Morphine	Methadone	Buprenorphine	
Methylated	0	0	0	0	0	0	0
Un methylated	8	8	8	8	8	8	48
The Chi-squared test and P				not applicable			



change was observed at the methylation status of +33 and -107 CpG sites in *Oprm1* promoter (Figure 2).

The Chi-squared analysis is a suitable test for the investigation of frequency differences between studied groups. The frequency of unmethylated cases in groups was less than 4. Therefore, the data did not apply to this analysis. Table 2 presents the frequency of the methylation status of the groups.

Discussion

The results of the present study showed that nicotine, morphine, methadone, and buprenorphine in the doses used here led to the addiction of the male Wistar rats, demonstrating particular symptoms and behaviors, including ecstasy, solace, disquietude, seeking behaviors, standing, grooming, anxiety, and other abnormal signs such as constipation and diarrhea. These results are in agreement with the previous findings [39]. Surprisingly no change was observed in the methylation of *Oprm1* gene promoter sites of blood lymphocytes. Our findings are contradictory to the previous studies carried out on the brain nucleus [31].

To the best of our knowledge, there is no study on methylation of *Oprm1* gene promoter in lymphocytes of animals addicted to nicotine, morphine, methadone, or buprenorphine. However, human studies revealed the positive results of methylation of *Oprm1* gene promoter in the blood (sites 13, 22, 24, and 25). In a recent study by Chorbov et al. on DNA methylation in 24 CpG sites of *Oprm1* promoter in male opioid abusers (extracted from their blood samples), significant DNA methylation was found at 7 CpG sites (sites 5, 9, 10, 11, 18, 23, and 24). They further reported a high level of DNA methylation only in 2 CpG sites in the DNA extracted from the sperm samples of the addicted individuals [22].

Although we found negative results from the methylation in CpG sites of *Oprm1* (-107 CpG and +33 CpG),

methylation might have occurred in other CpG sites. Then, the absence of methylation in our study could be accompanied with an inactive transcription of the gene [40, 41].

In addition, the study of DNA methylation on peripheral blood lymphocytes of heroin addicts under MMT of the Caucasian population showed that methylation is significantly associated with the two CpG sites (18 and 84) in the *Oprm1* gene promoter [13]. These observations were later replicated by Nielsen et al. in 2010. They reported that the level of DNA methylation in the *Oprm1* gene promoter was higher in heroin addicts than in the control group in African-American and Hispanic populations. A comparison between these two populations revealed that the level of methylation in the *Oprm1* gene promoter differs by geographical features [24].

Differences between human and animal studies might be related to the fact that addicted people often use a variety of drugs which can affect methylation rate cumulatively. The strong point of the present study relates to its capability to distinguish and specify the contribution of each drug to methylation. However, the limitation of our study was the evaluation of the methylation of other CpG regions or exons. Assessing all CpG sites in the promoter or exons might better explain the negative or positive epigenetic effect of nicotine, morphine, methadone, and buprenorphine.

One possible explanation for negative results on methylation of the *Oprm1* gene promoter in male Wistar rats following addiction drug usage might be the insufficiency of either the doses of drugs or duration of treatment. Also, these drugs might not have been capable of methylating *Oprm1* gene promoter in rats and probably cannot be considered as epigenetic substances in this species. Thus, these animals may have an unknown protective mechanism against epigenetic changes to addiction.

Conclusion

The current study shows that use of nicotine (0.4 mg/kg, SC injection for 5 days), morphine (10 mg/kg on days 1-3 and 20 mg/kg on days 4-6 and 40 mg/kg, IP injection on days 7-9), methadone (0.5 mg/kg, IP injection for 15 days) and buprenorphine (0.05 mg/kg, SC injection for 6 days) do not induce methylation of *Oprm1* gene promoter sites in the blood samples of the male Wistar rats.

The epigenetic mechanism is triggered by the environmental stimuli of the cells. Addiction is a multifactorial and polygenic condition; therefore, various factors and interactions must be considered in the study of epigenetics in addiction. For future studies, it is recommended to use a higher dose of drugs with different time courses to clarify the effect of addictive drugs on epigenetic changes.

Ethical Considerations

Compliance with ethical guidelines

All study procedures complied with the Ethical Guidelines of the Declaration of Helsinki 2013.

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Authors contributions

Design of the study: Parvaneh Keshavarz, Parvin Babaei; Collection of samples, and experimental procedure: Maedeh Mohammad Alizadeh; Interpretation of data, assembly, critical revision of the article for important intellectual content, and final approval of the study: Seyed Reza Kazemi Nezhad, Parvin Babaei, Parvaneh Keshavarz; Writing the article and its editing: Maedeh Mohammad Alizadeh, and Parvaneh Keshavarz.

Conflict of interest

The authors declared no conflict of interest.

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