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Investigation of Methylation Levels in OPRK1 Gene Promoter among Smokers and Opium-Addicts underwent Methadone Maintenance Treatment

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1. Abstract

1.1. Background: Previous studies reported the association of the OPRK1 gene with illicit substances, nicotine, and alcohol. The present study aimed to look at the methylation levels of OPRK1 gene promoter among smokers and addicts who underwent methadone maintenance treatment (MMT).

1.2. Methods: DNAs were extracted from the whole blood of all male samples including 30 smokers, 30 opium-addicted individuals undergoing methadone treatment, and 30 healthy people, and they were treated with a sodium bisulfite kit. The studied region included 53 CpG dinucleotides investigated by sequencing technique.

1.3. Results: Results of methylation levels in addicted individuals who underwent MMT compared with healthy people showed no difference. Also, there was no change in any CpG sites of OPRK1 gene promoter in both smokers and compared healthy controls. There was a significant difference in the mean age between opium-dependent people and healthy controls (P=0.017). According to the findings of the statistical analysis, resident situation and libido dysfunction were associated with methadone dose (P=0.032 and P=0.003, respectively).

1.4. Conclusion: Altogether, the study of methylation levels at OPRK1 gene promoter was not significant among smokers and individuals who underwent MMT compared to the healthy controls; additionally, methadone dosage had significant associations with demographical statuses in the MMT group.

2. Introduction

In accordance with the WHO, tobacco consumption will be the leading avoidable cause of death globally, killing about 6 million people annually [1]. By 2030, tobacco use will kill more than 8 million people worldwide if the present trends continue. Dependence and smoking are multifactorial, polygenic illnesses which

do not inherit according to the traditional Mendelian pattern, like other common, complex diseases [2, 3]. Because nicotine is an addictive substance, it quickly enters the system after smoking and travels less than 10 seconds to the brain. It has an impact on the brain's rewarding system, resulting in a feeling of contentment and satisfaction [4]. But nicotine is a deadly medication, and abusing it may be dangerous. In certain cases, excessive nicotine use might result in mortality because it lowers blood pressure, impairs breathing, and creates confusion [5]. The most popular synthetic drug used in opioid substitution treatment (OST) is methadone [6]. Due to a sharp fall in injecting drug usage and the sharing of injection facilities, methadone maintenance treatment (MMT) reduces the risk of HIV transmission [7]. Methadone has been found to reduce dependency on other sedatives and lower the rate of drug misuse. Although MMT is therapeutically beneficial, there is still considerable intra-individual variation in outcomes and no clear biomarker for opioid dependence treatment responses [8].

Previous researches have documented that the methylation of the cytosine in the genomic DNA, which is produced when DNA methyltransferases add a methyl group to the 5' of the cytosine ring in CpG nucleotides in the promoter region of the gene, is crucial for the gene expression regulation by affecting the interactions between transcription factors, chromatin proteins, and DNA [9-12]. Prior studies have demonstrated that nicotine alters the epigenome [13-15]. Studies on mice have indicated that cocaine use affects changes in DNA methylation, a crucial process of gene regulation [16].

The word opioid is used to describe a class of substances which have qualities similar to opium. These drugs can cause physical dependency and have effects on awareness, motor coordination, mood, and autonomic function. According to pharmacologic investigations, there are at least three primary kinds of opioid receptors: kappa (OPRD1; 165195), mu, and delta (OPRD1; 165195). (OPRM1; 600018) [17]. OPRK1 or KOR protein is a coding gene in human containing four exons which is located in 8q11.23 cytogenetic situation [18]. Based on the previous studies, opioid dependence is one of the major factors which increases the OPRK1 gene expression. Suzuki and colleagues reported that morphine increases OPRK1 gene expression [19]. Furthermore, drug exposure disrupts the KOPr/dynorphin pathway, which is induced by a change in the quantity of mRNA or the level of the aforementioned proteins [20, 21]. There are other studies demonstrating the relationship between OPRK1 and PDYN genes with alcohol dependence [22, 23]. On the other hand, there are remarkable studies reported the association of both drug addiction and smoking with single nucleotide polymorphisms (SNP) of the OPRK1 gene [24-26]. To the best of our knowledge, there is only a report stated that the OPRK1 promoter hypermethylation was associated with a higher risk of heroin and methamphetamine addiction [27]. Due to the importance of the OPRK1 gene, which includes its important role and high expression in the cells of the central nervous system, no study has been investigated the methylation of the OPRK1 promoter among smokers and addicts who underwent MMT. Therefore, the present study was designed with the aim of understanding and investigating the effect of methadone and nicotine usage on OPRK1 gene promoter through plausible methylation processes.

3. Material and Method

Active smokers engaged in Rasht's smoking cessation program. Subjects for MMT were chosen and recruited from three MMT clinics in Guilan Province, Iran. Furthermore, a Control (never smokes) group was enlisted in the Razi laboratory in Rasht, Iran. All of the individuals who satisfied the inclusion criteria for the current study were Iranian and lived in Guilan province. A total of 30 opioid addicts undergoing MMT, 30 smokers, and 30 healthy adults were chosen; all samples were male and ranged in age from 30 to 60 years. The minimal time period of opioid addiction for opium-dependent patients receiving methadone therapy was three months. Additionally, in the three months preceding the sample date, dependent patients seeking methadone therapy were taking 60 to 80 mg of methadone daily. Finally, each individual was asked about his educational background and employment title. Age over 18, a MMT treatment length of at least three months, regular attendance by patients in the seven days before to sample, a lack of concurrent use of other drugs, and a lack of continuous substance misuse other than opium were the inclusion criteria for addicts who took methadone. Minimum age of 18 years, daily cigarette usage of 10 or more, and continuous use for more than one year were inclusion criteria for the smoking group.

A total of 30 healthy controls were chosen from individuals who had no prior history of addiction at the time of the sampling. The inclusion criteria for the control group included the following: 1) no history of substance abuse, including other opioids; 2) no history of alcohol consumption; 3) age of over 18 years (to be consistent with the studied group); 4) male (to be consistent with the experimental group); 5) no use of CNS active drugs, including psychotropics that can affect sleep period and libido; and 6) absence of psychotic problems (since many psychotic problems induce libido dysfunction and insomnia problems). The lack of opiates or illicit substances was confirmed by urine toxicology tests. DNA was extracted from the entire blood of all subjects using the extraction technique (Qiagen Corporation kit). The extracted DNA quality for each sample was determined using 0.8% agarose gel electrophoresis, and the amount was determined using Nanodrop (NP1000). All individuals' extracted DNA was treated with sodium bisulfite utilizing an EPITEC kit (Qiagen Corporation, CAT. NO.59104).

The Meth Primer website designed primers for the amplification of CpG islands in the promoter region of the OPRK1 gene. Amplicons of CpG islands containing 667 bases and 53 CpG sites were produced by forward primer 5'-TAGGTGTATATTTGTAT-

TAAATAGG-3' and the reverse primer 5'-AAAATACCTCCCT-CACCAATTC-3' (Figure 1) (The aforementioned primers were utilized for all study groups, comprising addicts undergoing MMT, smokers, and healthy controls.) Thermo Science Corporation Master mix (CAT.NO. K0171), primers, free-nuclease water, and bisulfite DNA were used in PCR according to the instructions specified in the EPITEC kit. On the 1.5% agarose gel, PCR products (667 nucleotides long) were tested for sharpness, no smear, and no primer dimer. The PCR results were then evaluated using the sanger sequencing technique. All statistical analyses were calculated using SPSS22 software. For each CpG site, the methylation percentage and any changes between controls and cases that were significant or not were calculated. Chi-square and Fisher's exact tests were applied to compare OPRK1 gene methylation levels and examine participant characteristics.

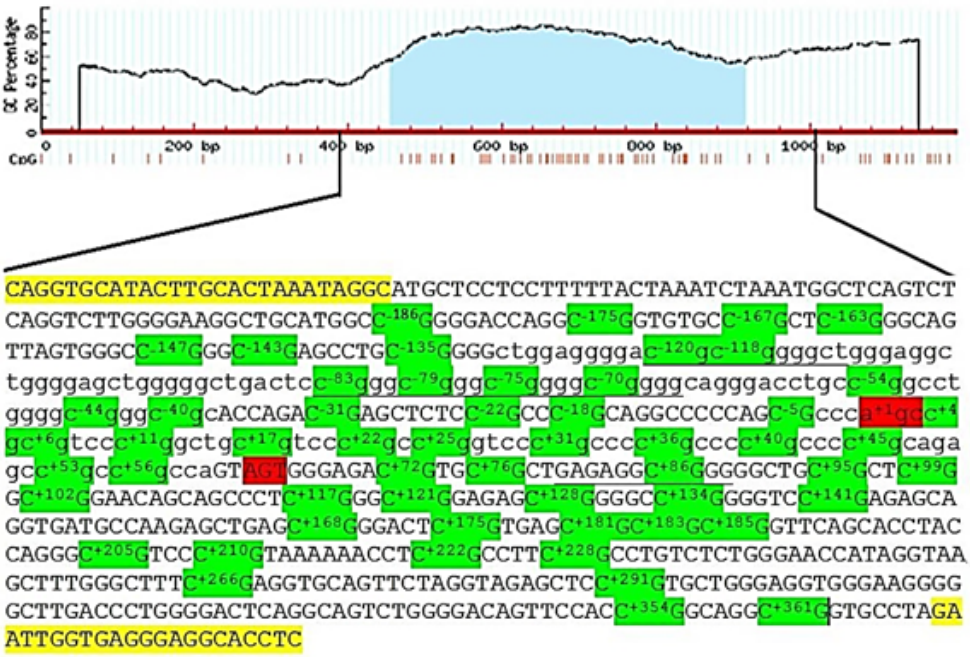

Figure 1: Two primers including a forward (25 bp) and a reverse (22 bp) designed for promoter sequence of OPRK1 gene. Meth primer web site indicated that this gene has one CpG Island with 461 bp which is displayed with grey color. The complete sequence that amplified by PCR was 667 bp. The CpG sites relatively numbered the transcription start point (A in AGC is +1). Underlined parts refer to SP1 transcription factor binding site.

4. Results

4.1. Samples

The current study included 90 patients separated into two case groups: 30 addicted persons receiving methadone therapy and 30 smokers, and 30 healthy people. The Ethics Committee for Human Genome/Gene Research at Guilan University of Medical Sciences approved the consent process for all participants in the current study (No. 1930400417, June 25, 2014). Data from methylation levels at 53 CpG sites were derived from different statistical examinations of healthy controls compared with addicted individuals who underwent MMT and healthy controls against smokers.

4.2. Methylation Results

The RCR reaction was accomplished with a total volume of 50 mL (PCR Master Mix- # K0171 25 µL, Forward Primer 2 µL, Reverse Primer 2 µL, bisulfited DNA 2 µL, nuclease-free water 19 µL) and annealing temperature of 56 °C. Next, PCR products were loaded on gel electrophoresis and the favored bands were detected by UV light (Figure 2).

The rate of total methylation from each CpG site was compared between addicted people who underwent MMT and healthy controls. Because of the lack of methylation in both addicted patients receiving methadone therapy and healthy controls, statistical analysis was not applicable for all 53 CpG sites. None of the 53 CpG sites had a statistically significant difference between the addicted and the control group to be analyzed by the Chi-Square test and Fisher's Exact test. Based on the lack of methylation in any of the 53 CpG site, the calculation of the difference in the rate of total methylation was not applicable among addicted individuals who underwent MMT (0% methylation) and controls (0% methylation). The comparison of the total methylation percentage of each CpG site was evaluated for smoker and healthy control groups. The statistical analysis of all CpG sites was impossible, due to the lack of methylation in both groups. Thus, no further statistical analysis with both Chi-square and Fisher's Exact test methods was possible.

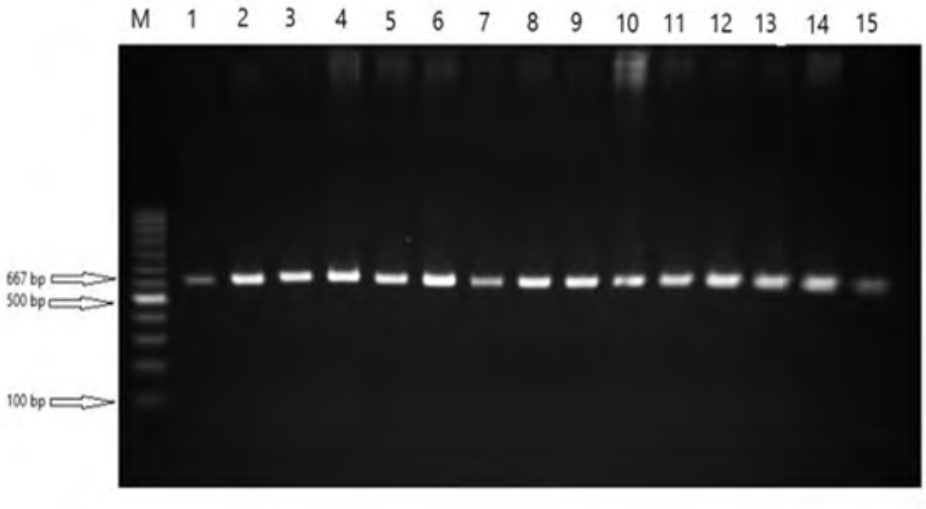

Figure 2: The result of primer-designed sequence of bisulfite-DNA of samples containing the whole CpG island of OPRK1 promoter (667 bp) on 1.5% gel agarose.

4.3. Demographical Sub-Analyses

Independent t-test results revealed a significant difference in mean age between opium-addicted individuals (47.37±14.06) and control subjects (38.40±14.28) (P=0.017); however, there was no statistically significant difference in mean age between the smoker group (41.74±10.08) and the control group (38.40±14.28) (P=0.294). Insomnia, libido difficulties, marital, educational, residency, and employment statuses were examined in opium-dependent people receiving methadone therapy, adjusting for methadone dose as a genetically influenced variable. According to the findings, resident situation and libido dysfunction were correlated with methadone dose (P=0.032 and P=0.003, respectively). A significant difference between urban and rural opium-addicted persons indicated a higher prescribed dosage among urban group (86.84±32.50 mg/day) compared to villagers (111.82±21.83 mg/day). When comparing addicted patients without libido dysfunction (60.00±44.72 mg/day) and addicted individuals with libido dysfunction (103.20±22.68 mg/day), the association of methadone dosage with libido dysfunction revealed that higher prescribed methadone dosage can have a damaging impact on libido dysfunction incidence (Table 1). Pack-year smoking was regarded as a customized characteristic associated with genetic variations, similar to the MMT group. Based on this, the subcategories of smokers' marriage, education, residency, and work status were examined, and no statistically significant difference was discovered (all P-values >0.05). (Table not shown).

Table 1: Demographic characteristics of opium-addicted individuals (n=30) according to their prescribed methadone dosage.

Categorical features	Subcategorical features	Mean±S.D.*	P-value
Marrage Status		98.00±14.83	0.979
Single		95.42±34.39	
Married		100.00±--	
Widowed			
Education Status		116.67±8.16	0.187
Illiterate		90.00±35.84	
Non Academic		95.00±5.77	
Academic			
Resident situation		111.82±21.83	0.032
Rural		86.84±32.50	
Urban			
Job status	No job	110.00±10.95	0.418
	Day worker	93.81±33.54	
	Day and Night worker	83.33±40.42	
Insomnia	Without Insomnia	101.33±22.32	0.357
	With Insomnia	90.67±38.07	
Libido dysfunction	Without L.D.**	60.00±44.72	0.003
	With L.D.	103.20±22.68	

Independent t-test and one-way ANOVA results are signified by mean, Standard vdeviation (S.D.*), and P-value. The significant level was < 0.05. L.D** means Libido dysfunction.

5. Discussion

Addiction is a complex and polygenic condition caused by the interaction of heredity and the environment. Several type of researches in both animal and human species have found a link between addiction and epigenetic alterations [28-30]. Remarkable epigenome-wide association studies (EWAS) have been conducted based on the diversity of study designs and investigations in different populations; these studies have revealed variations in repeatable associations of smoking with DNA methylation in whole blood DNA at CpGs related to genes such as ALPPL2, AHRR, F2RL3, IER3, and GPR153 [31-40]. Some studies have revealed that DNA methylation in genes encoding methylteransferase proteins is associated with smoking. Based on Fowler et al's research, overexposure to tobacco smoking can reduce human brain MAOA activities [41]. Nicotine injections, according to Satta et al., can decrease the mRNA and protein expression levels of the DNA methylation enzyme, DNMT1, in the mouse brain and hippocampus [14].

The OPRK1 proteins can cause dysphoria by triggering anti-reward effects [42]. In a rat study of adolescents, KOR was correlated to less social play [42]. Corticotropin-releasing factor (CRF) signaling can be enhanced by social or physical stresses, such as extended exposure to substances of abuse, and can promote relapse in addicts [43, 44] Furthermore, stress from long-term drug use might have a depressive impact. Indeed, KOR antagonists may be utilized to treat depressive disorders, particularly in addicts [45]. According to a recent study, a KOR antagonist did not affect naive rats who had not previously experienced alcoholism, but it did affect rats who had [46]. Ultimately, KOR exerts anti-reward effects throughout the addiction process and has the opposite impact of MOR. As addiction progresses, increased stress can improve the action of KOR, contributing to dysphoric mood during both withdrawal and abstinence phases and ultimately leading to relapse [47].

The current study was designed to investigate the status of OPRK1 gene promoter methylation in association with nicotine and methadone consumption among smokers and addicted males who underwent MMT. To study OPRK1 gene methylation levels, 53 CpG sites in the promoter of the OPRK1 gene were candied. Next, DNA was extracted from whole blood samples, bisulfited, and lastly, the PCR products were sequenced. Results of sequencing did not show a significant difference in methylation levels of the OPRK1 gene among studied groups; neither in addicted individuals nor in smokers. In contrast with the methylation investigation results, data represented a significant difference in the mean age between opium-dependent people and healthy controls (P=0.017). Based on the statistical analysis, resident situation and libido dysfunction were also associated with methadone dose (P=0.032 and P=0.003, respectively).

Unlike many studies on the association investigation of the different OPRK1 gene SNPs with drug addiction, smoking, and alcohol consumption [24-26, 48], there are very limited and few data about the effect of addiction and smoking on OPRK1 gene promoter methylation [27]. Based on Ji et al's study, they reported that OPRK1 promoter hypermethylation may promote the risk of Alzheimer's disease (AD) by its regulation of the OPRK1 gene expression [49]. Along with Ji et al's study, Liu et al's study represented the association of OPRK1 and OPRM1 methylation with mild cognitive impairment in Chinese populations [50]. Stress-related dysmnesia was shown to be correlated with kappa opioid receptor activation, and these receptors may modulate glutamate neurotransmission and alter synaptic plasticity underlying memory formation [51]. The opioid system has been identified as a pharmacological target for the development of novel pharmacotherapies for Alzheimer's disease, which is characterized by memory loss and mental decrease [52]. Opioid receptor kappa 1 (OPRK1) inhibits neurotransmitter release by decreasing calcium ion currents and increasing potassium ion conductance. A study found more kappa binding sites in AD brains at autopsy, raising the possibility of opiate receptor up-regulation in AD [53].

Ji et al's investigation, as the only similar and most related study with our study indicated that the levels of OPRK1 promoter methylation were considerably greater in drug users than in controls (P=2.43×10−4). In male heroin addicts, also there were strong associations between OPRK1 promoter methylation and the length and frequency of drug use (methamphetamine) [27]. Interestingly, this results can be considered as reliable clues for the negative effects of methadone consumption on promoter methylation of OPRK1 gene. This suggests unknown mechanism(s) involving in the molecular pathway of methadone which may have remarkable impacts on the unmethylation of OPRK1 gene promoter after opium addiction.

Smoking was considered as covariance in some studies with no significant impact on the addiction association of the OPRK1 gene [54, 55]. Prodynorphin interactions in the opioid system cause nicotine negative sensations that decrease the rewarding route of nicotine dependence via beta-endorphins and enkephalins [56]. Nicotine withdrawal promotes an increase in prodynorphin expression in mice, according to Isola et al [57]. Studies on the association between smoking and OPRK1 gene polymorphisms are not noticeable and to the best of our knowledge, there is no report on methylation impact of smoking on the OPRK1 gene promoter. A previous study conducted by Albonaim et al investigated the variant association and including haplotypes of OPRK1 gene with nicotine dependence among male smokers. They demonstrated the significant associations of two variants of OPRK1 gene including rs997917 and rs6985606 and four haplotypes between Iranian smokers and non-smokers [25]. Based on the negative impact of smoking on promoter methylation of OPRK1 gene, the association of potential variants might be more important to be considered; however, further investigations of plausible smoking impacts on

the methylation of other CpG sites in the OPRK1 gene are highly recommended.

According to the sub-analysis data and other publications, there is a link between MMT and libido dysfunction, which may be one of the adverse effects of opium treatment with methadone [58]. Previous researches shown that among opium-addicts receiving MMT, libido dysfunction may be influenced by various variants both in exonic and intronic locations of a gene [24, 59]. Higher rates of poverty, unemployment, high-risk behaviors, and a lack of services for prevention, treatment, and rehabilitation may also have had an impact on rural opium addicts [60].

6. Conclusion

Conclusively, according to the review of desired CpG sites in OPRK1 gene promoter region between studied groups of smokers, addicted individuals who underwent MMT, and healthy people, the current study investigated the methylation levels of CpG sites, but no significant difference was found. Consequently, methylation investigation of OPRK1 gene in other genetic locations and also other related genes in opioid pathways might be better to be designed and performed. It is recommended to investigate the methylation and expression of such genes in mouse brain tissues since the brain is the primary site where pharmacological effects and OPRK1 gene expression are elicited.

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8. Conflict of Interest

The authors report no conflict of interest to disclose.

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