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Investigation of Methylation Levels in COMT Gene Promoter among Smokers and Opium Addicted Individuals Undergoing Methadone Treatment

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1. Abstract

1.1. Background: Previous studies have demonstrated that the COMT gene is associated with alcohol, nicotine, and illicit substances. The aim of the present study was to examine the methylation status of a remarkable region in the COMT gene promoter in methadone-treated smokers and addicts. Methods: All male samples, including 30 smokers, 30 opium addicts receiving methadone treatment, and 30 healthy individuals, had their DNAs extracted from their whole blood and processed with a sodium bisulfite kit. 61 CpG dinucleotides were included in the study region and were sequenced.

1.2. Results: Results represented that within these CpG sites, only 25 CpG sites in the addicted group and 22 in the smoker group compared to the healthy controls indicated different methylation levels; however, none of these CpG sites had a statistically significant difference (P=0.281 and P= 0.329, respectively). The mean age of opium-addicted individuals and healthy controls had significant differences between the two groups (P=0.017). Demographical results revealed that methadone dosage correlated with the resident situation and libido dysfunction (P=0.032 and P=0.003, respectively).

1.3. Conclusion: In conclusion, the investigation of methylation levels at COMT gene promoter had no noticeable significance among smokers and methadone maintenance treatment (MMT) patients compared to the healthy controls; moreover, methadone dosage had significant correlations with demographical statuses in the MMT group.

2. Introduction

Addiction to illicit and psychotropic medications is one of the world's most serious problems [1, 2]. According to the WHO, tobacco smoking will be the leading preventable cause of death worldwide, killing nearly 6 million people each year [3]. Tobacco will kill more than 8 million people globally by 2030 if current trends continue. Addiction and smoking, like other common complex diseases, are multifactorial and polygenic disorders that do not follow the standard Mendelian inheritance pattern [4, 5]. Being an addictive chemical, nicotine instantaneously enters the bloodstream after smoking and reaches the brain in less than 10 seconds. It has an impact on the brain's reward system, resulting in feelings of fulfillment and pleasure [6]. But nicotine is a poisonous drug, and abusing it can be dangerous. In some circumstances, excessive nicotine use can result in death [7] because it lowers blood pressure, impairs breathing, and causes confusion.

The most popular synthetic drug used in opioid substitution treatment (OST) is methadone [8]. Methadone was approved by the FDA in 1972 as a synthetic opioid to treat sedative dependence [9]. Due to a sharp decline in injecting drug use and the sharing of injecting facilities, methadone maintenance treatment (MMT) lowers the risk of HIV transmission [10]. Methadone has been found to reduce reliance on other sedatives and lower the rate of substance abuse. Despite MMT's well-documented therapeutic efficacy, there is substantial intra-individual heterogeneity in outcomes and no accurate biomarker for opioid dependency therapy responses [11]. Epigenetic pathways greatly regulate cell development and differentiation, and deficiencies in this mechanism can have serious consequences [1, 2]. Numerous studies have demonstrated that the methylation of the cytosine in the genomic DNA, which is created by the addition of a methyl group to the 5' of the cytosine ring by DNA methyltransferases in CpG nucleotides in the promoter region of the gene, is essential for the regulation of gene expression by influencing the interactions between transcription factors and chromatin proteins and DNA [3-6]. The association between addiction and methylation of genes involved in the methylation process has been studied in both human and animal studies [7]. According to earlier studies, nicotine causes changes in the epigenome [8-10]. Studies on mice have shown that cocaine usage has an impact on alterations in DNA methylation, a key mechanism of gene control [11].

The Catechol O-methyltransferase (COMT) gene is involved in the neurobiological activities of nerve cells exposed to medications or cigarettes. The COMT protein is one of numerous enzymes that catalyze catecholamines including dopamine, adrenaline, and norepinephrine [23, 24]. Several studies have found a link between single nucleotide polymorphisms in the COMT gene and illegal drugs, nicotine, and alcohol; however, few studies on gene promoter methylation in smokers and addicted individuals have been conducted; thus, the current study was designed to investigate the methylation levels of a large region of the CpG island in the COMT gene promoter in smokers and addicted individuals receiving methadone treatment. Because of the country's geographical and economic circumstances, addiction and smoking are common. Previous studies have shown that nicotine increases dopamine release in the brain through the synaptic vesicles. Considering that methylation is a mechanism for modulating gene expression, we investigated changes in COMT gene promoter methylation to gain a better understanding of addiction and smoking mechanisms, as well as its association with response to MMT.

3. Material and Method

Active smokers enrolled in Rasht's smoking cessation program. Subjects for MMT were chosen and recruited from three MMT clinics in Guilan Province, Iran. Furthermore, the Control group (never smokers) was enrolled in the Razi laboratory in Rasht, Iran. All of the volunteers who met the inclusion criteria for the current study were Iranian and lived in Guilan province. A total of 30 opioid-addicted people undergoing MMT, 30 smokers, and 30 healthy people were chosen; all samples were male and ranged in age from 30 to 60 years. The minimum duration of opioid addiction for opium-addicted individuals undergoing methadone treatment was three months. Additionally, during the most recent three months following the sampling date, addicts receiving methadone treatment were consuming 60 to 80 mg of the drug daily. Each subject was then asked about his educational status and his job. Age over 18, an MMT treatment period of at least three months, regular attendance by patients in the seven days before sampling, a lack of concurrent use of other medications, and a lack of ongoing substance abuse other than opium were the inclusion criteria for addicts who used methadone. Minimum age of 18 years, daily cigarette consumption of 10 or more, and continuous use for more than one year were inclusion criteria for the smoker group. 30 healthy controls were selected from people without any addiction history at the time of sampling. Inclusion criteria for controls included 1) no history of drug abuse and other types of opioids, 2) no history of drinking alcohol, 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 psychotropic which can effect on sleep period and libido, 6) lack of psychotic problems (since many psychotic problems induce libido dysfunction and insomnia problems). Urine toxicology screens were performed to confirm the absence of opiates or licit drugs.

DNA was extracted from the whole blood of all samples using the extraction protocol (Qiagen Corporation kit). The extracted DNA quality for each sample was determined using 0.8% agarose gel electrophoresis, and the quantity was ascertained utilizing Nanodrop (NP1000). All subjects' extracted DNA was treated with sodium bisulfite using the EPITEC kit (Qiagen Corporation, CAT. NO.59104). The Meth Primer server was used to design primers for the amplification of CpG islands in the COMT gene's promoter

region. Using the primers 5'-ATAGGTGTAGTTAGTAGGA-3' as the forward primer and 5'-CCTCATCACAACAAATCTTCA-3' as the reverse primer, amplicons of CpG islands with 485 bases and 61 CpG sites were established (The aforementioned primers were applied to all studied groups, including smokers, addicted individuals undergoing MMT, and healthy controls.) Following the instructions in the EPITEC kit, PCR was carried out using Thermo Science Corporation Master mix (CAT.NO. K0171), primers, free-nuclease water, and bisulfite DNA. On the 1.5% agarose gel, PCR products (711 nucleotides long) were examined for sharpness, the absence of primer dimerization, and smear. The sanger sequencing process was then used to evaluate the PCR products.

All statistical analyses were done by SPSS20 software. Percentage of methylation and significant or insignificant differences between controls and cases were measured for each CpG site. To compare the methylation levels of COMT gene and analyze the personal characteristics of participants, Chi-square and Fisher's exact test were used.

4. Results

4.1. Samples

The present study consisted of 90 subjects divided into two case groups including 30 addicted individuals undergoing methadone treatment and 30 smokers, and a control group including 30 healthy people. All individuals who participated in the current study consented to a process approved by the Ethics Committee for Human Genome/Gene Research at the Guilan University of Medical Sciences [No. 1930400417]. Data from methylation levels of 61 CpG sites consisted of separate results of computational investigations among healthy controls compared to addicted individuals undergoing MMT and healthy controls compared to smokers.

4.2. Methylation Results

RCR reaction was performed 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 58 °C. Then, PCR products were electrophoresed, and the specific bands were observed with UV light. The comparison between addicted individuals undergoing MMT and healthy controls were assessed the rate of total methylation from each CpG site. Out of these 61 CpG sites, statistical analysis was not applicable for 20 CpG sites due to the lack of methylation in both addicted individuals undergoing methadone treatment and healthy controls (CpG numbers:8, 9, 12, 21, 24, 27, 29, 32, 33, 35, 36, 39, 48, 50, 51, 52, 53, 57, 59, 61). In addition, 16 CpG sites had a P-value of 1.000 due to similar methylation rates between case and control individuals (CpG numbers:1, 5, 6, 7, 10, 11, 13, 15, 16, 25, 26, 31, 38, 47,54, 58). Different methylation rates were seen in 25 CpG sites including CpG numbers 2, 3, 4, 14, 17, 18, 19, 20, 22, 23, 28, 30, 34, 37, 40, 41, 42, 43, 44, 45, 46, 49, 55, 56, 60 (Figure 1). Analysing data with Chi-Square test and Fisher's Exact test, it was determined that none of the 25 CpG sites had a statistically significant difference between addicted and control group. Statistical analysis on 61 CpG sites indicated that difference in the rate of total methylation was not statistically significant among addicted individuals undergoing methadone treatment (36.3% methylation) and controls (24.6% methylation) (P=0.281).

The comparison of total methylation percentage of each CpG sites were evaluated for smoker and healthy control groups. The statistical analysis of 20 CpG sites were impossible, due to the lack of methylation in both groups (CpG numbers: 8, 9, 12, 21, 24, 27, 29, 32, 33, 35, 36, 39, 48, 50,51, 52, 53, 57, 59, 61). In addition, 18 CpG sites had P-value about 1.000 because of their equal methylation percentage (CpG numbers: 1, 3, 5, 6, 7, 11, 13, 15, 16, 20, 22, 25, 26, 38, 47, 49, 54, 58). Finally, 23 CpG sites revealed differences in the percentage of methylation including CpG numbers 2, 4, 10, 14, 17, 18, 19, 23, 28, 30, 34, 37, 40, 41, 42, 43, 44, 45, 46, 55, 56 and 60 (Figure 2). After statistical analysis with both Chi-square and Fisher's Exact test methods, it was found that none of the 23 CpG sites had no significant differences of methylation levels between smoker (30% methylation) and healthy controlgroups (26.1% methylation) (P= 0.329).

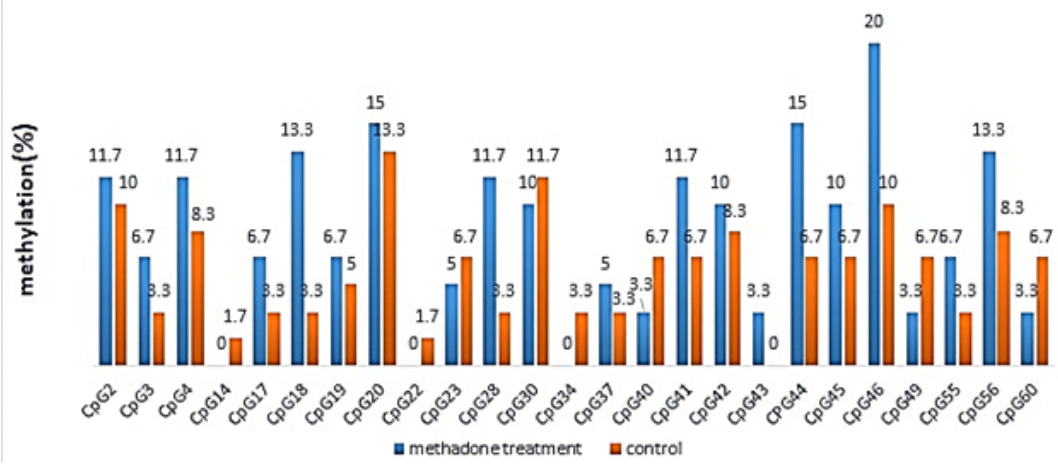

Figure 1: Percent methylation of CpG islands in COMT promoter among opium-addicted individuals undergoning methadone treatment.

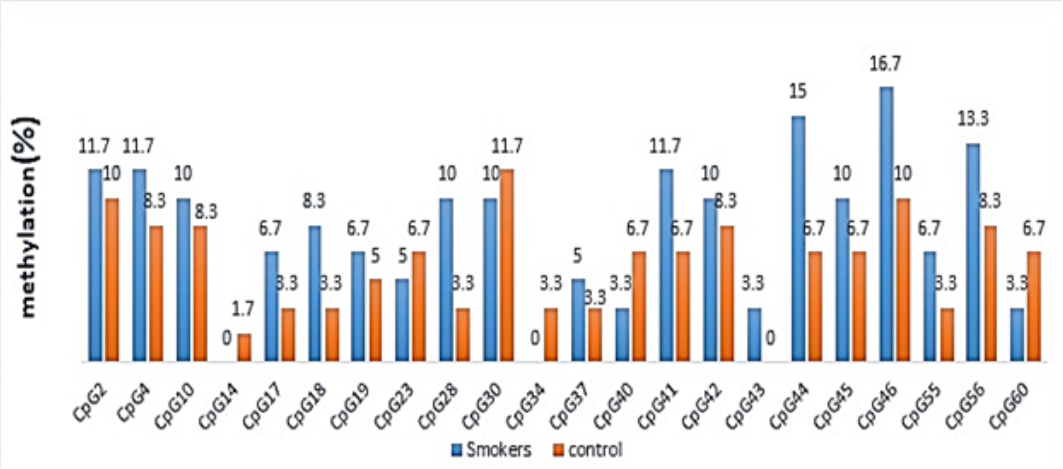

Figure 2: Methylation percentage of 23 CpG sites among smokers.

4.3. Demographical Sub Analyses

Independent t-test showed that the mean age of opium-addicted individuals (47.37±14.06) and healthy controls (38.40±14.28) had significant difference between the two groups (P=0.017); however, the mean age of the smoker group (41.74±10.08) and control group (38.40±14.28) had no statistical significance level (P=0.294).

Insomnia, libido dysfunction, marriage, education, resident, and job statuses were assessed among opium-addicted individuals undergoing methadone treatment, with methadone dosage considered a genetic-dependent variable. Methadone dosage was found to be associated with the resident situation and libido dysfunction (P=0.032 and P=0.003, respectively). There was a significant difference between rural and urban opium-addicted individuals, with villagers receiving a higher prescribed dosage (111.82±21.83 mg/day) than urban individuals (86.84±32.50 mg/day). When comparing addicted patients without libido dysfunction (60.0044.72 mg/day) and addicted individuals with libido dysfunction (103.2022.68 mg/day), the association of methadone dosage with libido dysfunction revealed that higher prescribed methadone dosage can have a disruptive effect on libido dysfunction occurrence (Table 1). Similar to the MMT group, pack-year smoking was considered a personalized variable related to genetic differences. Based on this, marriage, education, resident, and job statuses were compared among subcategories of smokers and no statistically significant difference was found (all P-values >0.05) (Table 2).

Table 1: Demographic characteristics of opium-addicted individuals (n=30) based on 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 represented by mean, Standard vdeviation (S.D.*), and P-value. The significant level considered lower than 0.05. L.D** means Libido dysfunction.

Table 2: Demographic characteristics of smokers (n=30) based on their pack-year smoking.

Categorical features	Subcategorical features	Mean±S.D.*	P-value
Marrage Status	Single	11.76±1069	0.39
	Married	25.09±22.80	
	Widowed	18.00±--	
Education Status		14.53±11.93	0.416
	Non Academic	26.75±27.57	
	Academic	20.93±5.53	
Resident situation	Rural	15.75±11.91	0.207
	Urban	25.88±24.33	
Job status	No job	9.64±8.03	0.37
	Day worker	23.64±22.17	
	Day and Night worker	37.50±--	

Independent t-test and one-way ANOVA results are represented by mean, Standard deviation (S.D.*), and P-value. The significant level considered lower than 0.05.

5. Discussion

Addiction is a multifactorial and polygenic disorder and results from the interplay between genetics and environment. Several studies have demonstrated the relationship between addiction and epigenetic changes in both animal and human subjects [1, 2, 12]. According to the diversity of study designs and investigations in various populations, remarkable epigenome-wide association studies (EWAS) have been performed; such these studies have identified differences in repeatable associations of smoking with DNA methylation in whole blood DNA at CpGs related to genes including ALPPL2, AHRR, F2RL3, IER3, and GPR15 [13-22]. According to some studies, DNA methylation in genes encoding methyltransferase proteins is associated with smoking. Based on the Fowler et al's study, acute exposure to tobacco smoking can reduce human brain MAOA activity [36]. Nicotine injections, according to Satta et al., can reduce the mRNA and protein expression levels of the DNA methylation enzyme, DNMT1, in the mouse cortex and hippocampus [20].

The current research aimed to study the association of COMT gene promoter methylation with smoking and opium addiction in smokers and addicted males receiving MTT. To investigate COMT gene methylation levels, 61 CpG sites in the COMT gene promoter were selected. The DNA was then extracted from whole blood samples and bisulfited before the PCR products were sequenced. Statistical analyses revealed no significant differences in COMT gene methylation levels among studied groups; however, methylation was observed in some CpG sites among smokers and addicted individuals undergoing methadone treatment, whereas no epigenetic change was observed in healthy controls.

Some studies showed a relationship between the low activity of COMT enzyme and nicotine dependence with greater sensitivity [37, 38]. Methadone affects multiple signaling pathways, such as the dopaminergic pathway by blocking dopamine receptors in the brain [39]. According to the importance of the COMT gene in neurological signaling pathways, such as the dopaminergic pathway, the methylation investigation of its regulatory regions, specifically its promoter, with both smoking and opioid addiction is important [23]. By direct sequencing, Xu et al. examined the methylation of 33 CpG sites at the COMT gene's promoter region in the blood of 50 smokers and 50 non-smokers. Two CpG sites in the study by Xu et al. showed a significant difference in methylation levels between smokers and non-smokers (P 0.01) [21]. Knaap et al. investigated the methylation correlation of the COMT gene with young people's substance use in a similar way to that of Xu et al. Knaap et al investigated the methylation correlation of the COMT gene with young people's substance use in a similar way to that of Xu et al They also examined the relationship between 463 teenagers' substance use (including smoking, drinking, and cannabis dependence) and the genotypes Val108/158Met of the COMT and the levels of methylation of its membrane-bound (MB) and soluble (S) promoters. They discovered an association between non-daily smoking and methylation of the MB-COMT promoter (P=0.03). However, their results showed no association between S-COMT promoter methylation with substance use. Moreover, their data indicated the correlation of the Val allele with high rates of MB-COMT promoter methylation[40]. Compared with the study by Xu et al. and Knaap et al., the present study had the advantage of being performed on more CpG sites (61 CpG sites) among smokers and addicted individuals undergoing methadone treatment, which out of 61 CpG sites, 28 CpG dinucleotides had not so far been studied in smokers as well in the previous study. Out of 61 CpG dinucleotide sites, statistical analysis did not apply to 20 CpG sites due to a lack of methylation in both smoker and control groups. In addition, 16 CpG sites had a P-value of 1.000 because of similar methylation rates among study groups. Finally, in 25 CpG sites, despite a difference in methylation rate, there was no statistically significant difference between the two groups of addicted individuals undergoing methadone treatment and controls, according to Chi-Square test and Fisher's Exact test. After comparison of Xu

et al.'s study and current investigation, it was concluded that the methylation level at all CpG sites in the two groups of smokers and controls among the US population was more than that of the present study (addicted individuals undergoing methadone treatment and healthy controls). Furthermore, CpG No. 4 and CpG No. 39, which had statistically significant differences in the American population, had no statistically significant differences in this study among Iranians at the methylation level. Other epigenetic changing factors, such as type and dosage of addiction, duration of consumption, lifestyle, and any other epigenetic and environmental factors, can explain this.

According to the studies mentioned, the frequency of methylation could differ from the current findings reported for the COMT gene promoter if the methylation of the COMT gene was examined in exons in the current study. The present study's access to brain tissue—the main site of opioid effects in men—was limited, which may help to explain why CpG site methylation levels were of little consequence. Another limitation of this research was that COMT gene expression was not examined. Since hypermethylation of the promoter regions affects gene expression level, the absence of notable changes in gene expression may provide evidence that there is no hypermethylation. All of the subject were male and there might be sex-specific methylation pattern for studied region.

Based on sub-analysis data and other reports, there is a correlation between methadone treatment and libido dysfunction that could be considered as one of the side effects of opium treatment with methadone [41]. Previous studies demonstrated that libido dysfunction may be linked to genetic variations among opium-addicted individuals undergoing methadone treatment [42, 43]. Also, higher rural opium-addicted individuals may have been influenced by factors like low educational attainment, poverty, unemployment, high-risk behaviors, and limited resources for prevention, treatment, and recovery [44].

6. Conclusion

Finally, concerning the review of desired CpG sites in the COMT gene promoter region between studied groups of smokers, addicted individuals undergoing methadone treatment, and healthy people, the present study represented the methylation in some CpG sites, but this difference was not statistically significant. Therefore, it is suggested to carry out these studies on a larger population and investigate promoter methylation patterns in other genes influenced by illegal drugs and nicotine. In addition, promoter methylation patterns can be examined in all parts of the COMT gene, including exons. Given that the brain is the main area for eliciting drug actions and COMT gene expression, it is suggested to examine the methylation and expression of such genes in mouse brain samples.

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8. Conflict of Interests

The authors report no conflict of interest to disclose.

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