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Original Article

Promoter Methylation of Two *HOXA9* and *NISCH* Genes in Opium Users

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Abstract

Background: Opiate abuse has been critically increased in the world, especially in Iran. Owing to the association of opiate use with multiple human cancers and neurological disorders, seeking for genetic and epigenetic effects of opium can pave the way for early diagnosis of major health defects in addicted users. Accordingly, the present study aimed to determine the methylation status of the promoter of two genes, which are actively involved in neurodevelopment and cancer evolution.

Methods: DNA was isolated from peripheral blood of 28 opium abusers and 19 healthy controls and then subjected to sonication. Sonicated DNAs undergone methylated DNA immunoprecipitation-real time polymerase chain reaction (MeDIP-Real Time PCR) using specific primer pairs designed for *HOXA9* and *NISCH* genes. Obtained data were analyzed using SPSS software.

Findings: *HOXA9* and *NISCH* genes were found to be significantly methylated in addicted users compared to controls ($P<0.001$) which was significantly associated with the mean of the age regarding *HOXA9* gene ($P=0.002$). Neither opium amount nor duration or route of using was associated with the methylation status of *HOXA9* or *NISCH* genes.

Conclusion: Hypermethylation of *HOXA9* and *NISCH* genes as tumor suppressor in opium-addicted individuals can be considered as confirmatory evidence for carcinogenesis of opium. Further studies are required to figure out the role of epigenetic alterations in cancer evolution among opium users.

Keywords: HOXA9, NISCH, Promoter methylation, Opium, MeDIP-real time PCR

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Introduction

Opiate abuse is a worldwide health and social problem. According to the latest report of the United Nations Office of Drug and Crime (UNODC), the frequency of opium and heroin users just doubled in the previous decade (2010-2019) and reached around 32 million individuals all over the world.[1] Although, illicit use of opium or its derivatives is mostly limited to some geographical areas, more than half of the world's opioid abusers live in Southwest Asia and South Asia.[1,2] Iran has the highest frequency of opium abusers in the world which might be due to sharing common geographical borders with Afghanistan, as the main producer of opium. Moreover, Iran is the most important route for transportation of opium to Europe.[3] Several studies have shown a positive association between opium use and the risk of respiratory diseases as well as laryngeal, esophagus, bladder, pancreatic, and stomach cancers.[4-7] In addition, devastating effects of long-term consumption of opium would be associated with defects in mesocorticolimbic dopamine system functions leading to some behavioral and serious psychological abnormalities.[8] Recently, epigenetic modifications induced by long-term use of opium and opioid have attracted great attention to find the mechanisms involved in predisposing opium addicts to cancers and neurological abnormalities especially those involved in psychological dependence.[9]

DNA methylation is one of the main epigenetic mechanisms of gene expression control whose aberrations within different genes have been shown to play a pivotal role in evolution of human cancers and various neurological diseases.[10] In this regard, identification of the abnormal pattern of hyper- or hypomethylation of the genes in opium abusers can disclose the possible

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mechanism of human cancers and neurological dysfunctions.

Homeobox A (HOXA) cluster genes, located on chromosome 7p15, serve fundamental roles in the development of normal organs such as central nervous system, axial skeleton, gut, and hematopoietic and urogenital systems.[11] HOXA9, as a member of HOXA family, encodes a DNA-binding transcription factor that is proposed to regulate gene expression, morphogenesis, and differentiation. Hypermethylation of *HOXA9* gene promoter has been recently found to be useful in the early diagnosis of various types of cancer including hepatocellular carcinoma (HCC), high-risk non-muscle invasive bladder cancer, as well as lung and cervical cancers.[12-16] Moreover, the association of *HOXA9* gene methylation and smoking has been demonstrated in various case-control studies.

Nischarin *(NISCH)* is another gene found to be hypermethylated and downregulated in the tissue and circulating cell-free DNA of lung, thyroid, ovarian, and breast cancers.[17-19] *NISCH* gene encodes for a nonadrenergic imidazoline-1 receptor protein which has been shown to play an important role in cytoskeletal organization, cell migration, neuronal growth, and differentiation. Interestingly, a significant association was found among *NISCH* gene expression, methylation, smoking, morphine tolerance, and physical dependence.[20,21] Owing to the limited studies on the effect of opium abuse on DNA methylation, the present study was conducted to identify the methylation status of CpG islands within the promoter regions of two *HOXA9* and *NISCH* genes in opium-addicted individuals in comparison to a healthy control group.

Methods

Case selection

A total of 28 cases were selected from among addicts who visited a private addiction treatment center in Tehran, Iran. The included samples were newly diagnosed and had not received any treatment for opium abuse. Cases with any history of medical and psychiatric diseases or other drug abuse (self-report and urinary testing) were excluded from the study. A total of 19 healthy controls were randomly selected from among healthy non-addicted and non-smoking residents of the same county.

DNA isolation and preparation

Genomic DNA was isolated from fresh peripheral whole blood samples using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The quality and quantity of extracted DNAs were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Ten nanograms of isolated genomic DNA was subjected to sonication using ultrasonic processor UP50H (Hielscher, Germany) and then enriched according to the previously described protocol.[22] Enriched sonicated DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, PH: 8) including 20 µg/mL RNAse A (Thermo Scientific, US) to avoid pseudo-detection of 5-methylcytosine in the RNA sequences.

Methylated DNA immunoprecipitation-real time polymerase chain reaction (MeDIP-Real Time PCR)

MeDIP reaction was performed on enriched DNAs using EpiQuik MeDIP Kit (EpiGentek, USA) according to the manufacturer's instructions. The real-time quantitative PCR was then carried out to amplify a part of the first CpG island sequence of *NISCH* and *HOXA9* genes promoter using their corresponding specific primer pairs. The *H19* and *GAPDH* genes were selected to be amplified as positive and negative internal control for methylated and unmethylated sequences, respectively (Table 1). The quantitative amplification reaction included 5 µl of 2x SYBR Premix Ex Taq master mix (TaKaRa Bio Inc., Japan), 5 ng of DNA, and 10 µM of each primer adjusted with ddH2O up to the total volume of 10 µl. Amplification was performed on a Rotor Gene 6000 Real-time machine (Corbett, CA) and the cycling condition was optimized through two steps including 10 seconds of holding at 95°C followed by 40 cycles of denaturation at 95°C for 10 seconds and polymerization and extension at 60°C for 20 seconds. Melt curve analysis was performed following each run of amplification to confirm the specificity of the reaction which included ramping temperature from 57°C to 95°C by the rate of 1°C per second.

Statistical analysis

The statistical analysis was performed using SPSS statistical software (IBM SPSS Statistics V22.0, USA). Chi-square, Fisher's exact test, and *t* tests were used to determine the association among different variables of enrolled samples. In all statistical analyses, a p-value of less than 0.05 was considered significant.

Results

The mean age of the participants was 37.41 ± 10.96 and 36.44 ± 9.81 years in the case and control groups, respectively ($P = 0.53$) (Table 2). All the cases used to consume opium every day and the average of the *duration* of opium *consumption* was 97.14 ± 13.75 months. *HOXA9* gene was methylated in 82.1% of addict cases and two healthy controls (Figure 1A). The *NISCH* gene was found to be methylated in 60% of addict cases and only one healthy control (Figure 1B). The *HOXA9* and *NISCH* genes promoter methylation was significantly higher in addicted cases compared to the healthy controls ($P < 0.001$). Unlike *NISCH*, the methylation status of *HOXA9* promoter was significantly associated with the mean age of opium users ($P = 0.002$). Promoter methylation of neither *HOXA9* nor

Table 1. Primer pairs sequences used in Real Time PCR reaction

Gene	Reverse	Forward
HOXA9	5′ CAGCCAGGAGCGCATGTA 3′	5′ GCAGCTTCCAGTCCAAGG 3′
NISCH	5′ GCAGACTAGCAGCAGCAGGG 3′	5′ AAGAAGGCGGGGCCAAGATG 3′
H19	5′ TTGGTGGAACACACTGTGATCA 3′	5′ GAGCCGCACCAGATCTTCAG 3′
GAPDH	5′ CAAGTTGCCTGTCCTTCCT A 3′	5′ CTCTCTCCCATCCCTTCTCC 3′

Table 2. Demographic and Clinical Data of the Case and Control Groups

Variable	Controls	Cases	*P* value
Age, years	36.15 ± 9.61	37.64 ± 11.09	0.4
Gender, no (%)			0.08
Male	19	28	
Female	0	0	
Marital status, no (%)			-
Married	-	12	
Single	-	14	
Divorced	-	2	
Educational degree, No. (%)			-
Elementary School	-	10	
High School	-	17	
BS.c	-	1	
Opium use history			-
Onset age of opium use (years)	None	30.11 ± 9.67	
Opium use time (month)	0	97.14 ± 72.75	
Amount of opium using per day	0	2.69 ± 1.96	
Opium manner, No. (%)			-
Inhalation	None	18 (30)	
Eating	None	9 (15)	
Both	None	1 (1.7)	

NISCH was associated with the duration and amount of opium consumption ($P=0.4$ and $P=0.69$, respectively). The routes of consumption were eating and inhalation in 9 and 18 users, respectively. One of the users consumed opium through both eating and inhalation. The route of consumption was not significantly associated with the methylation status of neither *HOXA9* nor *NISCH* genes (*P* value > 0.05) (Figure 2).

Discussion

Herein, it was found that *HOXA9* and *NISCH* genes were significantly methylated in opium users compared to healthy controls. In addition, *HOXA9* promoter methylation was significantly higher among older addicts while there was no association between *NISCH* gene methylation and the mean age of opium users. To the best of the researchers' knowledge, this is the first report on the association of a gene hypermethylation and mean of the age among opium users. Kozlenkov et al investigated hypermethylation of 454 CpG sites including genes involved in synaptic and axonal functions, which was significantly correlated with the mean age of the heroin users.[23] However, no significant association was found between neither age nor gender of the heroin users and, κ1 opioid receptor (*OPRK1*) gene methylation, which is in line with the results of the present study regarding the *NISCH* gene.[24] Moreover, no association was found between the duration of opium consumption and gene methylation status. Although it was not statistically significant, the hypermethylation of the studied genes in opium addicted persons can be assumed as a long-term effect since the average time of opium consumption was about 97 months. However, those changes should be analyzed and compared in a distinct sample size with short-term duration of use. The significant hypermethylation of mu (μ) opioid receptor (*OPRM1*) gene promoter, which has been frequently studied in epigenetic analysis of opium and opioids, as a short-term effect of opioids (< 10 days), may be indicating the earlier hypermethylation changes induced by opioids.[25] A study showed chronic in-vivo morphine treatment induced DNA methylation in most organs of male mice compared to female mice and this may open a new window towards interaction of sexual hormones and gene hypermethylation mediated by opioids beside age-specific alterations.[26] One of the limitations of the present study, and most previous similar studies, is that only male cases were included; therefore, the methylation status could not be compared in both genders.

It was demonstrated that the methylation of both *HOXA9* and *NISCH* genes was not significantly associated with the amount of opium used by every case. Among methylation studies of opium, opioids, and other substances abuse, a few studies investigated the effect of substance dosage on gene methylation. A positive correlation was found between opioids dosage and methylation of the *OPRM1* promoter gene.[25] Ji et al found a significant correlation between the frequency of heroin use and methylation of *OPRK1* gene promoter among male cases.[24] Two methylated single-nucleotide polymorphism (SNP)-CpG sites were identified in the Dopamine Receptor D1 (DRD1) gene among heroin abusers. However, the correlations between the methylation of the aforementioned sites and the frequency, amount, and duration of heroin use were not evaluated.[27]

In spite of limited studies investigating the role of opium abuse on genome methylation, genetic and epigenetic

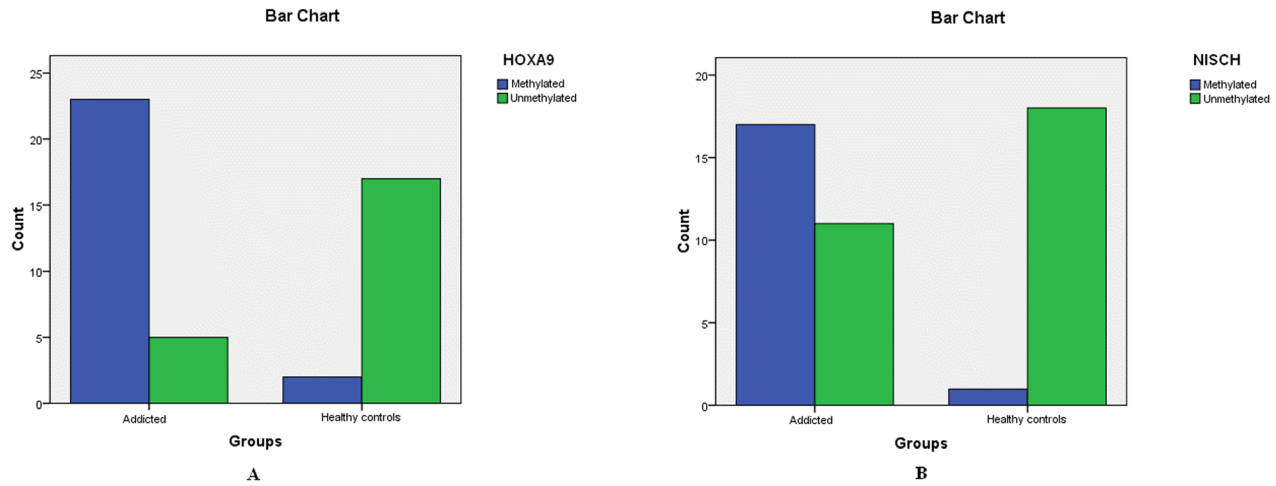

Figure 1. Methylation patterns of HOXA9 (A) and NISCH genes (B) in addicted opium users and healthy controls

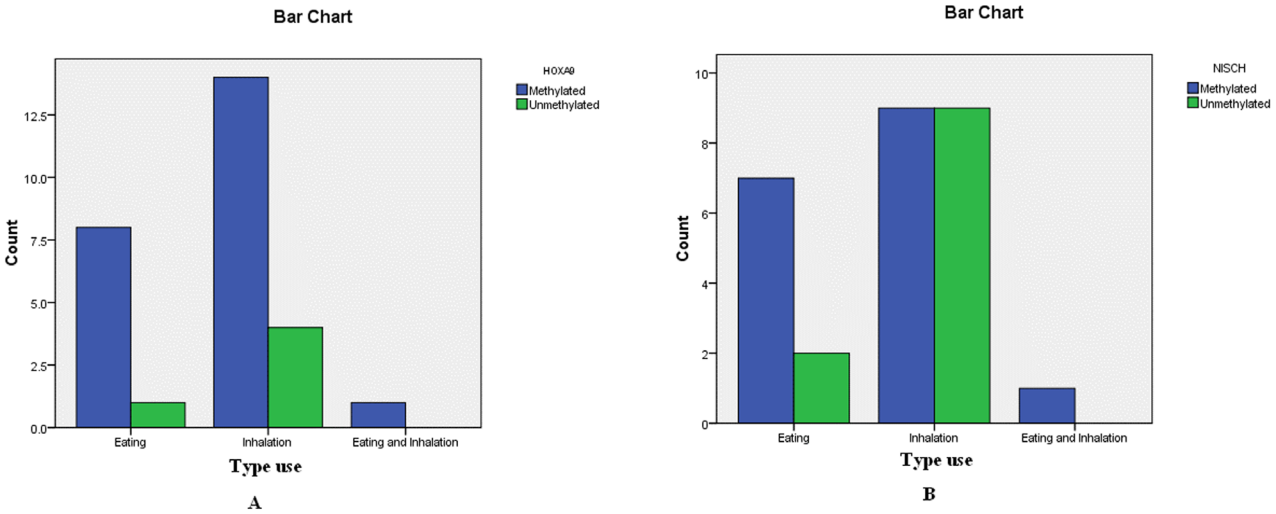

Figure 2. Methylation patterns of HOXA9 (A) and NISCH genes (B) in addicted opium users according to route of consumption

effects induced by opium and opioid have been extensively studied in various types of human cancer, so that the results may change the future of pain management.[7,28,29] In this regard, finding the hypermethylation of *HOXA9* and *NISCH*, as two potential tumor suppressor genes in the present study, presumably is another evidence of the tumor stimulating effects of opium which can be studied in opium-addicted cancer patients as well. Further studies are required to exactly clarify the role of dosage, duration, and frequency of opium use on the methylome of the human genome to figure out their correlation with risk of human diseases as well as various types of cancers.

Conclusion

This study indicated that *HOXA9* and *NISCH* genes promoter methylation was significantly high in addicted users. However, no significant association was detected among methylation status of these genes and the amount, duration, or route of use. Nonetheless, further investigation is required to precisely determine this.

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Authors' Contribution

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Competing Interests

The authors declare no conflict of interest.

Ethical Approval

All enrolled cases and controls filled the consent form according to the Ethical Committee Board of Kerman University of Medical Sciences and ethical standards of the Helsinki Declaration, as revised in 2013 (available at http://www.wma.net/en/30publications/10policies/b3/) (KNRC/95-56/EC).

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