Dopamine receptor D4 promoter hypermethylation increases the risk of drug addiction

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Abstract. Heroin and methylamphetamine (METH) are two addictive drugs that cause serious problems for society. Dopamine receptor D4 (DRD4), a key receptor in the dopaminergic system, may facilitate the development of drug addiction. The aim of the present study was to investigate the association between the promoter methylation level of *DRD4* gene and drug addiction. Bisulfite pyrosequencing technology was used to measure the methylation levels of *DRD4* promoter in 60 drug addicts and 52 matched controls. Significantly higher levels of *DRD4* CpG1 and CpG4 methylation were detected in METH and heroin drug addicts compared with controls (P<0.05). Male METH addicts exhibited significantly higher *DRD4* CpG1, CpG2 and CpG4 methylation levels compared with sex-matched controls (P<0.05). In heroin addicts, a positive correlation was observed between depression-dejection and *DRD4* CpG5 methylation (r=0.537, P=0.039) whereas there was a negative correlation between drug usage frequency and CpG1 methylation (r=-0.632, P=0.011). In METH addicts, methylation levels were not significantly associated with depression-dejection and drug usage frequency. In addition, luciferase assays demonstrated that the target sequence of the *DRD4* promoter upregulates gene expression. The results of the present study suggest that DNA methylation of *DRD4* may be responsible for the pathophysiology of drug addiction.

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Introduction

Drug addiction is a major health and social problem worldwide. The use of heroin (3,6-diacetylmorphine) is associated with the development of drug addiction, even in first-time users, and the symptoms of heroin withdrawal are difficult to endure and often painful (1,2). Long-term use of heroin has severe medical consequences, including scarred veins, bacterial infections of blood vessels, liver and kidney disease and lung complications (3). Methamphetamine (METH) is a novel drug that has increased in popularity over the past few years with an estimated 24 million users worldwide (4,5). It inhibits learning, processing speed and working memory and results in delayed recall (6). It has been reported that METH addiction may also damage dopamine neurons in human brain (7). Heroin was the most commonly used illicit drug in China between 2008 and 2012, followed by METH (8). Intravenous administration is the predominant way in which heroin and METH are used; however, frequent injections coupled with widespread sharing of needles increase the risk of contracting human immunodeficiency virus hepatitis B, C and other blood-borne diseases (3).

Genetic variants have been demonstrated to be involved in the onset (9), development (10) and dependence (11,12) of drug addiction. Furthermore, interactions between genetic and environmental risk factors contribute to the onset and development of drug addiction (13). Environmental risk factors tend to have a greater effect in children with genetic vulnerability (14). As a link between genetics and the environment, epigenetics may serve a crucial role in drug addiction (15).

The dopaminergic pathway, which serves an important role in the reward and reinforcing mechanisms of the brain, may be a target of therapeutic strategies to treat drug addiction (16,17). The dopamine receptor D4 gene (*DRD4*) encodes the D4 subtype of the dopamine receptor, which belongs to the dopaminergic system (18). The alternative methylation of *DRD4* is associated with various mental illnesses (19) and a male-specific association between *DRD4* methylation and schizophrenia has been identified (20). It has been reported that the *DRD4* promoter is hypermethylated in patients addicted to alcohol (21). However, little is known regarding the exact nature of *DRD4* promoter DNA methylation with regards to heroin and METH addiction.

The aim of the present study was to investigate the association between drug addiction and *DRD4* promoter methylation using pyrosequencing in drug addicts and healthy controls.

Materials and methods

Patients and clinical data collection. A total of 112 participants were recruited from the Ningbo Addiction Research and Treatment Center (Ningbo, China, n=60) and Ningbo Blood Bank (Ningbo, China, n=52) from June 2012 to June 2013. A total of 60 drug users, including 30 heroin (mean age, 30.90±0.97 years; male: female, 50:50) and 30 METH (mean age, 31.03±0.99 years; male: female, 50:50) users, were diagnosed by two experienced and professional psychiatrists according to the DSM-IV criteria (22) and the subject who had a history of psychiatric disease, severe disease, and aged less than 18 years or over 65 years was excluded. A total of 52 healthy control subjects (mean age, 30.90±0.73 years; male: female, 27:25) were included in the present study; those with any history of drug addiction or nervous system diseases, including mental illness, were excluded. All male patients were interviewed by the questions based on Profile of Mood State (POMS), which was designed to assess transient, distinct mood states (23). The POMS measures six different dimensions of mood, including tension-anxiety, anger-hostility, vigor-activity, fatigue-inertia, depression-dejection, and confusion-bewilderment over a period of time. A five-point scale ranging from 'not at all' to 'extremely' was used to assess the mood tastes of male addicts. Blood samples were gathered from subjects by the same investigators and all samples were stored at -80˚C with 1-2% EDTA-treated tubes. The present study was approved by the Ethics Committees of Ningbo University and Ningbo Addiction Research and Treatment Center. Written informed consent forms were obtained from all subjects.

DNA methylation assay. Genomic DNA was extracted from peripheral blood samples using a nucleic acid extraction kit (#605003) and a Lab-Aid 820 Automated Blood DNA Extraction system (both from Xiamen Zeesan Biotech Co., lnc., Xiamen, China) and the DNA methylation assay was performed as previously described (24). The following primers were used: Biotin forward, 5'-GGGAGGTTTTGT TAGATATTAGGT-3' and reverse, 5'-CCACCCTAAACC CAATATTTACTCATCTTA-3'; and the sequencing primer, 5'-ACCAAACCAAACCCT-3'. All oligomers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Construction of recombinant plasmids. Two fragments (DRD4L and DRD4 S) from *DRD4* were selected for the present study. DRD4L contained the target sequence whereas DRD4 S did not. The following primers were used for amplification: DRD4L forward, 5'-CTAGCTAGCAGATACCAG GTGGACTAGGGT-3' and reverse, 5'-GGAAGATCTCGG GGAAGGAGAAGAGG-3'; and DRD4 S forward, 5'-CTA GCTAGCTTCAGGGCCTGGTCTGG-3' and reverse, 5'-CTA GCTAGCAGATACCAGGTGGACTAGGGT-3' (Sangon Biotech, Co., Ltd.). A Gel Extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) was used to purify polymerase chain reaction products. The insert segments and pGL3 Basic vector (Promega Corporation, Madison, WI, USA) were both digested with *Nhe*I and *Bgl*II (New England BioLabs, Inc., Ipswich, England), purified using a Cycle Pure kit (Omega, Bio-Tek, Inc.) and the target DNA fragment was cloned into the pGL3 Basic vector using a DNA Ligation kit (Takara Bio, Inc., Otsu, Japan). The pRL-SV40 vector (Promega Corporation), containing the *Renilla* luciferase gene, was used as a reference.

Cell culture and transfection and reporter gene activity assay. Human HEK293T cells, purchased from the Chinese Academy of Sciences (Shanghai, China) were used in the present study. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, Utah) with 10% fetal bovine serum (FBS; TransGen Biotech Co., Ltd., Beijing, China) in a humidified 5% CO_2 incubator at 37˚C. When the cells were in exponential growth phase, they were rinsed twice with phosphate-buffered saline (PBS) and plated on 24-well plates at density of $0.5x10^4$/well in 500 μl DMEM supplemented with 10% FBS. Once cells reached 70% confluence, they were co-transfected with recombinant pGL3 vector and pRL-SV40 using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The medium was replenished 4-6 h following co-transfection. The *Renilla* and firefly luciferase activities were detected at 475 and 560 nm, respectively, using the SpectraMax 190 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) 24 h after co-transfection. A dual-luciferase reporter assay system (Promega Corporation) was used to evaluate gene expression levels according to the manufacturer's protocol.

Statistical analysis. Data are presented as mean ± standard deviation. PASW Statistical 18.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses in the present study. Pearson's χ^2 test was performed to compare categorical variables and Student's t-test was used to compare the differences of the mean values of continuous variables between the two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DNA methylation analysis of the different groups. As presented in Fig. 1, a fragment with five CpG sites was amplified to evaluate the methylation level of the *DRD4* promoter. *DRD4* CpG1 and CpG4 methylation levels were significantly higher in all heroin and METH addicts compared with 52 controls (CpG1, P=0.009; and CpG4, P=0.009; Fig. 2A and Table I). A further subgroup analysis of participants addicted to METH or heroin were compared with controls and elevated levels of *DRD4* CpG1 and CpG4 methylation were observed in heroin and METH users compared with controls (heroin: CpG1, P=0.049 and CpG4, P=0.045; METH: CpG1, P=0.014 and CpG4, P=0.026; Fig. 2B and C and Table I).

Correlation analysis between DNA methylation and sex. Sex differences are an important factor for drug addiction (25). Significantly higher *DRD4* CpG1 and CpG4 methylation levels

Table I. Comparisons of dopamine receptor D4 promoter methylation levels among different groups.

Groups	Subgroups (number ratio)	P-value				
		CpG1	CpG2	CpG3	CpG4	CpG5
Case vs. control						
Total	(60/52)	0.009	0.055	0.120	0.009	0.368
Male	(30/27)	0.030	0.074	0.051	0.015	0.140
Female	(30/25)	0.162	0.410	0.935	0.257	0.992
Heroin vs. control						
Total	(30/52)	0.049	0.214	0.091	0.045	0.643
Male	(15/27)	0.233	0.464	0.202	0.173	0.837
Female	(15/25)	0.129	0.260	0.307	0.153	0.732
METH vs. control						
Total	(30/52)	0.014	0.058	0.485	0.026	0.327
Male	(15/27)	0.041	0.021	0.101	0.024	0.074
Female	(15/25)	0.431	0.253	0.352	0.709	0.731
Heroin vs. METH						
Total	(30/30)	0.658	0.686	0.322	0.982	0.611
Male	(15/15)	0.240	0.226	0.976	0.444	0.112
Female	15/15)	0.435	0.404	0.096	0.288	0.447

METH, methylamphetamine.

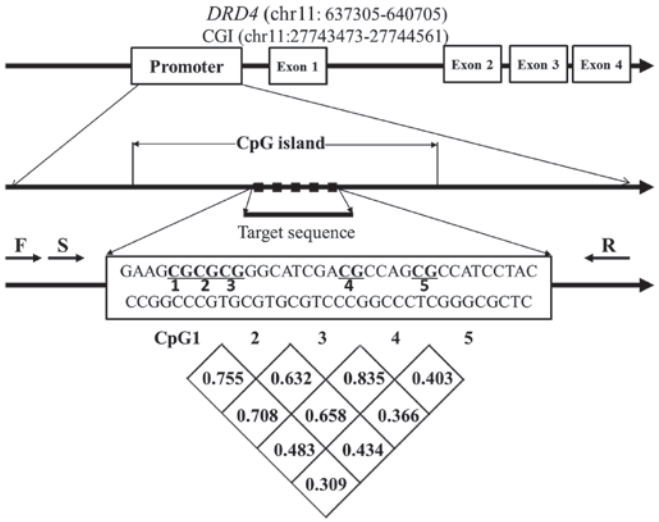

Figure 1. Correlations among five CpG sites in *DRD4* promoter. All of the five CpG sites are located in the target sequence, which makes DRD4 L different from DRD4 S. CGI, CpG island; F, forward primer; R, reverse primer; S, sequencing primer. DRD4, dopamine receptor D4.

were identified in male heroin addicts and METH addicts compared with healthy male controls (male: CpG1, P=0.030 and CpG4, P=0.015; Fig. 2A and Table I). Furthermore, levels of *DRD4* CpG1, CpG2 and CpG4 methylation were significantly higher in METH male addicts compared with male controls (male: CpG1, P=0.041; CpG2, P=0.021 and CpG4, P=0.024; Fig. 2C and Table I). No significant differences in DRD4 methylation levels were observed in male or female heroin addicts compared with sex-matched controls.

Correlation analysis between DNA methylation and phenotypic indices in males. A positive correlation between *DRD4* CpG5 methylation level and depression-dejection was observed in male heroin addicts (r=0.537, P=0.039; Fig. 3). Conversely, a negative correlation between *DRD4* CpG1 methylation level and usage frequency was identified in heroin addicts (r=-0.632, P=0.011; Fig. 3). No significant associations were observed between methylation levels and either usage frequency or depression-dejection in METH addicts.

Functional analysis of target fragment. As presented in Fig. 4A, to further validate the function of the target sequence, the two fragments DRD4 L and DRD4 S from the *DRD4* promoter were selected to insert into pGL3-basic vectors, respectively. Fluorescence activity was significantly higher in cells transfected with DRD4 L fragment compared with cells transfected with DRD4 S (P<0.001; Fig. 4B). Therefore, the target sequence may have the ability to upregulate the level of the expression of downstream genes.

Discussion

In the present study, the levels of *DRD4* methylation in drug addicts and matched controls were evaluated to determine the contribution of *DRD4* promoter methylation to drug addiction. *DRD4* CpG1 and CpG4 methylation levels were significantly higher in cases, including heroin and METH, compared with controls. Significantly elevated levels of *DRD4* CpG1 and CpG4 methylation were also observed in heroin and METH users compared with controls, separately. There were significantly higher levels of *DRD4* CpG1 and CpG4 methylation in

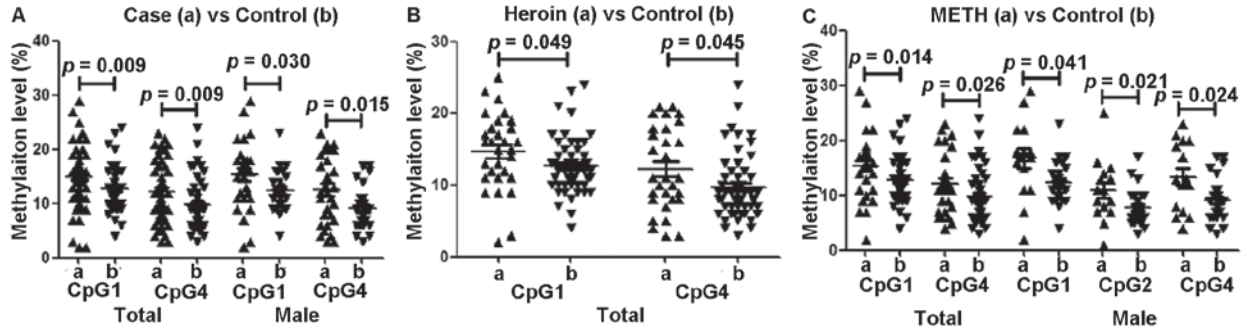

Figure 2. Differences in *DRD4* promoter methylation levels among different subgroups. Methylation levels of CpG1 and CpG4 were indicated in (A) METH and heroin cases. Total group includes heroin addicts and METH addicts and healthy controls. Male group includes total male addicts and male healthy controls. (B) Heroin cases alone compared with respective controls. Total group includes heroin addicts and healthy controls. (C) METH cases alone compared with respective controls. Total group includes METH addicts and healthy controls. Male group includes male METH addicts and male healthy controls DRD4, dopamine receptor D4; METH, methylamphetamine; a, METH cases; b, controls.

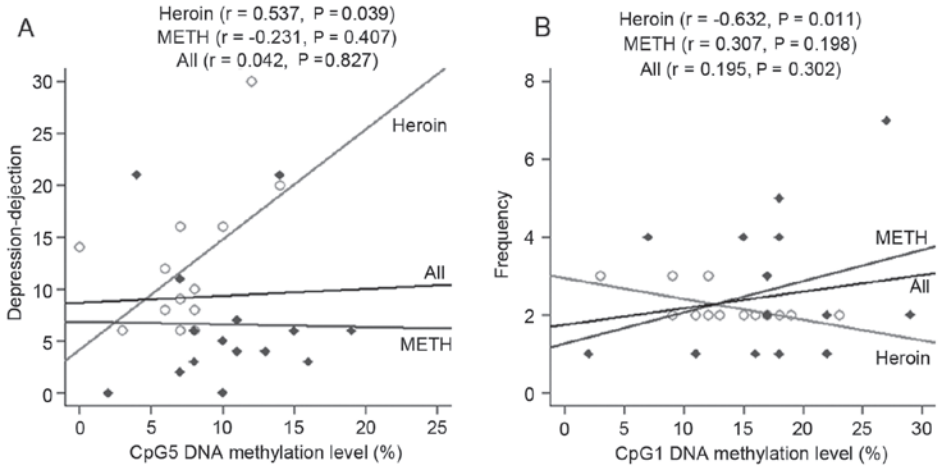

Figure 3. Correlation between *DRD4* methylation levels and phenotypes. Correlation analysis of CpG5 DNA methylation levels with (A) depression-dejection and (B) usage frequency in heroin and METH addicts. Circles and squares stand indicate methylation levels of *DRD4* promoter in heroin and METH addicts, respectively. DRD4, dopamine receptor D4; METH, methylamphetamine.

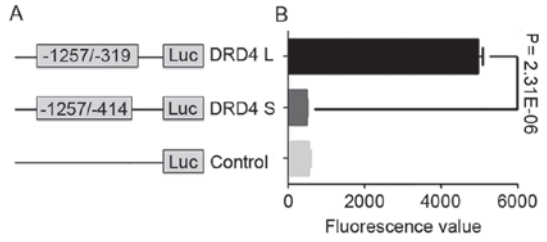

Figure 4. Luciferase assay of the pyrosequenced fragment in the *DRD4* promoter. (A) Locations of DRD4 L fragment and DRD4 S fragment in the *DRD4* gene. DRD4 L indicates the fragment containing the pyrosequenced sequence. DRD4 S indicates the fragment did not contain that sequence. (B) Fluorescence intensities in control, DRD4 S and DRD L.

male heroin and METH addicts compared with male healthy controls. Furthermore, significantly higher methylation levels of CpG1, CpG2 and CpG4 were detected in METH male addicts compared with male healthy controls. In addition, in heroin addicts, a positive correlation between CpG1 methylation and depression-dejection was observed, whereas a negative correlation between CpG1 methylation and usage frequency was identified. Gene promoter methylation level was reversely associated to its expression (26). The subsequent transfection experiment also suggests that the targeted sequence was able to upregulate levels of *DRD4* gene expression.

Drug addiction is a serious problem in many countries, including China. The dopaminergic system serves a crucial role in the reward and reinforcement mechanisms of the brain (16). DRD4 is an important member of the dopaminergic system and the association between the *DRD4* gene and heroin has been reported previously (27). A previous case-control study revealed that a promoter polymorphism (rs936462) of *DRD4* was indirectly associated with heroin addiction via the -521 C/T (rs1800955) polymorphism in promoter (11). DNA methylation is involved in the long-term memory formation (28) and serves a crucial role in a number of mental disorders, including those associated with drug addiction (29). The present study demonstrated that significantly increased CpG1 and CpG4 methylation levels were identified in heroin addicts compared with controls. According to subsequent functional experiments, the target sequence measured in the present study was able to upregulate the expression of downstream genes. The results of the present study indicate that DNA methylation alteration in *DRD4* may be associated with heroin addiction.

Similar to heroin addiction, the etiology of METH abuse is complex. The association between *DRD4* and METH abuse was determined by a previous study (30). In the present study, increased methylation levels of *DRD4* promoter were detected in METH addicts compared with normal controls. Furthermore, significantly higher levels of *DRD4* were identified in male METH addicts following subgroup analysis by sex. The sex of addicts may affect METH addiction. The dopaminergic system is sexually dimorphic and the physiological status and influence of drug abuse of the dopamine system differ between males and females (31). Thus, DNA methylation may serve different roles in males and females; however, the association between DNA methylation and sex requires further study.

Heroin addicts have lower levels of striatal dopamine transporter and more depressive symptoms compared with healthy controls (32,33). A previous study on genome-wide DNA methylation profiling identified that DNA methylation levels were lower in patients with major depressive disorder compared with controls and that the majority of the methylated sites (85.7%) were located on the promoter CpG islands (34). Additionally, a previous study suggested that maternal depression is associated with DNA methylation changes in cord blood T lymphocytes and adult hippocampi (35). In the present study, a positive correlation between CpG5 methylation and depression-dejection was detected in heroin addicts, indicating that the *DRD4* CpG5 methylation level may contribute to the onset of depression in heroin addicts. Depressive symptoms in addicts may be associated with heroin withdrawal, which compared with withdrawal from other opiates, is considered to be more uncomfortable, difficult and painful (1,2). By contrast, a negative correlation was identified between CpG1 methylation and usage frequency in heroin addicts. These observations suggest that the frequency of heroin usage may alter CpG1 methylation.

Although the present study identified a number of meaningful results, there were a number of limitations. Firstly, the sample size was relatively small for the associated study. Secondly, a fragment that contains only five CpG sites in the *DRD4* promoter was tested in the present study and this fragment may not be sufficient to reflect the DNA methylation state of the whole gene. Thirdly, tissue specificity between the peripheral blood and brain still needs to be taken into account, although a strong correlation of gene methylation between the blood and brain has previously been observed (36). Lastly, a functional study of the target sequence on the RNA and the protein expression levels was not performed due to a lack of samples. Further studies investigating the mechanisms regulated by the target sequence are required.

In conclusion, the present study demonstrates that *DRD4* promoter hypermethylation may be associated with heroin and METH addiction. The results suggest that changes in *DRD4* promoter methylation may affect *DRD4* expression and the behavior of drug addicts.

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