Promoter Methylation and *BDNF* and *DAT1* Gene Expression Profiles in Patients with Drug Addiction

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Key Words

Brain-derived neurotrophic factor · Dopamine transporter 1 · Expression · Gene · Methylation · Dopamine · Drug addiction

Abstract

Background: Drug addiction is a brain disorder that has negative consequences for individuals and society. Addictions are chronic relapsing diseases of the brain that are caused by direct drug-induced effects and persevering neuroadaptations at the epigenetic, neuropeptide and neurotransmitter levels. Because the dopaminergic system has a significant role in drug abuse, the purpose of this study was to analyze the methylation and expression profile of brain-derived neurotrophic factor (*BDNF*) and dopamine transporter (*DAT1*) genes in individuals with drug addiction. **Materials and Methods:** *BDNF* and *DAT1* promoter methylation were investigated with a methylation-specific polymerase chain reaction (PCR) technique in blood samples from 75 individuals with drug addiction and 65 healthy controls. The expression levels of *BDNF* and *DAT1* were assessed in 12 mRNA samples from the blood of patients and compared to the samples of healthy controls (n = 12) with real-time quantitative reverse transcription PCR. **Results:** No significant differences were found in the methylation of *BDNF* and *DAT1* between patients and controls, but the relative levels of expression of

BDNF and *DAT1* mRNA differed significantly in the patients compared to controls (p < 0.0001). **Conclusion:** These results showed that the methylation status of the *BDNF* and *DAT1* genes had no significant function in the processes of drug addiction.

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Introduction

Drug addiction is a chronic relapsing disorder that is characterized by compulsive drug seeking [1, 2]. Mechanisms involving dopamine and endogenous opioids have been investigated in many addiction studies [3]. The dopaminergic system has an important role in the reinforcing effects of drug abuse [4, 5]. The following 3 agents have been shown to be involved in the development of addiction: genetic, diverse environmental, and effects of drugs on gene expression or mRNA levels [6]. A recent study has shown that transcription factors, such as non-coding RNAs, histone modifications and chromatin structure, could change the transcriptional potential of genes. These transcription factors also contribute significantly to many neuroadaptations that result from chronic exposure to drugs [7]. Increasing evidence supports the hypothesis that every mechanism of epigenetic regulation

is directly affected by drugs of abuse and that these adaptations are among the main processes through which drugs induce the highly stable changes in the brain that mediate the addicted phenotype [8]. The important epigenetic mechanisms are DNA methylation, histone modifications and small noncoding RNAs [1, 9]. The present study focused on examining epigenetic variations, such as DNA methylation, which is a covalent modification of DNA that involves the addition of a methyl group to cytosine residues in dinucleotide CpG sequences by DNA methyltransferases. This causes gene silencing by several mechanisms. One of these mechanisms is direct interference of the binding to DNA of specific transcription factors, such as AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F and NF- κ B, which recognize sequences containing CpG residues [10]. The CpG-rich regions overlap with the promoters of 50–60% of human genes and are usually methylated more than the CpG dinucleotides that are found outside of islands [8, 11].

In this study, we examined the effects of DNA methylation on the expression of brain-derived neurotrophic factor (*BDNF*) and dopamine transporter (*DAT1*) genes. These genes are important in brain function. *BDNF*, which is a component of the nerve growth factor family, has an important role in the development of the central nervous system and in synaptic plasticity [12, 13]. Increased *BDNF* expression initiates changes in synaptic strength and neuronal signaling. Signal transduction is initiated by the binding of *BDNF* to two different transmembrane receptors: the tyrosine kinase receptor B (Ntrk2 or TrkB) and the neurotrophin receptor. *BDNF* binding to TrkB triggers the ligand-dependent dimerization of the receptor, the autophosphorylation of specific intracellular tyrosine residues and the activation of 3 different signal transduction cascades, including phosphoinositide 3-kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase and phospholipase C γ cascades. Some of these intracellular transduction mechanisms can also be activated by the stimulation of dopamine and glutamate neurotransmission. Some evidence has shown that crosstalk between these pathways might potentiate synaptic plasticity in drug addiction [14]. *BDNF* protein promotes the survival of nerve cells (neurons) by playing a role in the growth, maturation (differentiation) and maintenance of these cells. In the brain, *BDNF* protein is active at the connections between nerve cells (synapses) where cell-to-cell communication occurs. The *BDNF* gene is located in a region of chromosome 11p13 [13, 15]. The *DAT1* gene, which is also identified as *SLC6A3* [16], is located on chromosome 5p15

Table 1. Demographic data of patients and control group

	Patients (n = 75)	Controls (n = 64)	p value
Sex			0.051
Male	41	40	
Female	34	24	
Average age, years	28.11	27.59	>0.50
Education level			
Less than diploma	57	46	–
More than diploma	6	10	–
Unknown	13	8	–
History of substance abuse			
Less than 2 years	38	–	–
More than 2 years	27	–	–

and belongs to a family of Na/Cl-dependent transporters. This transporter is widely distributed throughout the brain in areas of dopaminergic activity. *DAT* is localized in the plasma membrane of axon terminals, and it reuptakes dopamine from the synapse [17] and controls the levels of dopamine in the extracellular space [18–20]. The goal of the present study was to investigate the effects of the promoter hypermethylation of *BDNF* and *DAT* and their expression profiles in individuals with drug addiction.

Materials and Methods

Study Subjects

The case-control study involved the collection of peripheral blood samples (2–4 ml) of 139 subjects (table 1). Seventy-five cases were newly diagnosed and untreated individuals with drug addiction [mean age $\pm$ standard deviation (SD): 28.11 $\pm$ 12.64 years]. The samples were collected from the Bahonar and Afzalipour Hospitals in Kerman, Iran, during 2013. The Ethics Committee of the Bahonar and Afzalipour Hospitals approved this study. It is noteworthy that all used drugs by our cases were part of the opioid class. Our control groups have been selected from individuals without any history of any drug addiction or even drug abuse. Control peripheral blood samples (n = 64, mean age $\pm$ SD: 27.59 $\pm$ 8.45 years) were collected from the same hospitals in an age range similar to our case group.

DNA Isolation, DNA Modification and Methylation-Specific Polymerase Chain Reaction

Genomic DNA was extracted from ethylenediaminetetraacetic acid-anticoagulated peripheral blood samples with the salting-out method from patient and healthy control samples, and its quality was estimated with a spectrophotometer. DNA bisulfate modification was prepared with a Promega kit. The annealing temperatures

Table 2. Primer sequences and annealing temperatures

Genes	Sequences (5'→3')	Annealing temperature, °C	Product size
<i>DAT1</i> M	F: TTTTTCGTTTAGTTGGGAGTC R: GAAAACCTAAAAATCCGAAACGT	51	162
<i>DAT1</i> U	F: TTTTTCGTTTAGTTGGGAGTTGT R: CAAAACCTAAAAATCCAAAACATC	58	162
<i>BDNF</i> M	F: GTTTTGTGGGTAATTAGTGGTTC R: AATCTCCAACCGATAAAATCGTA	53	192
<i>BDNF</i> U	F: TTTTGTGGGTAATTAGTGGTTTGT R: AAAATCTCCAACCAATAAAATCATA	58	193
<i>DAT1</i> (real time)	F: CTCTGCGAGGCGTCTGTT R: AGCTGGAGAAGGCGATCAG	60	89
<i>BDNF</i> (real time)	F: TAACGGCGGCAGACAAAAGA R: GAAGTATTGCTTCAGTTGGCCT	60	101

M = Methylated; U = unmethylated; F = forward; R = reverse.

Fig. 1. Methylation analysis of the *DAT* gene. Lanes U and M: amplified products with primers recognize unmethylated (U) and methylated (M) sequences (162 bp); lane L: DNA ladder (100 bp). **Fig. 2.** Methylation analysis of the *BDNF* gene. Lane U: amplified product with primers recognizes unmethylated sequence (193 bp); lane M: amplified product with primers recognizes methylated sequence (192 bp); lane L: DNA ladder (100 bp).

and primer sequences are listed in table 2. Each polymerase chain reaction (PCR) was prepared in a total volume of 25 μ l, which included 15 μ l of double-distilled water, 2.5 μ l of buffer, 0.5 μ l of Hot Star Taq (Pars Tous Biotechnology, cat. No. 101011, 2.5 U/ μ l), 1 μ l of dNTP mix (10 mmol/l), 2.5 μ l of $MgCl_2$ (25 mmol/l), 1.5 μ l of DNA (80 ng) and 1 μ l of each primer (10 mmol/l). The methylation-specific (MS) PCR amplification program was set as follows: 95°C for 5 min, which was followed by 40 cycles (95°C at 40 s);

annealing at a temperature of 53°C for *BDNF* (methylated primer), 58°C for *BDNF* (unmethylated primer), 51°C for *DAT1* (methylated primer) and 58°C for *DAT1* (unmethylated primer) for 30 s; extension at 72°C for 3 s. The final incubation was completed at 72°C for 10 min. Finally, 10 μ l of each PCR product was directly loaded into nondenaturing 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination (fig. 1, 2).

Table 3. Methylation status of *DAT1* and *BDNF* genes in individuals with drug addiction and healthy controls

Genes	Methylation status	Controls	Cases	p value	OR	95% CI
<i>DAT1</i>	MM	11	13	0.50	0.55	0.13–2.14
	MU	45	47	0.22	0.48	0.15–2.14
	UU	8	15	Ref.	–	–
<i>BDNF</i>	MM	12	8	0.28	0.45	0.12–1.63
	MU	39	48	0.84	0.84	0.33–2.06
	UU	13	19	Ref.	–	–

OR = Odds ratio; CI = confidence interval; M = methylated; U = unmethylated.

Extraction of RNA and Reverse Transcription PCR

First, the buffy coat fraction of blood was separated, and the total RNA was then extracted from blood with a Pars Tous Total RNA Extraction Kit. The RNA concentration was identified spectrophotometrically, and the quality of all of the samples was confirmed by electrophoresis in ethidium bromide-stained 1% agarose gel. One milligram of total RNA was converted to cDNA with a Vivantis cDNA synthesis kit according to the manufacturer's instructions.

Quantitative Real-Time PCR with SYBR Green

Real-time PCR of *BDNF* and *DAT1* was performed in order to examine the quantitative association between the PCR products that were obtained from the target gene and a housekeeping gene (RNA18s). Quantitative reverse transcription PCR assays were performed using the reverse transcription PCR System (Applied Biosystems) with SYBR green fluorescence. PCR amplification was done in 20 µl of the reaction mixture containing 10 µl of 2 × SYBR green, 2 µl of primers (forward and reverse), 2 µl of cDNA and 6 µl of H₂O.

The data analysis was performed with SPSS software version 19.0, and t tests were used to evaluate the association between the methylation status of the *BDNF* and *DAT1* genes and the clinical parameters [target cycle threshold (C_T)/housekeeper C_T]. For all of the tests, the level of significance was set at $p < 0.0001$.

Results

Detection of Methylation with MS-PCR

Methylation was detected in the *BDNF* and *DAT1* promoter genes with MS-PCR. As shown in table 3, there were no significant differences in the methylation percentages of both genes between the patients and controls. Methylation of *BDNF* was found in 75% (56 out of 75) of the patients with drug addiction and in 79.68% (51 out of 64) of the healthy controls. Similarly, *DAT1* gene methylation

Table 4. Comparison of relative gene expression for *DAT1* and *BDNF* between patients with drug addiction and healthy controls

Genes		n	Mean ± SD	Range	p value
<i>DAT1</i>	cases	12	1.20 ± 0.23	0.77 ± 0.35	0.0001
	controls	12	1.77 ± 0.25		
<i>BDNF</i>	cases	12	1.27 ± 0.28	0.87 ± 0.39	0.0001
	controls	12	1.91 ± 0.27		

was found in 80% (60 out of 75) of the patients and 87.5% (56 out of 64) of the controls. These differences were not statistically significant between the patients and healthy individuals in comparison to the unmethylated pattern.

Gene Expression Analysis

The analysis of relative gene expression (target C_T/housekeeper C_T) for *BDNF* and *DAT1* was done by normalizing the target C_T to housekeeping C_T genes. As shown in table 4, there was a significant difference between the patients and healthy controls in a Mann-Whitney test ($p < 0.0001$).

Discussion

Our results showed that the promoter methylation variations of the *BDNF* and *DAT1* genes were not significantly different between patients with drug addiction and healthy controls.

A number of studies have focused on the opioidergic and dopaminergic mechanisms of addiction [3]. Drug abuse plays an essential role in drug reward and reinforcement by activating the mesolimbic dopaminergic pathway [21]. Novel data have detected that genes that encode molecules that are involved in the dopaminergic system (dopamine receptors, transporter and enzymes) are linked to drug addiction. The methylation status of the promoters of some of these genes have been implicated in substance addiction, but this analysis was not performed for the dopamine transporter gene [22].

BDNF is an important regulator of the mesolimbic dopamine pathway, and this neurotrophic factor is expressed at high levels by dopaminergic neurons [23–25]. *BDNF* is a critical regulator of drug dependency. Certain studies in drug addiction, schizophrenia and Parkinson's disease have shown that *BDNF* regulates the expression of the dopamine D₃ receptor [26, 27].

Studies of the DNA methylation profiles of the CpG island of the *BDNF* gene have shown that this gene could be useful as a biomarker of major depression [13, 28]. The *BDNF* gene has been implicated as a susceptible locus for addiction to multiple drugs of abuse, such as alcohol. In addition, a small nucleotide polymorphism in this gene has been linked with an early onset of alcoholism [29]. The results of a study by Lubin et al. [30] have shown that DNA methyltransferase enzyme inhibition causes *BDNF* DNA demethylation, which leads to increased *BDNF* mRNA levels in the hippocampus in vivo.

DAT1 has a crucial function in the regulation of dopamine neurotransmission by drawing dopamine back into the presynaptic neurons and terminating its action. This transporter is encoded by the *SLC6A3* gene [13, 31]. Recent studies have indicated that epigenetic mechanisms are directly involved in the molecular mechanisms that influence the genetic events that are involved in development as well as in the heritability of addiction to drugs of abuse [32, 33].

Zhou et al. [34] have shown that drugs and alcohol are able to change the expression of genes through modifications of histone complexes in the human hippocampus.

Liu et al. [35] have also indicated that epigenetic mechanisms can inhibit genes, such as prolactin, through the activation of the dopamine D₂ receptor, which results in histone deacetylation. Frieling et al. [36] have observed hypermethylation of the dopamine D₂ receptor promoter in blood cells in individuals with drug addiction compared to healthy controls.

This study found no significant differences in the methylation of the *BDNF* and *DAT1* gene promoters in these two groups. Nevertheless, there were remarkable differences in the expression of the *BDNF* and *DAT1* genes in patients with drug addiction. These findings suggest that further investigation of other epigenetic agents are needed to highlight the exact reason underlying the change in gene expression.

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