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Analysis of CTLA4 Gene Methylation and Its Expression in Individuals with Drug Addiction

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A B S T R A C T

Substance abuse is known as a relapsing and chronic disorder. Drug abuse has a significant role in the dopaminergic system in which dopamine is an important neurotransmitter. Binding dopamine and dopamine receptors could activate major intracellular signalling through protein kinase A (PKA). Activation of intracellular signalling such CREB affect gene expression of some genes. As dopamine and dopaminergic system have an important regulation role in the inhibition of immune system we analysed the effects of drug addiction on the DNA methylation and expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4). We evaluate promoter methylation and mRNA expression of CTLA-4 in individual with drug addiction. DNA was extracted from blood of 74 patients with drug addiction and 59 healthy controls. Methylation of CTLA-4 promoter was assessed using a methylation-specific PCR technique. In addition the expression level of CTLA-4 was also indicated in 17 individuals with drug addiction and 18 people of controls. Although Promoter Methylation of CTLA-4 was not significantly different between these two groups, expression of CTLA-4 was remarkably different among them. We observed that each one of the drugs can increase the mRNA expression of CTLA_4. Therefore, we suggest that further research for other epigenetic factors is required to detect the underlying cause of changing gene expression.

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Introduction

Drug addiction is known as a chronic and relapsing disorder characterized by compulsory drug seeking (Vandaele and Janak, 2017; Vassoler and Sadri-Vakili, 2014). Many studies have investigated the mechanisms involved in dopamine and endogenous opioids (Smith and Laiks, 2017). One of the most important role of dopaminergic system is reinforcement the effects of drugs abuse (Volkow et al, 2004). Three factors play a part in the extension of substance abuse. These are genetic and environmental factors as well as the effect of narcotics on gene expression (Kreek et al, 2012). Increasing evidence emphasize the hypothesis that drug abuse directly affects every mechanism of epigenetic regulation and these adaptations are one of the main pathways in which narcotics induce extremely stable changes in the brain to mediate drug abuse phenotypes (Robison and Nestler, 2011). The main epigenetic mechanisms are small noncoding RNAs, histone modifications and DNA methylation (Vassoler and Sadri-Vakili, 2014; Abreu-Villaça et al, 2017). A methyl group was added to cytosine residues in dinucleotide CpG sequences by DNA methyltransferases. In such DNA methylation and covalent binding of

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methyl group, genes are silenced by several mechanisms.

Drug abuse with different mechanisms can increase dopamine in the synaptic space. When dopamine stays in synaptic space for a longer than normal period, it causes the binding of dopamine to dopamine receptors on the postsynaptic neuron for a long time. Thus, cAMP activates protein kinase A (PKA). The majority of intracellular signals like CREB and MAPK are activated by PKA. Furthermore, this activated signaling has an effect on the DNA and alters the expression of some genes. Such effects can be genetic or epigenetic. Recent evidence shows that the drugs can affect gene expression through epigenetic mechanisms (Ritz et al, 1987).

Dopamine is one of the fundamental neurotransmitters in the central nervous system (CNC). This neurotransmitters and its Neuronal pathways have several key functions in behavior (Hefco et al, 2003), endocrine regulation, immune response and control of movement (Fiserová et al, 2002). Diseases such as Parkinson and schizophrenia that revealed hypo and hyperactivity of central dopamine system are well-associated with severe malformation of immune missions (Birtwistle et al, 1988; Muller et al, 1993).

CTLA-4, Located on chromosome 2q33, is a single-spanning membrane protein that is positioned on the surface of T-cell molecule which interacts with the co-stimulatory molecule CD28 (Brunet et al, 1987). It should be noted that CTLA4 has a major role in peripheral control in immune respond (Doyle and Mamula, 2001). The functional magnitude of CTLA4 is characterized in knockout mice (CTLA-4-/-), which increases lethal self-reactive lymph reproducible disease. It seems likely that decreasing or increasing the expression of CTLA-4 might cause problem in immune system (Tamandani, Hashemi, and Shafiepour, 2013; Colucci et al, 1997) such as Tregopathies , hypogammaglobulinemia and other various autoimmune diseases, and lymphocytic infiltration into multiple organs(Noriko Mitsuiki et al, 2018).

Previous evidence indicated that dopamine and dopaminergic system have an important regulation role in the inhibition of immune system (Kordi-Tamandani, Tajoddini, and Salimi, 2015).

The present work aimed study the effects of drug addiction on the DNA methylation and expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4).

2. Materials and methods

2.1. Subjects

A total of 133 participants were recruited from the Afzalipour and Bahonar Hospitals in Kerman, Iran during 2012-2013. This case-control study was performed on blood samples (4 mL) of all subjects. 74 cases were newly diagnosed who persons have drug abuse [n = 74, mean ± standard deviation (SD) age: 28.11 ± 12.64]. All of them used opioid drugs at least for 2 years. The study was approved by the Ethics Committee of Afzalipour and Bahonar Hospitals. The clinical information including type and duration of drugs that used by patients, age and other related demographics was previously described17. It is important to mention that all patients used drugs that were part of the opioid class. Control peripheral blood samples [n = 59, mean ± SD age: 27.59 ± 8.45] were collected from the same hospitals and patients that were in the similar age range. These people were selected very carefully and without history of any substance abuse or even drug and alcohol addiction. They or a guardian (for children) also filled a "General information form", where participants are children, a parent or guardian.

2.2. DNA extraction and modification

Genomic DNA was extracted from peripheral blood samples with the salting-out method. Quality of DNA was estimated using spectrophotometery method. Modification of DNA bisulfite was performed using a (Promega kit, Germany cat#N1301) according to the manufacturer's protocol.

2.3 Methylation-Specific PCR (MSP)

To performed DNA methylation assay, methylated and unmethylated primers of preferred promoter sequence were designed by Perlprimer V1.1.21. Primers sequences and annealing temperatures are listed in Table 1. Each 25 µL PCR reaction was contain to the following construction: 15 µL of double distilled water, 2.5

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µL of buffer, 0.5 µL of Hot StartTaq (Pars Tous Biotechnology, Iran, cat#101011, 2.5 U/µL), 1 µL of dNTP mix (10 mmol/L), 2.5 µL of MgCl2 (25 mmol/L), 1.5 µL of DNA (80 ng), and 1 µL of each primer (10 mmol/ L). The MS-PCR Amplification program was set as follows: 94°C for 5 min, followed by 35 cycles of denaturation at 94˚C for 40 seconds, the variable step of cycle was 52˚C for methyl primer and 50˚C for unmethyl primer annealing for 30 seconds and extension step was at 72˚C for 30 seconds. The last extension was completed by incubation at 72°C for 10 min. Finally, 10 µL of each PCR product was directly loaded in 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination (Figure 1).

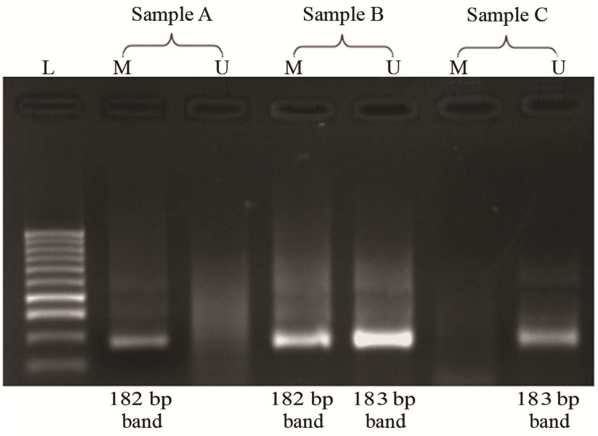


Fig. 1- Methylation analysis of the CTLA-4 gene. Lane U: Amplified product with primers recognize unmethylated sequence (183 bp) Lane M: Amplified product with primers recognize methylated sequence (182bp) L: DNA ladder (100pb)

2.4 RNA Extraction and Reverse Transcription PCR

RNA extraction was performed as previously describe by Kordi-Tamandani et al. 2015. The Vivantis Synthesis Kit (Fermentas, cat# K1621) was used to reverse transcription 1 mg of RNA to 20 µL cDNA by (M J Mini Termal Cycler, Iran, Serial N#PTC1148EDU).

2.5 Quantitative real-time PCR with SYBR green

To assay the quantitative expression between the target (CTLA_4) and a housekeeping gene (RNA18s) Real-time PCR was performed for both groups (17 participants in case and 18 participants in control group). The sample size for gene expression analysis is too low but it made by limitation of the study. Quantitative Real Time-PCR assays were executed using the Real Time-PCR System (Applied Biosystems Step one, USA Serial N: LS4376357). Total volume of each reaction was 20µL; included 6µL of double distilled water, 10 µL of 2X SYBR green, 2 µL of cDNA, and 1 µL of each primer which were indicated in Table 1. For confirmation gel electrophoresis pattern and melting curve analysis provided to ensure the absence of non-specific amplification.

SPSS software version 21 was used for data analysis and t-test was used to measure the association between the methylated status of the CTLA_4 gene and the clinical parameters. The level of significance was set at $P < 0.0001$ for all tests. [Target cycle threshold (CT)/housekeeper (HK) CT].

Table 1- Primers sequences and annealing temperatures

product	Sequences (5′ → 3′)	Annealing temperature (°c)	Product size (bp)
CTLA-4 M	F:GAGATTAGTTTGGTTAATATGGCGA R:CCAAATTAAAATACAATAACGCGAT	52	182
CTLA-4 U	F:GAGATTAGTTTGGTTAATATGGTGA R:CCCAAATTAAAATACAATAACACAAT	50	183
CTLA-4 (real time)	F:CACAAGGCTCAGCTGAACCT R: AGGTGCCCGTGCAGATGGAA	60	226

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3. Results

3.1. Detection of DNA Methylation using MS-PCR

MS-PCR detected methylation in the CTLA_4 promoter gene. As shown in table 2, there is no significant difference regarding the percentages of both methylation genes between the two groups. Methylation of this gene was found in 67.56% (50 out of 74) of the patients with substance abuse and in 77.96% (46 out of 59) of the healthy controls. Statistically, these differences were not significant between the cases and healthy controls in comparison to the unmethylated pattern.

Table 2- Methylation status of CTLA-4 gene in patients and healthy controls

Gene	Methylation status	Controls	patient	P value	OR	CI 95%
CTLA-4	MM	16	14	0.28	0.52	0.18-1.52
	MU	33	23	0.39	0.65	0.27-1.55
	UU	14	22	Reference	-	-

3.2. Analysis of Gene Expression

The analysis of gene expression (for CT/HK CT) in CTLA-4 was carried out by normalizing the CtTarget to CtHousekeeping genes by ΔΔCt analysis method. We observed significant differences among the two groups based on Mann-Whitney test (see table 3) ($P < 0.0001$). No differences were observed in the results based on different drug types used by patients. We declare that the same class of drugs can cause the same resuFlts. Drugs related to opioiFd class can increase the gene expression of CTLA4.

Table 3- Comparison of gene expression for CTLA-4 between case and control

Genes	participants	No.	Mean ± SD	Range	P_ value
CTLA-4	cases	17	.300±0.308	0.893±0.427	0.0001
	controls	18	1.961±0.463		

4. Discussion

In recent study we did not observe a significant difference concerning promoter methylation of CTLA-4 between the two groups. Nevertheless, expression of CTLA-4 is increased in case group in comparison to control group. This finding is similar to a previous study that examined BDNF and DAT genes (Kordi-Tamandani, Tajoddini, and Salimi, 2015).

As epigenetic mechanisms can inhibit genes expression, alcohol and drugs through histone complexes modifications in the human hippocampus could change expression of genes (Zhou et al, 2011; Liu et al, 2005). Frieling et al compared dopamine D2 receptor promoter hyper methylation in blood cells between individuals with substance abuse and healthy controls. Drug abuse has a fundamental function in substance reward and reinforcement as well as activating the mesolimbic dopaminergic pathway (Di Chiara and Bassareo, 2007). Furthermore, Evidence approved that dopamine has an important role in modulating, mainly suppressing immune functions and central peripheral nervous system regulated immune system (Qiu, Peng, and Wang 1996; Basu and Dasgupta, 2000).

Recent study in Parkinson disease, as a dopaminergic disease, indicated that the number of CD+4 lymphocytes, antibody production and T cell activity is declined in blood (Bokor et al, 1992). However, the number of CD+4 lymphocytes is increased in individuals with schizophrenia (Ganguli et al, 1995). In study by Nistico et al, they demonstrated that stimulating the receptors of dopamine in dopaminergic system of brain can stimulate and suppress activity of the NK cells. In the study conducted on animals with a dopamine agonist such as bromocriptine, Hugo et al showed that antibody against LPS and SRBC was product and lymphocytes activities was suppressed in mixed culture of lymphocyte.

Moreover, some studies suggest that neurological disorder with hyperdopaminergic or hypo

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dopaminergic activity participated in CNS dopaminergic system are correlated with substantial alteration in immune response (Basu and Dasgupta, 2000).

In addition the result of interesting study showed that decrease production of antibody in Parkinson could be result of remarkable decrement in the proliferative peripheral lymphocytes response, Concanavalin A (ConA), pokeweed mitogen (PWM) and phytohaemagglutinin (PHA). These disorders correlated with a decrement in the activity of killer cell and decreased the number of CD4+ lymphocyte in the blood (Perez, Guan, and Sutherland, 2016).

In conclusion, we shows that CTL-4 methylation statues was not different between drug abuse and healthy controls, however, expression of CTL-4 have significantly different in two subject groups. The main suggestion from our study is that further researches on other epigenetic factors are required to highlight the underlying cause of changing gene expression.

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بررسی متیلاسیون و بیان ژن های TAP1 و TAP2 در افراد با مسمومیت الکلی

منا موسوی[1]، شهراد تاج الدینی[2*]

چکیده

مطالعات نشان می دهد سو مصرف الکل، پاسخ های ایمنی را مهار می کند و بیماری های عفونی را در افراد الکلی افزایش می دهد ولی هنوز چگونگی این ارتباط به طور کامل مشخص نشده است. گزارشات نشان می دهد که تغییرات اپی ژنتیکی که در اثر مصرف الکل ایجاد می شود، بر بیان ژن هایی که در سیستم ایمنی دخیل هستند، تاثیر می گذارد. هدف از این مطالعه بررسی متیلاسیون و بیان ژن های TAP1 و TAP2 در افراد با مسمومیت الکلی در استان کرمان و مقایسه آن ها با افراد سالم بود. متیلاسیون پروموتر ژن های TAP2 و TAP1 توسط تکنیک PCR مختص متیلاسیون، بر روی DNA 60 بیمار مبتلا به مسمومیت الکلی و ۶۵ فرد سالم مورد بررسی قرار گرفت. بیان ژن های TAP1 و TAP2 نیز بر روی DNA 12c بیمار و ۱۵ کنترل توسط تکنیک Real time PCR بررسی شد. بر اساس یافته های این تحقیق در متیلاسیون پروموتور ژن های TAP2 و TAP1 از لحاظ آماری بین نمونه های خون افراد بیمار در مقایسه با گروه کنترل اختلاف معنی داری مشاهده نشد. علاوه بر این، در بیان ژن های TAP1 و TAP2 بین این دو گروه مورد مطالعه، از لحاظ آماری اختلاف معنی داری مشاهده گردید. این تحقیق نشان داد که مصرف الکل تاثیری در وضعیت متیلاسیون پروموتور ژن های TAP1 و TAP2 نداشته، ولی بیان ژن های TAP1 و TAP2 در نتیجه مصرف الکل کاهش یافته است. بنابراین می توان نتیجه گرفت که مصرف الکل بر روی سیستم ایمنی افراد مصرف کننده، اثر گذاشته است.

واژگان کلیدی: مسمومیت الکلی، اپی ژنتیک، متیلاسیون DNA، سیستم ایمنی، TAP1 و TAP2.

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