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Evaluation of microRNA let-7b-3p expression levels in methamphetamine abuse

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SUMMARY

OBJECTIVE: In this study, we aimed to identify a microRNA expression signature that could be used to distinguish methamphetamine from control samples. We also utilized the existing bioinformatics tools in order to predict the candidate microRNAs that could play potential key roles in regulating drug addiction-related genes.

METHODS: Methamphetamine samples from 21 ventral tegmental area and 21 nucleus accumbens samples and their control regions were obtained from the Council of Forensic Medicine (Istanbul). Quantitative analysis of let-7b-3p was studied using quantitative reverse transcription PCR. Statistical analysis was carried out using Student's t-test. The receiver operating characteristic curves were plotted with Statistical Package for the Social Sciences (SPSS 20.0).

RESULTS: Our quantitative reverse transcription PCR results revealed that let-7b-3p was significantly overexpressed in brain tissues of the methamphetamine-user group. Let-7b-3p had significant power to discriminate methamphetamine from control samples in the ventral tegmental area (AUC; 0.922) and nucleus accumbens (AUC; 0.899) regions.

CONCLUSION: We have shown for the first time in the literature the differential expression of let-7b-3p in samples from methamphetamine-addicted individuals. We suggest that let-7b-3p could be a powerful marker for the diagnosis of methamphetamine addiction. Our results showed that differentially expressed let-7b-3p in methamphetamine users could be used as a diagnostic and therapeutic marker.

KEYWORDS: Methamphetamine. MicroRNAs. Autopsy. Biomarkers. Brain.

INTRODUCTION

Methamphetamine, usually known as "crystal meth", is the most widely used psychoactive component of illicit drugs[1]. Similar to other amphetamines, methamphetamine increases the levels of neurotransmitters such as dopamine and norepinephrine and shows a notably strong serotonergic effect[2]. Methamphetamine is one of the most widely used illegal synthetic drugs, especially in Europe (nearly 15 million users), Oceania, and North America[3]. The European Drug Report has estimated that the availability and use of methamphetamine have increased and the trend will continue to rise. The report also highlighted that the number of methamphetamine deaths has dramatically increased among adolescents and young adults in recent years[4].

Drug addiction is a serious psychiatric disorder that is characterized by loss of control over drug consumption[5]. Addictive drugs converge on the mesocorticolimbic dopaminergic [DA] circuitry, which contains the nucleus accumbens (NAc) and ventral tegmental area (VTA) in the brain's limbic system[6]. Therefore, VTA and NAc regions are the major components of the brain reward system and also play a highly important role in response to drug addiction[7]. Drug addiction induces adjustment in neuroplasticity, which is regulated by permanent alterations in gene expression and protein function in the VTA and NAc[8]. As potent regulators of post-transcriptional gene expression, microRNAs (miRNAs) are poised to play key roles in the addiction-relevant reprogramming of neuronal gene expression in the corticostriatal system[9-11]. miRNAs are a class of non-coding 18–25 bp long nucleotide RNAs that can regulate the expression of hundreds of genes, either by translational suppression or by degrading mRNAs to bind their complementary sequences in the 3′ UTR[12,13]. Recent studies demonstrated that miRNA expression plays a key role in drug addiction in the NAc and VTA

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as well as in other regions of the mesocorticolimbic DA system[14,15]. Furthermore, the impact of drugs such as heroin, morphine, and alcohol on differentially expressed miRNAs has been shown both *in vivo* and *in vitro*. However, the role of miRNAs in methamphetamine-seeking behaviors and the specific targets of key regulatory miRNAs need to be identified. In our previous study, we have worked on unraveling the molecular mechanisms underlying 3,4-methylenedioxymethamphetamine (MDMA)-seeking behavior to develop specific biomarkers of therapeutic approaches in postmortem human brain tissues of MDMA users[16].

In this study, we have utilized diverse bioinformatics resources to predict potential miRNA regulators of drug addiction. Based on our literature search and bioinformatics analysis, we selected let-7b-3p as a top candidate biomarker for methamphetamine-seeking behavior. The family members of let-7 are extremely evolutionarily conserved across various animal species, including flies and mammals. Some known biological roles for let-7b, which was the first defined human miRNA, include the regulation of stem-cell differentiation, cell differentiation, and neuromuscular development. Many studies have shown that let-7b has putative target sites on several addiction-related genes and causes neurodegeneration diseases[17]. The findings of our study highlight a new role of let-7b-3p in methamphetamine-seeking behavior.

METHODS

Postmortem human brain tissue acquisition

The NAc and VTA regions of postmortem human brain tissues were collected from the Morgue Department, Council of Forensic Medicine, Istanbul, Turkey, and local ethical approval was obtained (approval number: 2020/38). The study consisted of 21 subjects (13 males and 8 females) whose deaths were ruled methamphetamine intoxication based on toxicology findings and 21 drug-free control subjects (13 males and 8 females) matched pairwise with methamphetamine users for age and gender. Post-hoc analysis elicited no meaningful differentiation between the study of methamphetamine users and the control subjects for any demographic parameter. The brain samples were collected under full ethical clearance. Cases with a history of poly-drug abuse or other complicating conditions such as HIV/AIDS were excluded. Brain specimens of VTA and NAc were collected as part of the routine autopsy process, and the tissues were flash-frozen in isopentane in liquid nitrogen. The frozen samples were stored at -80°C until further use.

Total RNA extraction

An amount of 50–100 mg of NAc and VTA brain tissues were homogenized in 1 mL TRIzol reagent (Invitrogen, USA). Total RNA was extracted following the manufacturer's instructions. Total RNA concentrations and purities of the samples were determined by spectrophotometry using a NanoDrop ND-2000 system (Thermo Fisher Scientific Inc., Wilmington, DE).

Validation by real-time RT-PCR analysis

Validation of let-7b-3p was studied from the VTA and NAc regions of 21 methamphetamine users and 21 matched controls. Let-7b-3p and RNU43 assays were purchased from Applied Biosystems, Foster City, CA. Single-stranded complementary DNA (cDNA) was synthesized from total RNA using the TaqMan® MicroRNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Following reverse transcription, quantitative reverse transcription PCR (qRT-PCR) reactions were carried out in duplicate using TaqMan® MicroRNA Assays (Applied Biosystems) in a Light Cycler 480-II real-time thermal cycler (Roche, Switzerland, Basel). RNU43 was used for the normalization of miRNA expression analyses. Relative quantification analysis was performed by delta-delta-Ct ($2^{-\Delta\Delta CT}$) calculation as described previously[18].

Statistical analysis

Data presented as means±standard error and Student's t-test (unpaired, two-tailed) were used for statistical analysis of the qRT-PCR. A two-tailed p-value of 0.05 or below was considered statistically significant. The receiver operating characteristic (ROC) curves were plotted using SPSS 21.0 (Statistical Package for the Social Sciences) to determine the power of control and validated let-7b-3p to differentiate between the samples.

Target analysis and miRNA target prediction

We used miRDB (http://www.mirdb.org/), TargetScan 6.0 (www.targetscan.org), and mirTarBase (http://mirtarbase.mbc.nctu.edu.tw/) to identify the predicted let-7b-3p targeting mRNAs with p-value smaller than 0.01 involved in addiction. Using STRING (http://string-db.org/) to demonstrate the interrelationships between genes and their interactive functional networks, we demonstrated the common let-7b-3p targets in protein-protein interaction (PPI) networks.

RESULTS

A total of 21 methamphetamine tissues (21 VTA and 21 NAc) and 21 normal tissues (21 VTA and 21 NAc) were obtained

from the Council of Forensic Medicine, Istanbul, Turkey. We performed qRT-PCR to investigate the expression levels of let-7b-3p in order to determine the differences between methamphetamine and control subjects in VTA and NAc parts of human postmortem brain tissues. The mean age of methamphetamine subjects was 27.61±9.08, whereas controls had an average age of 30.09±10.43 years (Table 1). The methamphetamine group was found to have methamphetamine in the blood ranging from 569.3 to 1025.6 ng/mL with a median of 798 ng/mL (Table 1).

Table 1. Age, brain pH, postmortem interval, and methamphetamine in the blood level of methamphetamine and control postmortem samples that were involved in the study.

	Methamphetamine (n=21)	Control (n=21)	p-value
Age	27.61±9.08	30.09±10.43	0.42
Brain pH	6.61±0.25	6.48±0.23	0.09
PMI	17.38±4.55	16.9±4.47	0.74
Blood level of methamphetamine	798 ng/mL (569.3–1025.6 ng/mL min–max)	–	–

PMI: postmortem interval.

When the two groups were compared in terms of the postmortem interval (PMI) samples, the difference between the mean PMI levels of the two groups was not significant. We investigated the expression profiles of let-7b-3p in methamphetamine samples and controls in VTA and NAc parts of brain tissues using qRT-PCR analysis. The results showed that the expression levels of let-7b-3p in VTA samples and NAc samples in Figure 1A ($p<0.002$ and $p<0.004$, respectively) were significantly upregulated in methamphetamine-addicted brain tissue samples compared to normal tissues.

To test the power of let-7b-3p for distinguishing the methamphetamine group from controls, we plotted ROC curves and the results showed that let-7b-3p had a higher area under the curve for NAc (AUC; 0.899) and VTA regions (AUC; 0.922) (Figure 1B).

The biological process, molecular function, cellular component, and KEGG pathways analysis of the potential targets of let-7b-3p with functional enrichments in the PPI network showed that the genes shown in Table 2 play an important role in the mechanisms of neurexins and neuroligins and in the neuronal system (Figure 1C).

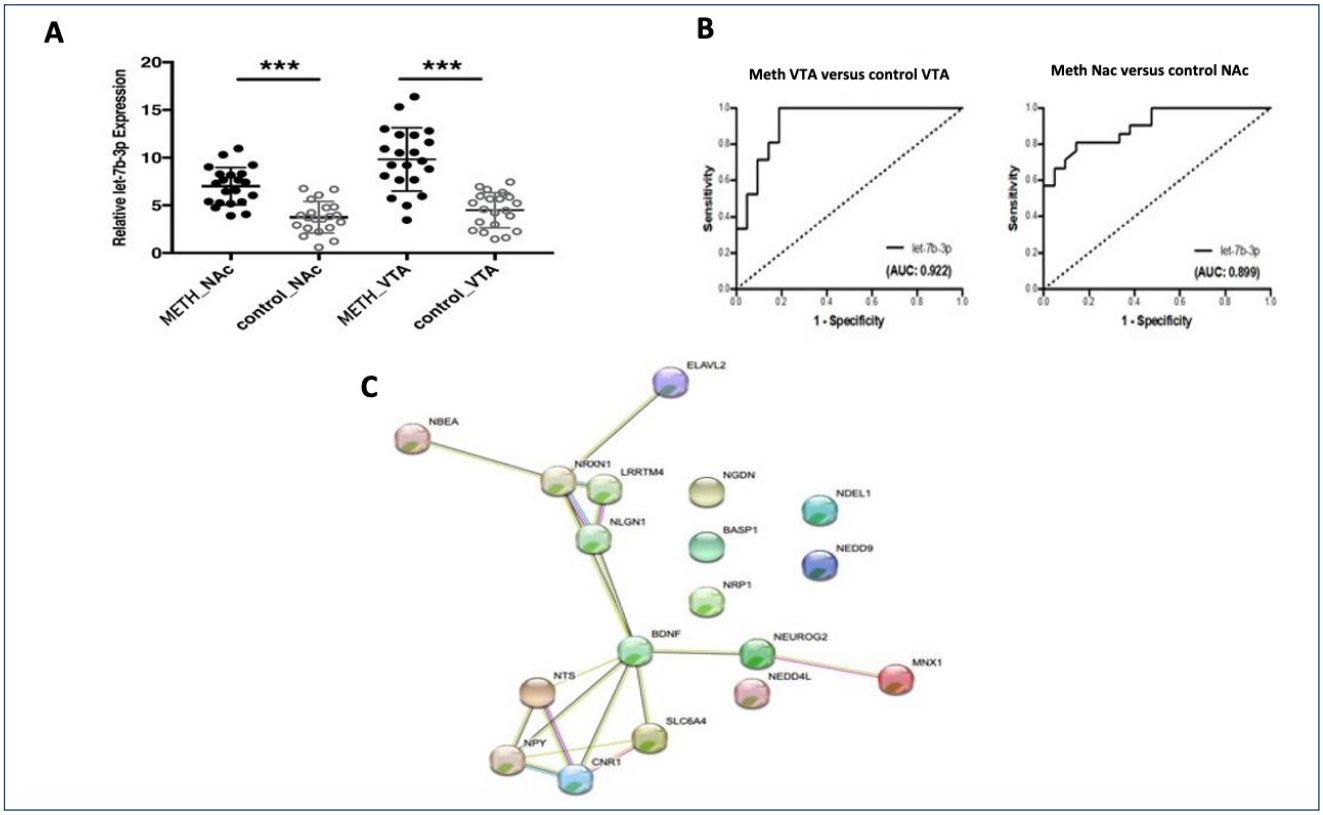

Figure 1. (A) Relative expression levels of let-7b-3p in control versus methamphetamine-addicted brain tissues. (B) ROC analysis of let-7b-3p in methamphetamine versus control samples (NAc and VTA regions). Let-7b-3p cooperative power to discriminate two sets of samples composed of 21 methamphetamine and 21 control samples. (C) PPI network of commonly deregulated let-7b-3p targets. Pink: experimentally determined (known interactions); blue: from curated databases (known interactions); yellow: text mining; green: gene neighborhood (predicted interactions); black: co-expression. The interaction score was set to high confidence of 0.49. ***$p<0.01$.

Table 2. Biological, molecular, cellular functions gene ontology, and Kyoto Encyclopedia of Genes and Genomes pathway analysis of let-7p-3p potential targets for functional enhancements in the protein-protein interaction network.

Pathway ID	Biological process (GO)	Count in network	False discovery rate
	Pathway description		
GO:0048699	Generation of neurons	11	8.43e-06
GO:0031175	Neuron projection development	8	1.54e-05
GO:0030182	Neuron differentiation	9	1.60e-05
GO:0010976	Positive regulation of neuron projection development	6	2.16e-05
GO:0051962	Positive regulation of nervous system development	7	3.63e-05
	Molecular function (GO)		
	Pathway description		
GO:0099106	Ion channel regulator activity	3	0.0177
GO:0005184	Neuropeptide hormone activity	2	0.022
GO:0098772	Molecular function regulator	7	0.0248
GO:0005246	Calcium channel regulator activity	2	0.0248
	Cellular component (GO)		
	Pathway description		
GO:0150034	Distalaxon	6	1.43e-05
GO:0098793	Presynapse	6	2.28e-05
GO:0043005	Neuron projection	9	2.28e-05
GO:0030426	Growth cone	5	2.28e-05
GO:0030424	Axon	7	2.28e-05
	KEGG pathways		
	Pathway description		
GO:6794361	Neurexins and neuroligins	3	0.0016
GO:112316	Neuronal system	4	0.0078

GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

DISCUSSION

Drug addiction is believed to be a neurological dysfunction because altered gene expression affects neuronal function and eventually behavior. Early detection and correct diagnosis are especially important for methamphetamine addiction in the therapy decision-making process. miRNAs have become a popular research subject among scientists in recent years. It has been shown that miRNAs can be used to explain several addiction processes, and determining the levels of miRNAs is proposed as an important approach to overcome therapeutic resistance in drug addiction. They are potential diagnostic and therapeutic biomarkers that have been shown to be present in the use of various types of drugs in recent years[19].

MicroRNAs, which are among the non-coding RNAs, have a critical role in gene expression. The first known human miRNA, *let-7,* is expressed in both embryonic and adult mammalian brains. *Let-7b* serves as a key regulator of neural stem cell proliferation and differentiation. Overexpression of *let-7* elicits neuronal cell proliferation and accelerated neural differentiation[20].

The let-7 miRNA family was identified as a top candidate based on the number of assumed target sites. This study experimentally validated the *in silico* prediction that *let-7b-3p,* which is a member of the *let-7* miRNA family, can interact with methamphetamine addiction.

Downregulation of let-7 was found to increase morphine and related μ-opioid receptor (MOR) expression in a human neuroblastoma cell line. This suggests that MOR is a target of let-7 because the expression of MOR is under constitutive suppression by let-7. Accordingly, morphine treatment causes an increase in let-7. Chronic morphine treatment notably upregulated let-7 expression in sensory neurons and brain stem nuclei[21]. The brain expression of the

level of let-7 increases after morphine treatment, temporally correlating with the development of tolerance to morphine. Treatment with a let-7 inhibitor decreases brain let-7 levels and opioid tolerance. Let-7b has been previously proposed as an important factor for distinguishing morphine exposed from non-morphine-exposed brain tissues[22]. In parallel with this finding, our results showed that let-7b-3p is significantly overexpressed in brain tissues.

Recent studies have identified the role of several miRNAs in mammalian midbrain dopaminergic neurons and that they are related to addictive behaviors. Deregulation of let-7 seems to play a key role in neurological disorders[23]. Also, cocaine addiction affects the expression of let-7d, highlighting the possibility that some miRNAs are important regulators of the brain reward pathway and likely implicated in addiction[21]. Toll-like receptor (TLR) signaling is known to be a key component of neurodegeneration, and TLR7 responds to miRNAs in promoting immune responses leading to neurodegeneration[22]. Moreover, an alcoholic individual's brain contains more ATP (adenosine triphosphate) than a non-alcoholic individual's brain, and alcohol dependence is associated with hippocampal degeneration. Studies of postmortem human alcoholic brain hippocampal tissues have shown that increased expression of TLR7 and let-7b causes neurodegeneration[24].

CONCLUSION

Our findings show that let-7b-3p is differentially expressed in methamphetamine users and let-7b-3p could serve as a potential biomarker for predicting methamphetamine abuse and treatment response. Let-7b-3p has been linked to mechanisms of drug abuse, and further studies would be very important in developing preventive strategies and new therapeutic interventions for methamphetamine abuse.

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AUTHORS' CONTRIBUTIONS

EGT: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation. Methodology, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. **GD:** Conceptualization, Data curation, Funding acquisition, Investigation. Methodology, Software, Supervision, Writing – original draft, Writing – review & editing. **HA:** Conceptualization, Formal Analysis, Project administration, Resources, Software, Supervision, Validation, Writing – original draft.

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