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Increased expression of plasma hsa-miR-181a in male patients with heroin addiction use disorder

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Abstract

Background: Drug addiction is an uncontrolled, chronic, and recurrent encephalopathy that presently lacks specific and characteristic biomarkers for diagnosis and treatment. As regulators of gene expression, microRNAs (miRNAs) are increasingly used for diagnostic and prognostic purposes in various disease states. Previous studies indicated that miRNAs play important roles in the development and progression of drug addictions, including addiction to methamphetamine, cocaine, alcohol, and heroin.

Methods: We identified significant miRNAs using the microarray method and then validated the hsa-miR-181a expression levels in 53 heroin addiction patients and 49 normal controls using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Finally, the potential associations between transcriptional levels in heroin addiction patients and their clinicopathological features were analyzed.

Results: A total of 2006 miRNAs were differentially expressed between heroin addiction patients and normal controls. The top 10 up-regulated miRNAs in patients were hsa-miR-21a, hsa-miR-181a, hsa-miR-4459, hsa-miR-4430, hsa-miR-4306, hsa-miR-22-3P, hsa-miR-486-5P, hsa-miR-371b-5P, hsa-miR-92a-3P, and hsa-miR-5001-5P. The top 10 down-regulated miRNAs in patients were hsa-miR-3195, hsa-miR-4767, hsa-miR-3135b, hsa-miR-6087, hsa-miR-1181, hsa-miR-4785, hsa-miR-718, hsa-miR-3141, hsa-miR-652-5P, and hsa-miR-6126. The expression level of hsa-miR-181a in heroin addiction patients was significantly increased compared with that in normal controls ($P < .001$). The area under the receiver operating characteristic curve of hsa-miR-181a was 0.783, the sensitivity was 0.867, and the specificity was 0.551.

Conclusions: The increased expression of hsa-miR-181a in the plasma of heroin patients may be a consequence of the pathological process of heroin abuse. This study highlights the potential of hsa-miR-181a as a novel biomarker for the diagnosis of heroin addiction.

KEYWORDS
biomarker, diagnosis, Heroin addiction, hsa-miR-181a, substance abuse

1 | INTRODUCTION

Drug abuse is a huge burden on public health and the world economy. As one of the typical representative drugs in addiction, heroin abuse is a chronic and relapsing brain disorder that is characterized by uncontrolled and compulsive drug use and continued high relapse after withdrawal.[1] Long-term abuse of heroin affects the plasticity of the central nervous system, leading to lesions of the central nervous system that develop into chronic encephalopathy.[2] Currently, an effective clinical treatment of heroin use disorder is still not available. Thus, it is necessary to identify reliable biomarkers to improve the diagnosis and treatment of heroin addiction. Such biomarkers would be important in the analyses of heroin addiction, including occurrence, development, and prognosis.

Like other drug addiction disorders, heroin addiction is a chronic recurrent encephalopathy caused by the interaction of individual factors (genetic susceptibility), the drug itself, and social environmental factors.[3-5] Drug abuse can induce changes in neuronal structure and functional plasticity, and changes in molecules and cells. These changes ultimately lead to individual abnormal behavior.[6] However, the molecular mechanisms leading to addiction are poorly understood.

Epigenetic changes leading to the sustainability of gene expression are among the most important mechanisms underlying drug addiction. Epigenetics has provided a new perspective for exploring the nature of drug addiction. An increasing number of studies have indicated the crucial role of epigenetics in drug addiction.[7-9] As one of the primary methods of epigenetic regulation, microRNAs (miRNAs) are a crucial class of endogenous non-coding small (~22 nucleotides) RNAs that regulate negative post-transcriptional repression of gene expression by targeting the RNA 3'-untranslated region to degrade or inhibit the translation of mRNA.[10,11] Through this mechanism, miRNAs can silence or prevent the translation of their target genes. Many studies have demonstrated the involvement of miRNAs in the regulation of addiction-related synapse plastic changes.[12-14] As a major regulator of gene expression, miRNAs are increasingly used for the diagnosis and prognosis of various disease states, such as cancer, cardiovascular disease, and schizophrenia,and neuro degenerative disorders.[15-17] Recently, the role of miRNAs dysfunction in addiction has attracted widespread research attention. Studies have indicated that miRNAs are involved in substance abuse disorders.[10,18] Additionally, animal experiments have shown that exposure to central stimulant drugs that include cocaine and amphetamines changes the expression levels of miRNAs. For instance, miR-124 and let-7 are reportedly consistently down-regulated in the ventral tegmental area and nucleus accumbens (NAc) brain regions in rats exposed to cocaine for 15 days.[12] The role of miRNAs in amphetamine addiction has also been reported. A methamphetamine (MA) self-administration study involving rats identified 28 differentially regulated miRNAs in the prefrontal cortex (PFC) in the controlled MA self-administration group (1 hour, 0.05 mg/kg) and escalated MA self-administration group (6 hour, 0.05 mg/kg).[19] Compared with the saline control group, miR-186, miR-195, and miR-329 were up-regulated in rats with controlled MA use (1 hour, 0.05 mg/kg). Otherwise, miR-127, miR-222, miR-186, and miR-24 in the rats with escalated MA use (6 hour, 0.05 mg/kg) were up-regulated, while miR-329 was down-regulated compared to the controls.[19] Moreover, miRNAs were also shown to be involved in addiction to other drugs, such as alcohol, nicotine, and morphine.[20-22]

The diagnosis of drug use disorders is still dominated by subjective reports in the absence of objective biomarkers. A growing body of research has demonstrated the presence of detectable levels of miRNAs in the blood.[23,24] Since Mitchell et al[25] first extracted and detected free miRNAs in the blood circulation, the important roles of miRNAs in various diseases have been widely applied. miRNAs extracted from the blood are very stable and resistant to RNase degradation, making them ideal potential biomarkers. Recently, plasma-based miRNAs screening has revealed that four miRNAs were significantly down-regulated in patients with MA use disorder.[26] However, there have been few studies of miRNAs in clinical drug addiction, especially heroin abuse.

The possibility of using miRNAs as biomarkers for certain diseases provides a new direction for exploring the biological basis of drug abuse and identifying possible biomarkers for clinical diagnosis and outcome evaluation. We hypothesized that plasma-based miRNAs can be used as useful biomarkers for heroin addiction diagnosis, disease progression monitoring, and prognosis prediction. In this study, we provide the first evidence that hsa-miR-181a is significantly increased in heroin abuse patients. These results indicate that hsa-miR-181a might be a novel biomarker for heroin addiction.

2 | PATIENTS AND METHODS

2.1 | Recruitment of subjects and data collection

We enrolled 53 Chinese male heroin addiction patients from June 2015 to June 2016 at the Ningbo Addiction Research and Treatment Center. All subjects were un-medicated prior to admission. The inclusion criteria were as follows: (a) Han Chinese; (b) no major infectious diseases, other major chronic diseases, or family history of such diseases; (c) informed consent provided after routine outpatient laboratory testing; and (d) positive for heroin addiction by DSM-V diagnosis and urine test. The exclusion criteria were as follows: (a) non-Han Chinese; (b) clear or existing medical history, which could include a history of major infectious diseases; (c) inability to participate in the study or complete informed consent; (d) and significant emotional disorders or mental illness related to heroin addiction, excluding the merger of other drug abuse. Forty-nine patients that were age- and gender-matched were selected as normal controls from August 2015 to June 2016 at the Ningbo Blood Bank. This study was approved by the Ethics Committee of Ningbo Addiction Research and Treatment Center. The general demographic data of these subjects were also collected. We collected detailed information about drug use history (such as use methods, years of drug use, and daily dose) by interview.

2.2 | Plasma sample collection and RNA extraction

A total of 5 mL of blood was collected from each participant. Each sample was placed in an ethylenediamine tetra-acetic acid containing tube and centrifuged at 12 000 × g for 15 minutes within 2 hours of collection. Detection was carried out by visual inspection and spectrophotometry (NanoDrop 2000 instrument; Thermo Fisher Scientific). The plasma was transferred to a new RNase/DNase tube and stored at −80°C.

Total RNA was extracted from 200 μL plasma using the miRNeasy Serum/Plasma Kit (QIAGEN, USA) according to the manufacturer's protocol. RNAs were eluted with 20 μL elution solution. The concentration of all RNA samples was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.3 | Microarray screening of candidate miRNAs

The total RNA of five heroin-dependent patients and five normal controls was extracted from blood samples for miRNA microarray screening. The expression of miRNAs was measured using the Human miRNA Microarray V19.0 (Agilent Technologies, Beijing, China). The array contains probes for a total of 2006 human miRNAs. Sample labeling, microarray hybridization, and washing were performed according to the manufacturer's standard protocols. A total of 173 differentially expressed miRNAs were identified by microarray screening. All these miRNAs showed significantly increased or decreased signals in the miRNA profiling stage ($P < .05$).

2.4 | Real-time quantitative-reverse transcription-polymerase chain reaction of candidate miRNAs

Based on the microarray results and literature review, candidate miRNAs were selected for further validation by real-time qRT-PCR. Complementary DNA was synthesized using the miScript®II RT Kit (QIAGEN, USA) according to the manufacturer's instructions. All reactions were run in triplicate, and results were normalized to those for U6. The sequences of primers were as follows: 5'-AACAUUCAACGCUGUCGGUGAGU-3' for *hsa-miR-181a*, 5'-CTCGCTTCGGCAGCACA-3' (forward primer) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse primer) for U6. Primers were used in conjunction with miScript SYBR Green PCR Kit (QIAGEN). The expression levels of the genes were expressed as $\triangle C_t$ values, which were determined by subtracting the U6 C_t values from the miRNA C_t value as previously described, in which smaller $\triangle C_t$ values indicated higher expression levels of the miRNA.[27]

2.5 | Statistical analyses

All data were analyzed using the SPSS16.0 software package (IBM, USA). Continuous variables were described using mean values with standard deviation or median values and quartiles, depending on whether the data were normally distributed. Student's t test was used to analyze the continuous variables. The categorical variables were analyzed by chi-square test or Fisher's exact test. Pearson or Spearman rank correlation was used to determine the relationship between the expression of hsa-miR-181a and clinical features. The receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic value for differentiating between heroin addiction and normal. GraphPad Prism 5 software was used for graphic rendering (GraphPad Software). A P-value ≤ .05 was considered statistically significant.

3 | RESULTS

3.1 | Demographic characteristics of the participants

The mean ages of the heroin addiction patients and the control group were 33.62 ± 8.22 years and 33.53 ± 8.46 years, respectively. Among the heroin addiction patients, 21 (39.62%) had little education and 34 (64.15%) did not have a stable job. The education level differed significantly among the two groups ($\chi^2 = 10.514$, $P = .005$). However, there were no significant differences in age groups or occupation between heroin addiction patients and the control group (Table 1).

3.2 | Drug use of heroin addiction group

The majority of the heroin addiction patients had a history of heroin use longer than 2 years, accounting for 86.79%. Thirty (56.60%) patients used heroin with a daily dose of more than 1 g.

TABLE 1 Demographic characteristics of the participants

Variables	Control group	Heroin addiction	χ^2	P
Age group				
20-	17	14	1.929	.627
30-	21	21		
40-	10	16		
50-60	1	2		
Education (years)				
≤9	12	21	10.514	.005
912	21	28		
>12	16	4		
Occupation				
Public institution staff	5	4	0.551	.765
Company employee	26	15		
Freedom and others	18	34		

TABLE 2 Drug use of heroin addiction patients

Variables	Cases	Proportion (%)
Duration of use (mo)		
<12	3	5.66
12-24	4	7.55
24-48	12	22.64
48-96	13	24.53
≥96	21	39.62
Daily dose (g)		
<0.5	9	16.98
0.5-1	14	26.42
>1	30	56.6
Usage		
Snorting	26	49.06
Intravenous injection	25	47.17
Else	2	3.77
Frequency of use		
<1 times/d	5	9.43
1-3 times/d	7	13.21
>3 times/d	41	77.36
Frequency of detoxification		
<2	21	39.62
2-4	15	28.3
4-6	3	5.66
>6	14	26.42

Most of the patients took heroin by snorting (49.06%). The drug use frequency of 41 patients (77.36%) exceeded three times a day. In total, 17 (32.08%) patients had been detoxified more than four times (Table 2).

3.3 | miRNA expression profiles of heroin-addicted inpatients relative to control group

The expression levels of miRNAs between the heroin addiction and control groups were significantly different (Figure 1). We selected miRNAs that were significantly differentially expressed ($P < .005$) more than twofold in patients with heroin addiction compared with controls.

3.4 | Expression of hsa-miR-181a is up-regulated in patients with heroin addition

hsa-miR-181a was significantly higher in the heroin addiction group than in the control group ($\triangle C_t$: 8.28 ± 2.03 vs 10.52 ± 1.76, $t = 5.93$, $P < .001$). The finding was consistent with the miRNAs microarray data (Figure 2).

3.5 | Correlation of clinical features with expression of hsa-miR-181a

Pearson correlation or Spearman rank correlation was performed to analyze the relationship of the expression of hsa-miR-181a in each group. The expression of hsa-miR-181a was negatively correlated with daily dose, in the heroin addiction group ($r_s = -0.288$, $P = .037$), but a positive correlation in frequency of drug use was observed ($r_s = 0.294$, $P = .033$) (Table 3). The correlations between hsa-miR-181a expression and other clinicopathological factors of subjects were also analyzed. No statistically significant correlations were detected.

### 3.6 | Potential diagnostic values of hsa-miR-181a

ROC curve analysis was performed to assess the diagnostic values of hsa-miR-181a for heroin addiction patients. The area under the ROC curve was 0.783 (95% $CI = 0.696\text{-}0.869$), with a sensitivity and specificity of 0.867 and 0.551, respectively. This indicated that hsa-miR-181a could act as an effective predictor for heroin addiction patients (Figure 3).

4 | DISCUSSION

At present, there is no effective method for the clinical diagnosis or treatment of heroin addiction with a high relapse rate. The development of simple, rapid, accurate diagnostic, and treatment methods is important and urgently required. Several studies have reported differences in plasma or serum miRNA expression levels between patients with mental disorders and healthy subjects.[28,29] These findings could have important clinical implications. However, the peripheral profile of miRNAs in drug use disorder is not well understood.

miRNAs are highly enriched in the brain relative to other tissues. The number of neuronal miRNAs is amplified with increasing brain complexity across species, particularly in humans.[30] Emerging evidence suggests that brain and peripheral miRNAs can be biomarkers of psychiatric disorders and addiction states.[26,31,32] Therefore, identification of humanspecific miRNAs associated with heroin addiction will have important clinical implications. In the present study, we investigated the peripheral levels of miRNAs in patients with heroin use disorder and found that the expression of hsa-miR181a in male heroin addiction patients was higher than that in the normal control group. Some in vivo and in vitro data partly support our findings and suggest that miR-181a may be involved in drug addiction.[12,33] For example, local knockdown of miR-181a in the nucleus accumbens (NAc) attenuates cocaine-induced conditional position preference in rats. Other studies have shown that miR-181a targets many genes (including *BDNF*, *CREB*, and *D3R*) involved in cocaine addiction, and that miR-124, let-7d, and miR-181a may be involved in a complex feedback loop in conjunction with cocaine-responsive plasticity genes.[12,33] Previous studies have demonstrated that *BDNF*, *CREB*,

FIGURE 1 Alterations in miRNAs expression profiles between patients with heroin addiction and control group. The result from hierarchical clustering shows miRNAs expression profiling among plasma samples. "Red" indicates high relative expression; "blue" indicates low relative expression
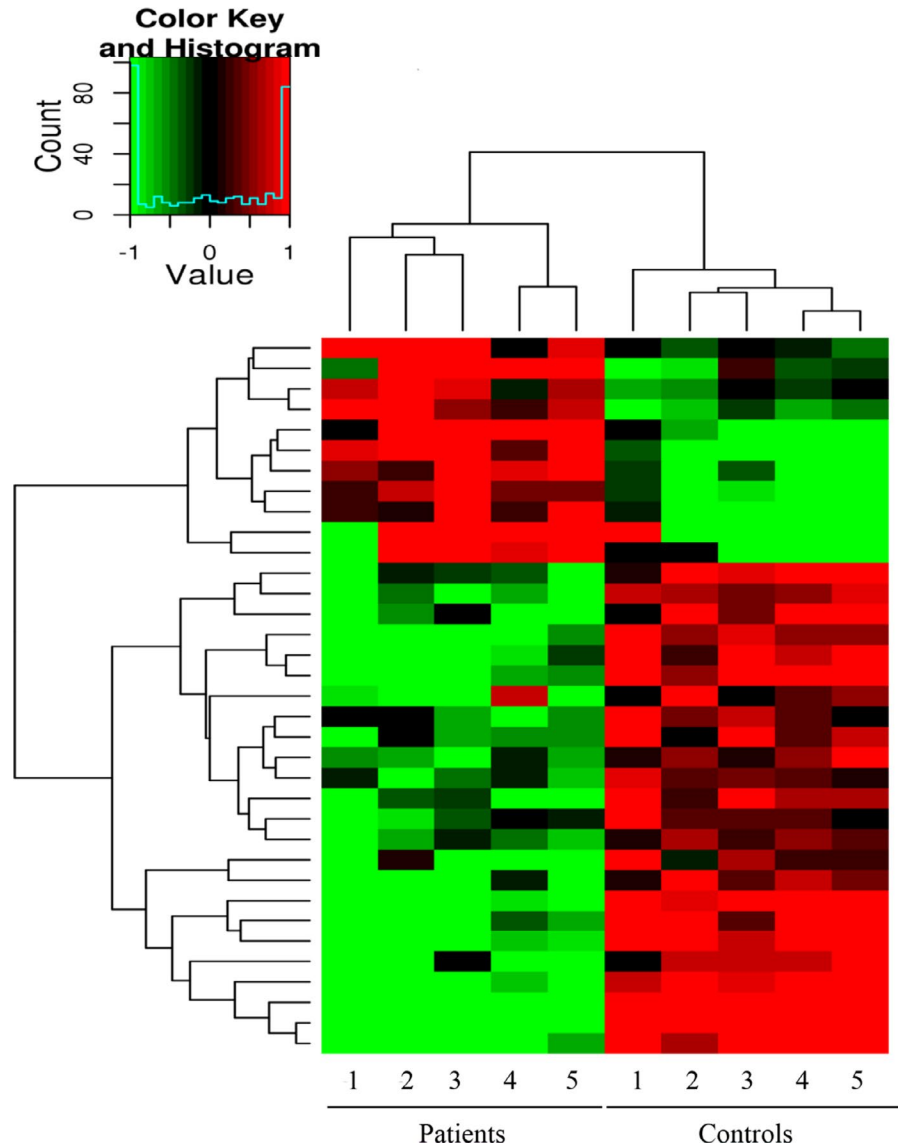
and *D3R* are associated with cocaine and alcohol abuse.[34] *BDNF* initiates the mitogen-activated protein kinase (MAPK) pathway in long-term potentiation (LTP) and learning.[35] In addition, *CREB* is recruited and phosphorylated by the MAPK pathway, and *CREB* activity was observed in NAc after opiate withdrawal and chronic amphetamine administration.[36-38] Furthermore, the functional role of the *D3R* in drug reward and addiction has been demonstrated, which has been shown to play an important role in the formation of addiction and the development of neuroplasticity.[39,40] Additionally, Saba[41] found that the expression of miR-181a is up-regulated by dopamine signaling in cell culture and by psychotropic drugs in mouse models of chronic cocaine addiction. miR-181a has seen implicated as a key regulator of mammalian AMPA-type glutamate receptors, with potential implications for the regulation of synaptic plasticity induced by cocaine and amphetamine. These results suggest that the pathway and target genes regulated by miR-181a are involved in the formation and development of neural plasticity and addiction. However, little has been known of the relevance of miR-181a in heroin addiction.

Ma and colleagues identified miR-181a decrease in the NAc after chronic exposure to heroin in rats using microarray analysis, but the underlying mechanisms were not illustrated.[42] These results indicate the relevance and importance of investigations of miR-181a as a possible target for treatment of drug addiction. The aberrant expression of miR-181a is involved in the neuronal apoptosis((p53 signaling pathway, MTOR, BCL-2, and MCL-1),[43] synapse plasticity and neurogenesis (neurotrophin signaling pathway, BDNF, D3R, and GluA2), and immune function (TGF-β signaling pathway, TGFBR1 and TGFBRAP1).[12,41,44] Accumulating studies have revealed that these pathways are involved in the development of drug addiction.[45] For example, acute treatment and systemic administration of MA reduce the LTP of hippocampal CA1 pyramidal neurons. *BDNF* is a regulator of LTP and neurogenesis, and neurotrophin signaling pathway changes may contribute to development of psychosis in MA users.[46,47] In addition, chronic exposure to heroin produces remarkable neurotoxicity and central nervous system dysfunctions, such as damage to neuronal cell bodies and terminals, neuronal apoptosis,
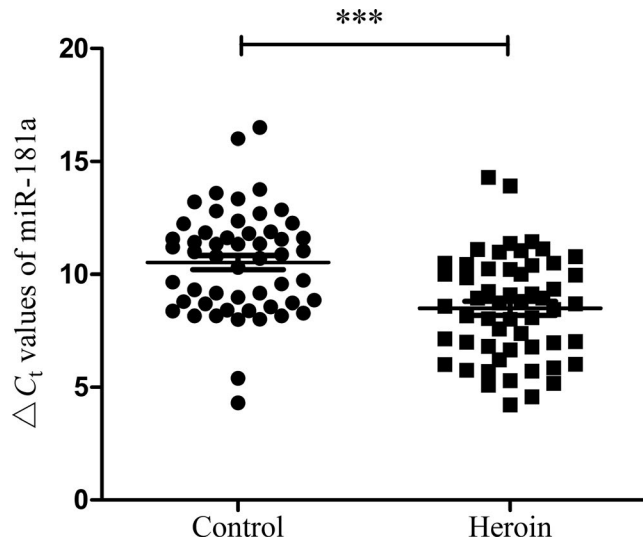
FIGURE 2 The expression level of hsa-miR-181a between normal controls and heroin addictions, $\triangle C_t = C_t$ (hsa-miR-181a) - C_t (U6). A smaller $\triangle C_t$ value indicates higher expression, [***]$P < .001$
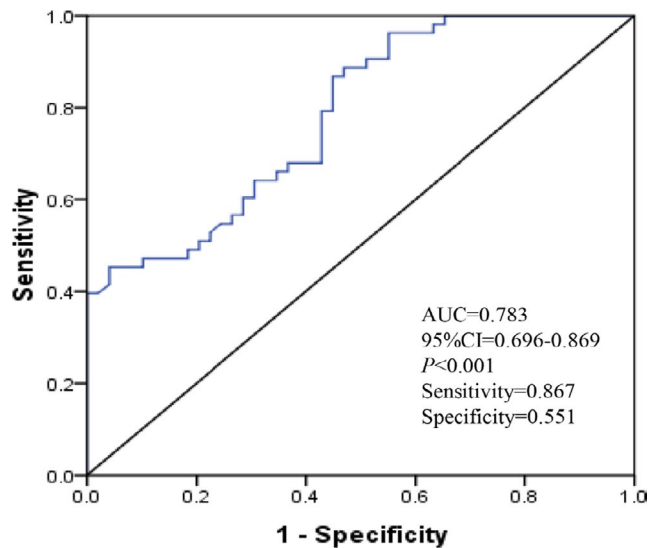

FIGURE 3 ROC analysis of the expression of hsa-miR-181a in the diagnosis of heroin addiction. ROC: receive operating curve; AUC: area under the curve. CI: confidence interval

TABLE 3 Hsa-miR-181a expression ($\triangle C_t$) in subjects with different clinicopathological features

Variables	Control group		Heroin addiction	
	r_s	P	r_s	P
Age	0.011	0.942	0.035	.803
Education	0.217	0.134	0.056	.689
Occupation	−0.197	0.175	−0.007	.962
Duration of use (mo)			−0.042	.767
Daily dose (g)			−0.288	.037
Usage			0.036	.8
Frequency of use			0.294	.033
Frequency of detoxification			0.089	.527

and impaired learning and memory, which contribute to the maladaptive plasticity and the transition into addiction.[48,49] Moreover, miR-181a plays a critical role in modulating T-cell and B-cell differentiation.[50] Peripheral miRNAs have been used as a diagnostic tool for various related neurological disorders, including cancer, schizophrenia, and Alzheimer's disease.[51-53] Human studies have reported changes in peripheral miRNAs, including miR181a, miR15b, let-7e, and let-7d, in patients with MA use disorder.[26] However, there have been no reports of biomarkers related to heroin addiction. The present finding that the expression levels of hsa-miR-181a in male heroin addicts were significantly higher than that of the normal control group (Figure 2) is novel. Whether the increased level of

hsa-miR-181a was induced by heroin addiction or whether its expression leads to addiction remains unclear and further studies are necessary. Heroin addiction can lead to changes in many miRNAs, which we presently confirmed. The expression of hsa-miR-181a is also changed in other diseases.[54-56] For example, miR-181a and miR-181d both inhibit the expression of the *PHLPP2* and *INPP4B* phosphatase genes, then increase the phosphorylation of growth factor-induced Akt, and finally lead to growth of breast cancer cells.[57] Interestingly, we found the potential diagnostic value of has-miR-181a in heroin addiction (Figure 3). Age, daily dose, and usage are important factors affecting prognosis of drug addiction.[58,59] In this study, we focused on the relationship between hsa-miR-181a and these factors. The increased expression of hsa-miR-181a in heroin addiction was related with daily dose ($r_s = -0.288$, $P = .037$) and frequency of heroin use ($r_s = 0.294$, $P = .033$) (Table 3). These findings indicate that a higher frequency of drug use might be correlated with aberrant expression of miRNAs. This may suggest that more severe heroin use induces more neurological impairment. This means that peripheral hsa-miR-181a should be considered as a noninvasive, plasma-based biomarker of heroin addiction. Conversely, Zhao et al[26] found a negative correlation between the expression of hsa-miR-181a and the drug use days in the past month of MA abuse ($r_s = -0.230$, $P = .012$). This discrepancy could be attributed to different regulatory mechanisms in various drug addictions.

In summary, we found that the expression of peripheral hsa-miR181a in male heroin addiction patients was higher than that of the normal control group, by expanding sample number after using bioinformatics methods to screen plasma miRNAs expression profiles associated with heroin abuse. Although a single miRNAs may be used as a predictive biomarker, the sensitivity and specificity of such a method are not strong. Therefore, it is necessary to reveal more miRNAs that are differentially expressed in heroin patients to further explore the use of miRNAs as biomarkers of heroin addiction. Importantly, several limitations should be noted in this study. Firstly, multiple miRNAs should be established as biomarkers to predict heroin addiction in future studies. Secondly, some of the participants in

the study were abstinent for some time before entering the drug rehabilitation center. It was unclear whether the expression changes in the hsa-miR-181a existed when patients were actively using drugs. Thirdly, other factors, such as smoking, may impact plasma miRNAs expression. Smoking was very popular among those with drug use disorders, but we did not quantify their smoking status. Finally, our study involved 53 heroin addictions and 49 normal controls. The small number of experimental samples may have influenced the results. Further studies with larger sample numbers should be performed to verify the findings.

In conclusion, our data indicate that hsa-miR-181a may represent a potential novel biomarker for the diagnosis of heroin addiction. The present study provides an important theoretical basis for the clinical application of heroin addiction, which is clinically significant.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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