Expression quantitative trait locus rs6356 is associated with the susceptibility of heroin addiction by potentially influencing the TH gene expression in the hippocampus and nucleus accumbens

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Abstract

Opioid addiction is a complicated and highly heritable brain disease. Dysfunction of dopaminergic signaling is involved in the pathogenesis of addictive disorders. Encoding the synthetase of dopamine, tyrosine hydroxylase (TH) gene has long been an interesting candidate in the genetic association studies for opioid addiction. However, the underlying mechanism of the association of risk gene variants and opioid addiction remains unknown. In the present study, we first analyzed the association between *TH* gene variants and susceptibility and traits of heroin addiction in 801 patients with heroin addiction and 930 healthy controls. The methylation level in the promoter region of the *TH* gene was detected and compared between the heroin addiction and healthy control groups. To reveal the potential mechanism of the association of *TH* gene variants and heroin addiction, correlation between the risk *TH* SNPs for heroin addiction and the methylation as well expression level of *TH* gene were examined. Our results demonstrated that SNP rs6356 was associated with the susceptibility of heroin addiction. CpG *TH*_15 was hypermethylated in the heroin addiction group compared with the healthy control group. Notably, SNP rs6356 was allele-specific correlated with the expression of *TH* gene in the hippocampus and nucleus accumbens, but not the methylation level of CpG *TH*_15. Our findings suggest that the eQTL rs6356 was associated with the risk of heroin addiction by potentially affecting the expression of *TH* gene in the mesocorticolimbic dopamine system brain regions including hippocampus and nucleus accumbens.

1. Introduction

Substance addiction is a complex brain disease, characterized as compulsive substance seeking, abstinence, and repeated relapse for substance use (Chisholm et al., 2021; Huang, Chen, Lane, Ho, & Chung, 2021). Opioid, one of the most abused prescription medicines for pain management and illicit drugs of addiction, causes the most harm in substance misuse-related deaths, killing tens of thousands people per year alone in America (Browne, Godino, Salery, & Nestler, 2020). In China, more than 730 thousand people have exposed to illicit opioid, mainly heroin in 2020. Mounting evidence has suggested that genetic underpinnings and environmental factors contribute to the development of substance addiction (Gerra et al., 2021). Twin studies reveal that the heritability of opioid addiction is about 70% (Goldman, Oroszi, & Ducci, 2005). Genome-wide association studies have proposed a large amount of risk gene variants for opioid addiction (Song et al., 2020; Zhou et al., 2020). However, the underlying mechanism of the association of risk gene variants and opioid addiction remains unknown.

Prolonged use of addictive substances could lead to adaptive alterations in neural plasticity in discrete brain regions including the mesocorticolimbic dopamine system which processes the reward and motivation effects (Hyman, Malenka, & Nestler, 2006), resulting in the transition to addicted states (Stewart, Fulton, & Maze, 2021). DNA methylation, one of the epigenetic components, represents a mechanism that affects the plasticity process and then the development of substance addiction by involving environmental stimuli and regulating gene expression patterns (Stewart et al., 2021). DNA methylation, especially in the promoter region of the gene where is rich with CpG sites and transcriptional

factors binding sites, is an important mechanism that regulates gene expression (Pathak, Miller, Morris, Stewart, & Greenberg, 2018). Genetic variants can be associated with the methylation and expression level of the gene. These variants are known as the methylation quantitative trait locus (mQTL) (Villicaña & Bell, 2021) or expression quantitative trait locus (eQTL) (Degtyareva, Antontseva, & Merkulova, 2021). Previous studies have revealed that the mQTLs and eQTLs are enriched in risk genetic variants in many neuropsychiatric diseases such as schizophrenia (Perzel Mandell et al., 2021; Zhao et al., 2018), depression (Ciuculete et al., 2020; X. Wang et al., 2020), and substance addiction (Lin et al., 2020; H. Zhang et al., 2014). Thus, risk genetic variants that correlated with the methylation or expression of the gene may be important mechanism underlying the association with susceptibility and traits of substance addiction.

Tyrosine hydroxylase (TH) is a key rate-limiting enzyme in dopamine and norepinephrine biosynthesis (Boundy et al., 1998; Walters, Kuo, & Blendy, 2003). By regulating the level of the above neurotransmitters with essential roles in neuronal activity, TH has been shown involved in many neuropsychiatric disorders including opioid addiction (Jalali Mashayekhi, Rasti, Khoshdel, & Owji, 2018; Vaillancourt et al., 2021). Repeated morphine administration increased the TH expression in hippocampus (Fang et al., 2017) and locus coeruleus (Jalali Mashayekhi et al., 2018), which are important brain regions in mesocorticolimbic dopamine system. Aldehyde dehydrogenase 2 (ALDH2) inhibitor could prevent the reinstatement of cocaine and alcohol intake by suppressing the activated (phosphorylated) tyrosine hydroxylase signaling (Diamond & Yao, 2015). Hence, *TH* gene has long been an interesting candidate for the genetic association studies for opioid addiction. However, few studies have elucidated the underlying mechanism in it.

In the present study, we first determined the association between *TH* gene variants and susceptibility of heroin addiction. Then, the association of *TH* gene variants and traits of heroin addiction were analyzed. To reveal the potential mechanism of the association between risk SNPs in the *TH* gene and heroin addiction, we detected and compared the methylation level in the promoter region of the *TH* gene. Association analysis between the risk SNPs and the methylation level of CpG sites with differential methylation between patients with heroin addiction and healthy controls were performed. Finally, the risk SNPs were screened in the genotype-tissue expression (GTEx) database to demonstrate whether they were eQTLs in human brain regions.

2. Materials And Methods

2.1 Study subjects

A total of 1731 unrelated Northwestern Han Chinese participated in the present study. In the heroin addiction group, 801 patients in the methadone maintenance treatment (MMT) Program were recruited from Xi'an Mental Health Center. All patients were diagnosed by senior psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, Five Revision (DSM-VR). Patients with other substances addiction including tobacco and alcohol, or subjected to other psychiatric disorders were

excluded. Demographic information including age, gender, marital status, education, occupation, income and family relation (Table S1 and S2), as well as traits of heroin addiction including the age of onset for heroin use, heroin dose per time, heroin intake times per day, duration from first heroin exposure to addiction and effective methadone dose (Table S3) were collected by questionnaire survey to the patients. Regarding the healthy control group, 930 healthy subjects were enrolled from the Health Examination Centers of the First Affiliated Hospital of Xi'an Jiaotong University and Tangdu Hospital. They had no substances (including tobacco and alcohol) addiction, no personal or family history of psychiatric disorders, or severe organic diseases. There were no age and gender distribution differences between groups (Table S1).

All study subjects were well informed and provided written informed consent for participating in the study. The protocol of our study was approved by the Medical Ethics Committees of Xi'an Jiaotong University and the Xi'an Mental Health Center, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2 SNP selection and DNA extraction

For the analysis of association between *TH* gene variants and risk of heroin addiction, tag SNPs in the functional region (promoter region, untranslated regions, exons, and intron-exon boundaries) of the *TH* gene that with minor allele frequency (MAF) > 0.05 and minor genotype frequency > 0.01 in the Han Chinese population of the 1000 Genomes database were selected. For the association analysis of *TH* gene variants and traits of heroin addiction, all functional SNPs with frequencies meeting the above criteria were genotyped in the heroin addiction group. In addition, all SNPs should meet Hardy-Weinberg equilibrium (HWE) in the healthy control group.

Genome DNA was extracted from the peripheral blood with the EZNA™ Blood DNA Midi Kit (Omega Bio-TeK, Norcross, GA, USA) according to the manufacturer's instructions. Following quality control, the DNA was subjected to SNP genotyping and detection of DNA methylation.

2.3 SNP genotyping and detection of DNA methylation

SNP genotyping was performed with the standard SNaPshot assay by Genesky Biotechnologies (Shanghai, China). The corresponding primers was first designed according to the sequences around the SNPs. Then, a fragment of DNA surrounding the SNPs was amplified by the polymerase chain reaction (PCR). The final product was separated by capillary electrophoresis with the ABI 3130XL Genetic Analyzer (Applied Biosystems Co. Ltd., CA, USA). GeneMapper 4.1 software was used to obtain the genotypes of the SNPs. To ensure the accuracy of genotyping, five percent of the samples were randomly selected and replicated.

For the methylation detection, DNA was first bisulfite conversed with the EZ DNA Methylation Gold™ Kit (Zymo Research Corporation, CA, USA). Then, the target region of the *TH* gene promoter, containing the CpG sites, was amplified with the corresponding primers (Table S4). Finally, the amplified products of the target fragments were analyzed using the Illumina Hiseq X Ten platform. The methylation level of the

CpG site was defined as the percentage of methylated sites in all sequences containing this site. DNA methylation detection was also conducted by Genesky Biotechnologies.

2.4 Statistical analysis

PLINK (version 1.09) was used for the association analysis of *TH* gene variants and risk of heroin addiction, with age and gender as covariates. The association between *TH* gene variants and traits of heroin addiction was performed using Matrix eQTL (website at: http://www.bios.unc.edu/research/genomic_software/Matrix_eQTL) with the demographic information (age, gender, marital status, education, occupation, income per month and family relation) as covariates. For the comparison of DNA methylation between groups, student *t* test was used. Linear regression analysis with SPSS 22.0 was conducted for the association of genotypes of *TH* SNPs and level of DNA methylation. The significance threshold was set at adjusted *P* value < 0.05 after the Bonferroni correction.

3. Results

3.1 Association between *TH* gene variants and susceptibility of heroin addiction

To determine the association of *TH* gene variants and risk of heroin addiction, two tag SNPs (rs10770140 and rs6356) were genotyped in 930 healthy controls and 801 patients with heroin addiction. Our results revealed that SNP rs6356 was associated with the susceptibility of heroin addiction. The distribution of genotypes of SNP rs6356 was different between groups (χ^2 = 8.636, *P* = 0.013). In the dominant inheritance model, the GG and GA genotype frequency was lower in the heroin addiction group (26.8%) than the healthy control group (33.0%) (χ^2 = 7.763, *P* = 0.005). For the minor allele frequency, the frequency of the G allele was lower in the patients with heroin addiction (14.8%) compared with the healthy controls (17.7%) (χ^2 = 5.660, *P* = 0.017). All the above significances survived after the Bonferroni correction (α = 0.05/2 = 0.025) (Fig. 1 and Table 1).

Table 1
Association of *TH* variants and risk of heroin addiction

SNP	Minor allele	Major allele	Model	Frequency in HA group	Frequency in control group	Chi-square	*P* value
rs10770140	A	G	Genotypic	2/102/697	5/119/806	0.890	0.641
	A	G	Trend	106/1496	129/1731	0.140	0.709
	A	G	Allelic	106/1496	129/1731	0.138	0.710
	A	G	Dominant	104/697	124/806	0.046	0.830
	A	G	Recessive	2/799	5/925	0.886	0.461
rs6356	G	A	Genotypic	22/193/586	24/282/621	8.636	**0.013**
	G	A	Trend	237/1365	330/1524	5.672	**0.017**
	G	A	Allelic	237/1365	330/1524	5.660	**0.017**
	G	A	Dominant	215/586	306/621	7.763	**0.005**
	G	A	Recessive	22/779	24/903	0.041	0.839

HA: heroin addiction.

3.2 Association between *TH* gene variants and traits of heroin addiction

To reveal the association between the genotypes of *TH* SNPs and traits of heroin addiction, five functional SNPs were genotyped in the heroin addiction group and linear regression analysis was conducted between the genotypes of SNPs and five traits of heroin addiction. According to our findings, only the polymorphism of rs10770140 was nominally associated with the age of onset for heroin use (Fig. 2, beta=-1.347, $P = 0.028$). Unfortunately, it did not remain significant after the Bonferroni correction ($\alpha = 0.05/5 = 0.010$). The detailed results of the association of *TH* SNPs and traits of heroin addiction were shown in Table S5.

3.3 Differential methylation in the promoter region of the *TH* gene between the healthy control and heroin addiction groups

DNA methylation plays vital roles in substance addiction (Browne et al., 2020). To explore the potential mechanism of the association between *TH* gene variants and heroin addiction, we first detected and compared the methylation level in the promoter region of the *TH* gene between groups. Student's *t* test revealed that the methylation level of CpG *TH*_15 was significantly higher in the heroin addiction group than the healthy control group (Fig. 3, t=-3.465, $P < 0.001$), even after the Bonferroni correction (Table 2, $\alpha = 0.05/15 = 0.003$). However, the methylation level other CpG sites and the mean methylation level of the promoter region of the *TH* gene did not show difference between groups (all $P > 0.05$).

Table 2
Differential methylation in the promoter region of the *TH* gene between healthy control and HA groups

CpG site	Mean methylation level in control group	Mean methylation level in HA group	*t*	*P* value
TH_01	0.851	0.839	1.060	0.281
TH_02	0.970	0.972	-0.439	0.672
TH_03	0.920	0.917	0.412	0.716
TH_04	0.867	0.864	0.354	0.792
TH_05	0.934	0.937	-0.538	0.646
TH_06	0.884	0.887	-0.346	0.737
TH_07	0.579	0.571	1.401	0.278
TH_08	0.866	0.867	0.061	0.854
TH_09	0.968	0.967	0.896	0.465
TH_10	0.880	0.883	-0.964	0.340
TH_11	0.902	0.899	0.867	0.524
TH_12	0.956	0.955	0.466	0.803
TH_13	0.753	0.757	-0.354	0.580
TH_14	0.137	0.145	-2.383	0.014
TH_15	0.139	0.151	-3.465	**< 0.001**
TH_promoter_mean	0.735	0.759	-1.757	0.080

HA: heroin addiction.

3.4 Association of SNP rs6356 and methylation level of CpG *TH*_15

Previous studies suggest that mQTLs, the SNPs that have an association with the level of gene methylation, convey more vulnerability in human diseases including substance addiction than randomly selected SNPs (Lin et al., 2020; Min et al., 2021). Based on the results of differential methylation above, we then analyzed the association between the genotypes of rs6356 and the methylation level of CpG *TH*_15. Linear regression analysis demonstrated that there were no association between them ($P > 0.05$).

3.5 Expression quantitative trait locus of rs6356 in human brain regions

To further reveal the potential function of rs6356 in heroin addiction, we screened it in the GTEx database (website at: www.gtexportal.org). SNP rs6356 was found allele-specific associated with the expression level of the *TH* gene in human brain regions including hippocampus (P = 3.83E-04), caudate (P = 6.50E-03), nucleus accumbens (NAc, P = 8.22E-03), hypothalamus (P = 1.59E-02) and putamen (P = 1.91E-02). The subjects with AA genotype had the highest expression level of *TH* gene in the above brain regions, then came the GA and GG genotype subjects. The detailed outcome of eQTL of rs6356 was shown in Table 3.

Table 3
Expression quantitative trait locus of rs6356 in human brain regions.

SNP	eQTL in brain regions	Frequency of CC/CT/TT genotypes	*P* value
rs6356	Hippocampus	73/76/16	3.83E-04
	Caudate	82/91/21	6.50E-03
	Nucleus accumbens	84/95/23	8.22E-03
	Hypothalamus	64/86/20	1.59E-02
	Putamen	72/84/14	1.91E-02

eQTL: expression quantitative trait locus.

4. Discussion

TH is involved in the rewarding effects of addictive substance (Fang et al., 2017; Vaillancourt et al., 2021). *TH* gene has long been an important candidate in genetic association studies in substance addiction. Further investigations of the potential mechanism in the correlation between *TH* gene variants and susceptibility and traits of opioid addiction are required. In the present study, we first determined the association between *TH* gene variants and susceptibility and traits of heroin addiction. The methylation level in the promoter region of the *TH* gene was detected and compared between heroin addiction and healthy control groups. Finally, mQTL and eQTL analysis was performed for the risk SNPs in heroin addiction to explore the potential mechanism or function of them in opioid addiction.

Long term addictive substance exposure induces neuronal adaptations including structural, functional and molecular changes in brain (Evangelou et al., 2021; Fernàndez-Castillo, Cabana-Domínguez, Corominas, & Cormand, 2021; Salery, Trifilieff, Caboche, & Vanhoutte, 2020; Snyder & Silberman, 2021). Epigenetic and gene expression alterations are essential molecular changes responsible for the neuronal adaptations that contribute to the development of substance addiction (Fernàndez-Castillo et al., 2021). Previous studies indicated that hypermethylation at several CpG sites in the *TH* gene is found in the postmortem NAc samples in the patients with cocaine addiction compared with the healthy controls (Vaillancourt et al., 2021). In accordance with the above findings, we observed that the methylation level of CpG *TH*_15 was higher in the heroin addiction group than the healthy control group. DNA methylation

is one of the mechanisms that could inhibit the gene transcription and expression (Li, Gonzalez, Inzé, & Dubois, 2020). With potential role in regulating DNA methylation, mQTLs were believed to constitute more liability than random SNPs in diseases including heroin addiction (J. Zhang et al., 2021). Unfortunately, we failed to detect any association between the risk SNP rs6356 for heroin addiction and the methylation level of *TH*_15. Hence, other mechanisms rather than methylation patterns may underlie the association of rs6356 and risk of heroin addiction.

Our results demonstrated that SNP rs6356 is an eQTL that allele-specific correlated with the expression level of *TH* gene in the hippocampus and NAc. The A allele of rs6356 was associated with higher expression of *TH* gene in the above two brain regions. In addition, the A allele frequency in the patients with heroin addiction was found significantly higher than that in the healthy controls. This was consistent with previous findings in rodents that repeated morphine exposure caused upregulation of *TH* gene expression in the hippocampus (Fang et al., 2017) and locus coeruleus (Jalali Mashayekhi et al., 2018), two important brain regions in substance addiction. Hippocampus is engaged in the formation of pathological context memory in substance addiction (Z. Wang et al., 2020). NAc is responsible for the addictive substances-induced rewarding and reinforcement effects (Al-Hasani et al., 2021; Amaral et al., 2020). Increased dopamine release in brain regions of the mesocorticolimbic dopamine system, including hippocampus and NAc is shown in all kinds of addictive substance use (Morales & Pickel, 2012; Pinheiro et al., 2015). The increase of dopamine synthesis may be associated with the upregulation of TH expression in these brain regions. Thus, we speculate that the eQTL rs6356 is associated with heroin addiction by implicating the *TH* gene expression in brain regions including hippocampus and NAc.

Several limitations should be considered when interpreting the results of our study. First, we used candidate gene method to elucidate the role and potential function of the *TH* gene variants in heroin addiction. Genome-wide association studies with large amounts of participants have the highest resolution in identifying risk gene variants for addictive diseases. Second, the eQTL results were obtained from the GTEx database, we did not examine the expression level of *TH* gene in brain regions due to lack of postmortem brain samples. Third, functional studies should be conducted to verify the precise role of the *TH* gene variants in the development of heroin addiction.

In summary, we found the eQTL rs6356 was associated with the susceptibility of heroin addiction. CpG site *TH*_15 in the promoter region of the *TH* gene was hypermethylated in the heroin addiction group compared with the healthy control group. SNP rs6356 was allele-specific associated with the expression level of *TH* gene in mesocorticolimbic dopamine system brain regions including the hippocampus and NAc, but not the methylation level of CpG *TH*_15 in peripheral blood. Our findings suggest that the eQTL rs6356 is associated with opioid addiction, potentially by influencing the *TH* gene expression in the hippocampus and NAc. It will further extend the mechanism underlying the association of genetic variants and addictive disorders.

Declarations

Data Availability Statement

The sequencing data of the methylation assaying has been deposited into Sequence Read Archive (SRA) under accession number PRJNA752473.

Ethics Approval and Consent to Participate

Our study was approved by the Medical Ethics Committees of Xi'an Jiaotong University and the Xi'an Mental Health Center, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All study subjects were well informed and provided written informed consent for participating in the study.

Consent for Publication

The authors affirm that all participants provided informed consent for publication.

Availability of Data and Materials

All data and materials will be available upon reasonable request.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Authors' Contributions

Jianbo Zhang and Yongsheng Zhu conceptualized this study. Kena Wang and Hongbo Zhang analyzed the data and drafted the manuscript. Jinshan Ji and Rui Zhang contributed to the methodology. Wei Dang and Qiaoli Xie reviewed and edited the manuscript. All the authors reviewed and approved the final version of publication.

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Figures

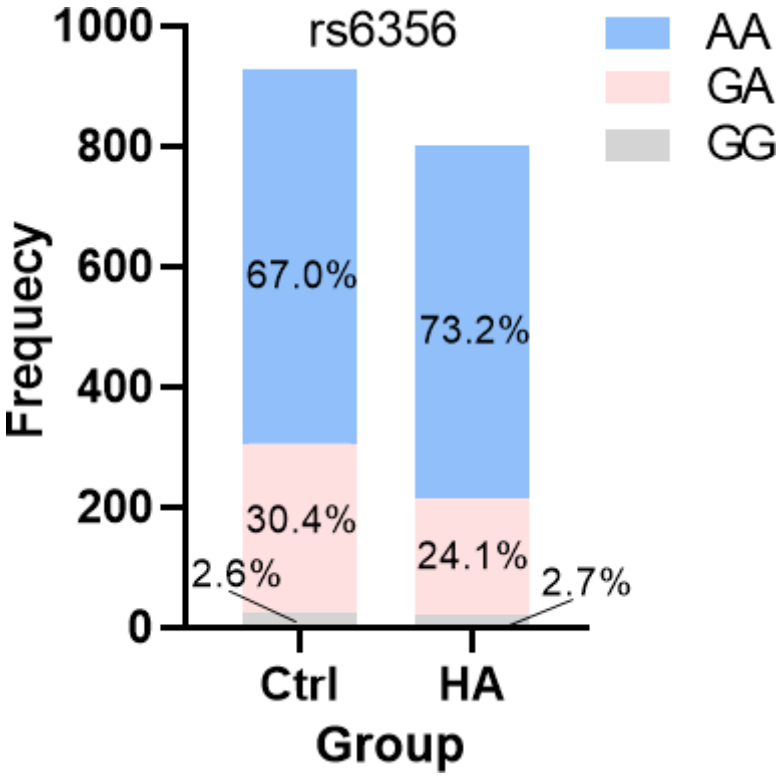

Figure 1

Distribution of genotypes in SNP rs6356 between heathy control and heroin addiction groups. Ctrl: healthy control group; HA: heroin addiction group.

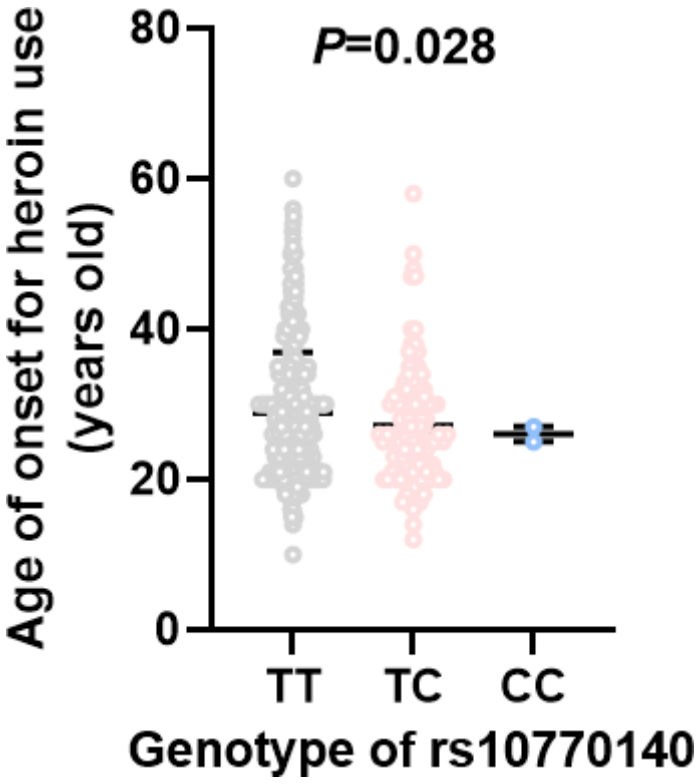

Figure 2

Association of SNP rs10770140 and age of onset for heroin use.

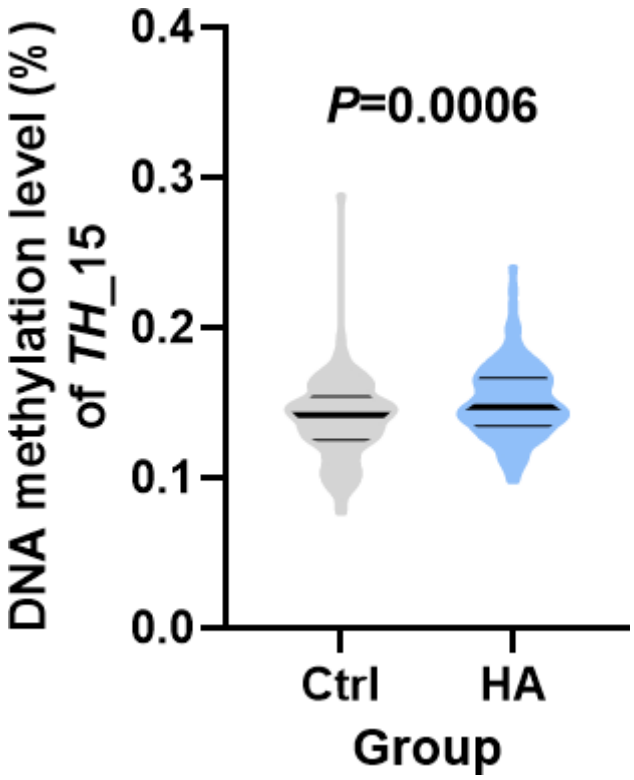

Figure 3

Differential methylation in CpG *TH*_15 between patients with heroin addiction and healthy controls. Ctrl: healthy control group; HA: heroin addiction group.

Supplementary Files

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- SupplementaryTables.xlsx