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•ORIGINAL RESEARCH ARTICLE•

CHN2 Promoter Methylation Change May Be Associated With Methamphetamine Dependence

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Background: Methamphetamine (MA) abuse is becoming increasingly serious in China. The mechanism of MA dependence remains unclear. CHN2 gene encodes chimeric protein-2 that regulate axonal pruning via the Rac-GTPase system and play a pivotal role in the formation of nervous circuits. Genetic studies suggest that the polymorphism of the CHN2 gene was related to substance dependence.

Aims: The aim of this study was to investigate the association between the methylation of CHN2 gene promoter with MA dependence.

Methods: According to SCID-I (Structured Clinical Interview for DSM-IV Axis I Disorders, SCID-I) used for investigating MA dependence, 224 male MA addicts were recruited into the case group. In addition, 109 healthy men were recruited into the control group. Blood samples were collected with the purpose of detecting the methylation levels of CHN2 gene promoter by methylight qPCR. The association between the methylation of CHN2 gene promoter with MA dependence was analyzed.

Results: The mean (sd) methylation levels of CHN2 gene promoter in the case group were significantly higher than in the control group, which were 2795.55 (733.19) and 1026.73 (698.73), respectively, showing significant differences between the two groups (t=21.25, P<0.001). Pearson analysis showed no significant correlation between the methylation levels of CHN2 promoter and other factors (the age of initial MA use, the duration of MA use, combination with K powder, tobacco and alcohol).

Conclusions: The abnormal methylation of CHN2 gene promoter was significantly correlated with MA dependence.

Key words: Methamphetamine dependence; DNA methylation; epigenetic; CHN2 gene

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1. Introduction

Since the beginning of the 21st century, synthetic drugs, represented by amphetamine-type stimulants, have become the most widely-abused and rapidly-spread drugs in the world. In China, the abuse of synthetic drugs represented by methamphetamine (MA) is becoming increasingly serious. By the end of 2016, there were 255,000 drug abusers in the country (excluding deaths, departures, and those who did not suffer a relapse for three years), among which there were 151,500 synthetic drug abusers, accounting for 60.0% of the total.[1] Moreover, the abuse of amphetamine-type stimulants occupied the first place among synthetic drugs being used.[2]

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With high lipid solubility, MA can easily pass though the blood-brain barrier. In the central nervous system, MA is the indirect receptor agonist of dopamine, norepinephrine and serotonin, promoting the release of these monoamine neurotransmitters in a variety of ways.[3,4] These neurotransmitters have physiological and psychological effects, such as euphoria, the increase of alertness, vigor and attention, and the decrease of fatigue. However, MA also leads to cardiac acceleration and hypertension.[3,4]

Long-term and large-scale abuse of MA can result in addictive behavior, and patients often suffer a slow change from recreational use to compulsive use. The mechanism of MA addiction remains unclear. Studies have found that effecting synaptic plasticity and inducing the changes of neuronal adaptability may be the neurobiological mechanisms for MA addiction.[5] One of the important mechanisms involved in changes of neuronal adaptability is the remodeling of actin cytoskeleton. This mechanism is regulated by multiple genes, and genomic association analysis shows that the CHN2 gene is involved in the formation of addiction by participating in the remodeling of actin cytoskeleton.[6,7] The CHN2 gene, with a length of 318 kb, is located on chromosome 7 at p15.3, and contains 13 exons and 12 introns.[8] CHN2 gene expresses chimeric protein-2 that selectively deactivate Rac. Moreover, Rac is a small molecule GTPase, which is the key enzyme to regulate the remodeling of actin.[9] In the central nervous system, animal model studies showed that chimeric protein-2 plays an important role in the regulation of hippocampal axonal pruning.[10] And, human genetic association analysis indicated that the polymorphism of CHN2 gene was related to tobacco addiction and schizophrenia.[11-13]

Based on the important role of CHN2 in the changes of neuronal compensatory adaptation and substance addiction, we hypothesized that there was a difference in the methylation level of CHN2 gene between MA addicts and normal individuals. With the purpose of finding out the epigenetic mechanism of MA-inducing psychotic disorder, methylight qPCR was used to detect the methylation levels of CHN2 gene in 224 MA addicts and 109 healthy controls.

2. Materials and Methods

2.1 Subjects

365 male patients with MA addiction were surveyed using SCID-I (Structured Clinical Interview for DSM-IV Axis I Disorders, SCID-I, Chinese version) from DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edition, American Psychiatry Association, 1994) at the Xinkaipu detoxification center in Changsha, Hunan Province and the Baini Lake compulsory detoxification center in Xiangyin, Hunan Province from March 2013 to January 2014 (see Figure 1). Patients aged 16-60 were recruited into the case group if they matched the diagnostic criteria for MA addiction of DSM-IV. We excluded those with severe physical diseases, organic brain diseases, primary mental disorder or other substance addiction. According to the recruitment criteria, 224 MA addicts were recruited into the case group. Healthy patients (109 male) matched by age were recruited into the control group from the health management center of the Second Xiangya Hospital of Central South University. We excluded those with severe physical illness, organic diseases of the brain, primary mental disorder or other substance addiction. This study was approved by the ethics committee of Second Xiangya Hospital of Central South University. Participants (patients and healthy controls) were informed of the research process and provided written informed consent.

2.1 Reagents and instruments

The probes and primers were synthesized by Genzyme Biotech Co., Ltd. The rest of the major reagents were provided by Changsha Kangkang Century Biotechnology Co., Ltd. Fluorescence quantitative PCR instrument produced by Thermo Co., Ltd, and desktop refrigerated centrifuge is produced by Xiangyi Co., Ltd.

2.3 Experimental procedure

2.3.1 DNA extraction and bisulfite modification

10 ml of peripheral blood was extracted and DNA was extracted. The DNA was modified with the EZ DNA Methylation-GoldTM Kit to convert the unmethylated cytosine (C) in the DNA sequence to uracil (U).

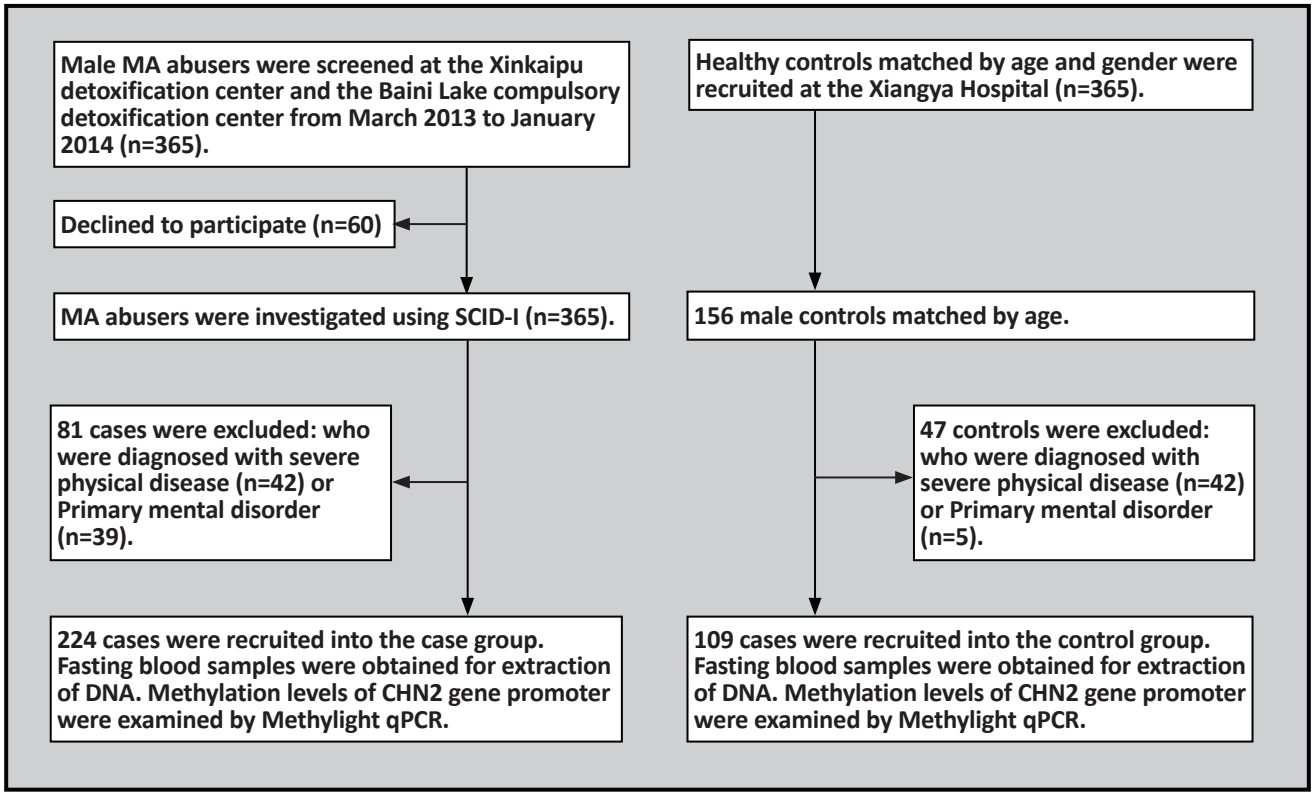
2.3.2 Design of methylation primer and probe

The CHN2 gene sequence was referenced to GenBank (http://www.ncbi.nlm.nih.gov), which is as follows: GGAAACAGAGAAGCCTGGGGCTGGTGAGGGCCAGGACA GAAGGCCGGCGTGGGCAGATGGCGAAGGCTGCGAAGGG TAATGAGCGCTTCCTGAGGACTCTCAGAAGGCGGGGGTG GCTGGGT. Methylated primers and probes were designed by Beacon Designer (Version 7.0). The CHN2 gene upstream primer is 5'-TAGTTTAGGATGGGGTTTA-3'; the downstream primer is 5'-ACAC ATTTCACACTCAAA-3'; the methylation probe FAM is 5'-CCCAACCAACTCT AACGTTCAAA-3', the unmethylated probe ROX is 5'-CCCAACCAACTC TAACATTCAAA-3', the size of target fragment is 111 bp.

2.3.3 Preparation of DNA standard

2xES Taq Master Mix reagent instructions were followed. The reaction system was constituted by the modified DNA template 1 uL, the upstream primer 1 uL, the downstream primers 1uL, 2x ES Taq Mix 25 uL, and ddH_2O 20 uL. The reaction conditions were as follows: denaturation at 94°C for 4 min, denaturation at 94°C for 15 s, annealing at 56°C for 30 s, extension at 72°C for 10 s, extension at 72°C for 7 min, and 31 cycles.

Figure 1. The flowchart of the study



The 2% agarose gel was prepared by electrophoresis to detect whether the PCR products contained the target fragments (111bp). The target fragments were recovered and dissolved, and the solution of DNA was collected. The concentration of DNA recovered was measured with a spectrophotometer, and the DNA sample was used as DNA standard. The OD value (260 nm) of CHN2 was 0.05 with a concentration of 210 ng/uL, and the Copies value was 2.1E + 12 Copies/uL. DNA Copies/uL = (mass/molecular weight) x NA = NA x [DNA concentration (ng/uL) x 1uL] x 10^{-9}/(DNA molecular weight x 660).

2.3.4 Quantitative PCR detection of CHN2 gene promoter methylation

S0 was obtained by diluting 100-fold the DNA standard, then S0 was diluted by a constant 10-fold gradient to prepare S1, S2, S3, S4, S5, S6 and S7 samples, which were served as standard curve samples. The reaction system was constituted by the modified DNA template 3uL, the upstream1uL, the downstream primers 1uL, the methylated probe 1uL, the unmethylated probe 1uL, 2 x Goldstar TaqMan mixture 15 uL, and RNase-Free Water added to 30 uL. The reaction conditions were as follows: denaturation at 95 ℃ for 10min, denaturation at 95 ℃ for 10 s, annealing at 55 ℃ for 30 s, extension at 72 ℃ for 30 s and circulation 40 times. The samples were repeated 3 times and the mean values were accepted. After part of the PCR products were connected to plasmids that were serving as carriers, they were sequenced by universal primers. The results showed that the sequences of the target sequences were consistent with the sequences designed by Beacon Designer software.

2.4 Statistical analysis

Statistical analyses were conducted using SPSS for Windows (Version 19.0). The counting data, such as methylation and non-methylation values, were compared using t-tests, and the measurement data were compared using chi-square tests. The Pearson method was used to analyze the correlation between CHN2 methylation levels and other factors, including the age of starting taking MA, the total duration of MA use, and combination with other psychoactive substances.

3. Results

3.1 Demographics and situations of substances use

224 male patients with MA addiction were recruited into the case group with a mean (sd) age of 30.7 (6.0), and 109 healthy men were enrolled into the control group with a mean (sd) age of 30.0 (5.7). There were

no significant differences in age between the two groups (*t=-7.9, p=0.43).* Married or cohabitating adults accounted for 47.32% in the case group and 55.05% in the control group, and no significant differences in marital status between the two groups were found. The circumstances of substance use for 224 MA addicts is shown in Table 1.

Table 1. Characteristic of substance use for 224 patients with MA addiction

Variable	Cases	Percentage (%)
Age of initial MA use		
<20	52	23.21
20~29	105	46.88
30~39	41	18.30
>40	26	11.60
Duration of MA use (months)		
≤12	44	19.64
13~36	88	39.28
37~60	55	24.55
>60	37	16.51
Approaches of MA use		
Iron absorption	224	100.00
Others	0	0.00
Accompanied with psychotic symptoms		
Yes	95	42.41
No	119	53.12
Substances use		
K power	93	41.52
Tobacco	97	43.30
Alcohol	84	37.50
Family history of substance use disorder		
Positive	11	4.91
Negative	213	95.09

3.2 The methylation levels of CHN2 gene promoter

As shown in Figures 2 and 4, the mean (sd) methylation levels of the CHN2 gene promoter in the case group were significantly higher than in the control group, which were 2795.55 (733.19) and 1026.73 (698.73), respectively, showing significant differences between the two groups **(***t=21.25, p<0.001).* As shown in Figure 2, the mean (sd) non-methylation levels of the CHN2 gene in case group were 1165.56 (814.26), whereas the mean (sd) non-methylation levels were 4872.42 (1089.83) in control group, showing significant differences between the two groups *(t=26.52, p<0.001).* Pearson analysis showed no significant correlation between the methylation levels of CHN2 promoter and other factors (the age of initial MA use, the duration of MA use, combination with K powder, tobacco and alcohol) with *p*>0.05.

Figure 2. Comparison of methylation levels of CHN2 gene between those with MA addiction and healthy controls

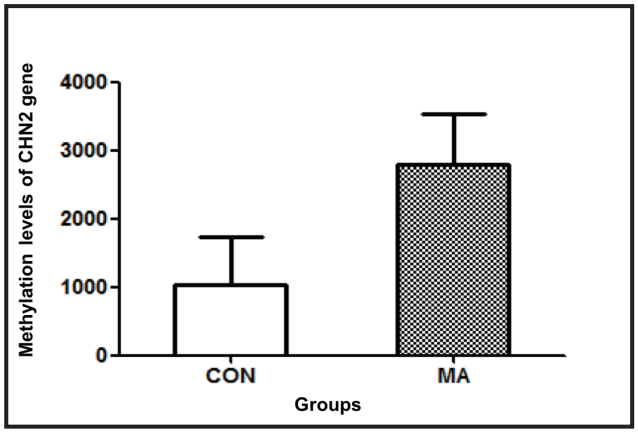
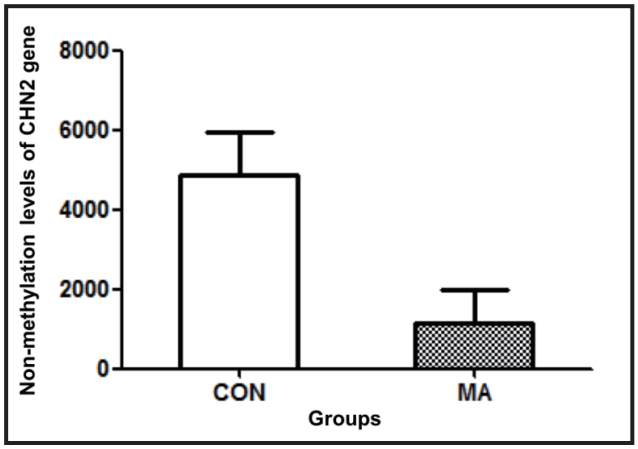


Figure 3. Comparison of non-methylation levels of CHN2 gene between those with MA addiction and healthy controls

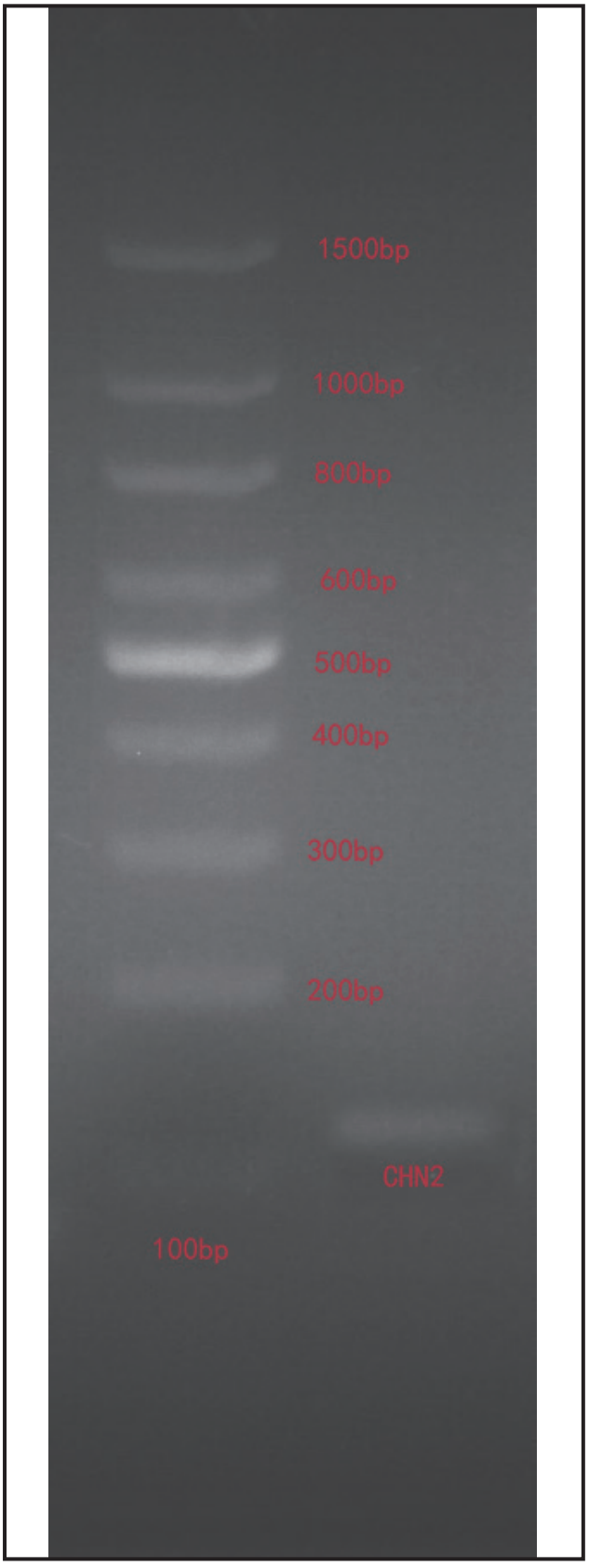


4. Discussion

4.1 Main Findings

Long-term large-scale use of MA can cause adaptive changes in neurons through epigenetic mechanisms, leading to the occurrence of addiction behavior.[14,15] DNA methylation is an important epigenetic mechanism. DNA methylation prevents transcription factors from getting close to the gene promoter region, leading to inhibiting gene expressions and rendering a gene into a silent state. Abnormal methylation of CHN2 gene can lead to the expression changes of chimeric protein-2. This research studied the relationship between the methylation levels of CHN2 gene promoter for the first time. The results showed that the methylation levels of CHN2 gene promoter in MA addiction group were significantly higher than in the control group, the levels

Figure 4. Electrophoresis profiling of CHN2 gene promoter sequences



of non-methylation were significantly lower than in the control group. The findings supported the fact that the abnormal methylation of the CHN2 gene was associated with MA addiction. At present, the relationship between CHN2 gene and MA dependence has not been reported, but the relationship between CHN2 gene and substance dependence has been confirmed. Barrio-Real's studies on smokers have shown that the polymorphism of the CHN2 gene was significantly correlated to nicotine dependence.[11]

4.2 Limitations

DNA methylation are closely related to environmental factors, such as stress, lifestyle, and occupational environment. The case group in our research came from compulsory detoxification centers, while the control group came from common living environments, which might affect the results. Secondly, this study could only match the factors of age and gender between the control group and the case group, however, there might be other factors influencing the results. In addition, the samples selected for this study were all male, and the methylation levels of CHN2 gene promoter may have differences between genders. Therefore, the interpretation of the results may not apply for women as well.

4.3 Implications

Chimeric protein 2 (CHN2) regulating Rac GTPase activity is the receptor for diglyceride and phorbol ester.[9] Rac GTPase is a member of the Rho-GTPase family that exert important influences on the development of neural networks and the formation of neurites and growth cones. Rac plays an important role in the regulation of actin skeletal structure, migration, metabolism, transformation, gene expression, and cell cycle progression.[9] We hypothesized that long-term abuse of MA would induce the abnormal methylation of CHN2 gene, change the expression of chimeric protein 2, and affect the activity of Rac signaling system. This mechanism resulted in the remodeling of the actin cytoskeleton and the adaptive changes in neurons, which eventually led to the formation of addictive behavior.[16] In addition, abnormal methylation of the CHN2 gene could also lead to addiction behavior by affecting JNK signaling pathway. JNK signaling pathway in the hippocampus is involved in learning and memory mechanism and is related to addictive behavior.[17, 18] In addition, it has been reported that chimeric protein 2 might be involved in the regulation of JNK signaling in the hippocampal neurons.[19]

Chimeric protein-2 interfering with the remodeling of actin skeleton by Rac GTPase system led to the abnormal formation of neurites and growth cones, which is considered one of the important mechanisms of addictive behavior. This research showed that long-term MA addiction caused the methylation changes

of CHN2 gene promoter. All of the findings provide a theoretical basis for further exploring the correlations between CHN2 gene methylation with MA addiction.

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Conflict of interest statement

The authors declare no conflict of interest related to this manuscript.

Ethical Approval

The study was approved by the ethics committee of Xiangya hospital of Central South University

Authors' contributions

Liu Hao and Wei Hao participated in the design of the study and coordination and helped to draft the manuscript.

Tao Luo and Huiqian Dong performed the data collection.

Aiguo Tang performed the statistical analysis.

嵌合蛋白 -2 基因启动子区甲基化改变可能与甲基苯丙胺成瘾有关

郝柳，罗涛，董慧茜，唐爱国，郝伟

背景：我国甲基苯丙胺滥用形势十分严峻，已成为我国第一大毒品。甲基苯丙胺依赖的机制尚不完全清楚。CHN2 基因编码嵌合蛋白 -2，该蛋白可通过 Rac GTP 酶系统调控轴突修剪，在神经系统功能环路的形成中起关键作用。遗传学研究显示该基因的多态性与物质依赖形成相关。

目的：本研究旨在研究 CHN2 基因启动子区甲基化改变与甲基苯丙胺依赖的关系，以探索甲基苯丙胺依赖的新机制。

方法：采用美国精神障碍分类诊断标准第四版（DSM-IV）轴 I 障碍定式检查工具中文版对强制戒毒所的甲基苯丙胺依赖者进行调查，筛选出 224 名男性依赖者纳入病例组。另外选择年龄匹配的 109 名身体健康的男性作为对照组。采取受检者静脉血液，采用 Methylight qPCR 技术测定 CHN2 基因启动子区甲基化状态，统计分析比较两组的检测结果。

结果：甲基苯丙胺依赖组 CHN2 基因启动子区甲基化程度为 2795.55（733.19），正常对照组为 1026.73（698.73），两组存在显著差异，P 值为 0.00。相关分析显示 CHN2 基因启动子区甲基化程度与开始使用甲基苯丙胺的年龄、使用甲基苯丙胺的总时间、合并使用其它精神活性物质无明显关联。

结论：CHN2 基因启动子区甲基化改变与甲基苯丙胺依赖显著相关。

关键词：甲基苯丙胺依赖；DNA 甲基化；表观遗传；CHN2 基因

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