Association of human serotonin receptor 4 promoter methylation with autism spectrum disorder

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Abstract

Human serotonin receptor 4 (HTR4) encodes a 5-HT4 receptor involved in learning, memory, depression, anxiety, and feeding behavior. The aim of this study was to investigate the association between the deoxyribonucleic acid (DNA) methylation of HTR4 promoter and autism spectrum disorder (ASD), a disease characterized by communication disorder and repetitive or restrictive behavior.

Peripheral blood DNA was obtained from 61 ASD children and 66 healthy children, and the DNA methylation of HTR4 promoter was assessed by quantitative methylation-specific polymerase chain reaction. We used percentage of methylated reference (PMR) to represent DNA methylation level.

Due to significant age differences between ASD cases and controls (3 [2, 5] years and 6 [5, 6] years, \( P = 3.34E-10 \)), we used binary logistic regression analysis for adjustment. Our results showed that the DNA methylation levels of HTR4 promoter were significantly lower in children with ASD than in healthy children (median PMR: 66.23\% vs 94.31\%, \( P = .028 \), age-adjusted \( P = .034 \)). In addition, the DNA methylation of HTR4 promoter was inversely associated with age in male ASD cases (total cases: \( r = -0.283 \), \( P = .027 \); male cases: \( r = -0.431 \), \( P = .002 \); female cases: \( r = -0.108 \), \( P = .752 \)). Dual-luciferase reporter gene assay showed that the reporter gene expression in the strain with recombinant pGL3-promoter-HTR4 plasmid was significantly higher than that in the strain with pGL3-promoter plasmid (fold change = 2.01, \( P = .0065 \)), indicating that the HTR4 promoter fragment may contain transcription factors to upregulate promoter activity.

Our study suggested that hypomethylation of the HTR4 promoter is a potential biomarker for predicting the risk of male ASD.

Abbreviations: ABC = the autism behavior checklist, ASD = autism spectrum disorder, CARS = child autism rating scale, HTR4 = human serotonin receptor 4, PMR = percentage of methylated reference, qMSP = quantitative methylation-specific polymerase chain reaction.

Keywords: autism spectrum disorder, DNA methylation, HTR4, promoter, qMSP

1. Introduction

Autism spectrum disorder (ASD) is a progressive neurological disorder characterized by social communication disorder and special repetitive behavior that can ultimately lead to permanent disability.\cite{1,2} Recent epidemiological studies have shown that ASD is estimated to be 1\% of the general population in the United Kingdom.\cite{3} In China, among children aged 18 to 36 months, the prevalence of ASD in Tianjin city of China is 0.275\%, and the
ratio of male to female is 4:1. Although drug and behavioral training program are used to treat ASD, side effects and excessive cost limit the prognosis and recovery of ASD. Therefore, it is important to find biomarkers for the early diagnosis and treatment of ASD in the context of increased social burden.

The etiology of ASD is complex, including genetic and environmental factors. The global incidence of soaring ASD may be caused by the deterioration of environment. Epigenetic modifications are mediators of genetic and environmental elements. Among them, deoxyribonucleic acid (DNA) methylation has been shown to play an important role in the development of ASD. Studies have shown that in nearly 15% of ASD brain tissues, the CpG island at the SHANK3 promoter was found to be hypermethylated, and this subsequently altered the expression of SHANK3. As a member of the Shank gene family, SHANK3 codes Shank proteins which are multidomain scaffold proteins of the postsynaptic density. Mutations in this gene are a cause of ASD. OXTR encodes protein which acts as a receptor for oxytocin. And the oxytocin-oxytocin receptor system plays an important role in the uterus during parturition. ASD is associated with preterm birth, and the promoter methylation of OXTR (a ASD candidate gene) was found to be higher in premature fetal membranes. Immunohistochemical analysis showed that increased methylation and decreased protein expression of RORA and BCL2 genes were found in the brain of ASD patients. The protein encoded by RORA is a member of the NR1 subfamily of nuclear hormone receptors and BCL2 encodes a outer mitochondrial membrane protein blocking apoptosis.

Human serotonin receptor 4 (HTR4) encodes a member of the serotonin receptor family that can stimulate cAMP production and plays an important role in the regulation of releasing various neurotransmitters in the peripheral and central nervous systems. Serotonin in the brain promotes prosocial behavior and correctly assesses social emotional information. Peripheral high serotoninemia is one of the few consistent findings in autism, since it may deplete the serotonin in the central nervous system. Serotonin receptor 4 controls many physiological effects, including the central nervous system. HTR4 has been found to be involved in many genetic studies of neuropsychiatric disorders, including bipolar disorder and schizophrenia. However, there are few studies of HTR4 with autism.

The serotonin system was found to be associated with ASD. A disruption of the serotonin system in ASD leads to elevated levels of serotonin. Selective serotonin reuptake inhibitors have been reported to successfully treat a variety of symptoms of ASD, including repetitive behavior and compulsions, depression and insomnia. The serotonin concentrations in the brains of ASD patients were significantly lower than those in the brains of controls. The serotonin depletions may be due to genetic abnormalities in the serotonin related genes. The ITGB3 gene encodes the integrin β3 subunit, and variations in this gene reduce the integrin β3 signaling that subsequently affect serotonin function via its modification of the 5-HT level in the blood. A chromosomal break-point near the HTR4 gene was found in the genome of an ASD male patient.

However, there is no study on the DNA methylation of HTR4 promoter in ASD. Based on the above findings, we aimed to investigate the association between the DNA methylation of HTR4 promoter and ASD, and to explore whether age is an influencing factor for ASD related to the methylation level of the promoter of HTR4.

2. Methods and materials

2.1. Subjects

A total of 61 ASD children and 66 healthy children were collected from the Kangning Hospital of Ningbo City, Zhejiang Province. The ASD cases consisted of 30 males and 11 females. The age of the case was 4.02 ± 2.83 (mean ± standard deviation) years. The age of control group was 5.76 ± 0.72 years. The age differences between ASD cases and controls is significant (P = 3.34E-10). According to the diagnostic and Statistical Manual of Mental Disorders 5 (American Psychiatric Association, 2013), patients were diagnosed with ASD by at least 2 psychiatric physicians in Ningbo Kangning Hospital (Ningbo, Zhejiang, China). At the same time, the child autism rating scale (CARS) and the autism behavior checklist (ABC) were used to confirm the accuracy of the diagnosis. The tested phenotypes included 20 clinical features including sensory ability, communication ability, exercise ability, language ability, self-care ability, interpersonal relationship, imitative ability (words and actions), emotional response, physical exertion, relationship with non-living objects, adaptation to environmental changes, visual response, auditory response, near-sensory response, anxiety response, verbal communication, nonverbal communication, activity level, intellectual function and overall impression. The scores of the ABC scale of the 61 ASD children were 74.98 ± 34.91, and the score of the CARS scale was 34.18 ± 14.57. The study case had an ABC score of >31 and a CARS score of >30. Patients with mental retardation, congenital/hereditary disease or severe physical illness were excluded from the study. In the control group, 66 children (35 males and 11 females) had complete physical examination and no family or individual history of neurological or psychiatric illness. Besides, the sex distribution between cases and controls is not significant (P = .135). Our research was approved by the ethics committee of Ningbo university. Written informed consent forms were signed by the guardians of all participants.

2.2. Methods

Quantitative methylation-specific polymerase chain reaction (qMSP): Peripheral blood was collected from tubes treated with 3.2% sodium citrate and stored at −80°C. Our previous article described the details of blood DNA separation and bisulfite conversion. qMSP was used to detect the DNA methylation of HTR4 promoter. The forward and reverse sequences of HTR4 were 5′-GTGTGGTATAGGGTGATAGTT-3′ and 5′-GATATCGAAGGCACTCCTTAA-3′, respectively. The forward and reverse sequences of ACTB were 5′-TGTTAGGGAGGTGTATGATT-3′ and 5′-AACCAATAAAACCTATCCCTCCCTTAA-3′, respectively. Our previous work showed the details of qMSP. Each 384 plate contained the participant’s DNA sample, a positive control, and multiple water sample as a blank control. The percentage of methylated reference (PMR) value was calculated using the method described previously.

Plasmid transfection and dual luciferase reporter assay: We chemically synthesized a fragment of the HTR4 promoter (657 bp to −566 bp) and digested with Xhol and Kpn1 (New England Biolabs, Ipswich, MA). Purified by cycle pure kit (Omega, Norcross, GA), the target DNA fragment was cloned into the pGL3 promoter vector (Promega, Madison city, WI) using a DNA ligation kit (TaKaRa, Japan). We used the empty pGL3 Basic vector as a negative control and used the pGL3 promoter
vector containing the SV40 promoter upstream of the luciferase gene as a positive control. We prepared cells using 96-well plates, and the details of plasmid transfection of human embryo kidney 293T (HEK293T) cells were as previously described. The culture method of the HEK293T cell line was as described previously. After 18 to 72 hours of HEK293T cell transfection, luciferase activity was determined using a dual luciferase reporter assay system (dual-luciferase reporter assay systems, Promega). Renilla and firefly luciferase activities were measured by SpectraMax 190 (Molecular devices, Sunnyvale).

2.3. Statistical analysis

The non-parametric Mann–Whitney U test was used to compare the DNA methylation of HTR4 promoter, age, and sex between ASD cases and healthy controls. Binary logistic regression analysis was used for adjustment of age differences. The Spearman correlation coefficient method was used to quantify the relationship of HTR4 DNA methylation with age and HTR4 expression. A P value of less than .05 was considered to be significant.

3. Results

As shown in Figure 1, we selected a fragment of the promoter region (hg38, chr5: 148032968–148033058) for HTR4 DNA methylation assay, which contained 2 CpG sites. Due to age differences between cases and controls (3 [2, 5] years and 6 [5, 6] years, P = 3.34E-10), binary logistic regression analysis was used to adjust age differences. Our results showed that the DNA methylation levels of HTR4 promoter were significantly lower in ASD cases than in healthy controls (Fig. 2, median PMR: 66.23% versus 94.31%, P = .028, age-adjusted P = .034).

We also observed gender differences in the DNA methylation of HTR4 promoter. The level of the DNA methylation of HTR4 promoter in male ASD cases was significantly lower than in the male control group (Fig. 2, median PMR: 65.38% vs 93.07%, P = .008, age-adjusted P = .014). However, we did not find a link between the DNA methylation of HTR4 promoter and ASD in females (P > .05, age-adjusted P > .05, Fig. 2). The DNA methylation of HTR4 promoter was found to be significantly higher in female cases than in male cases (Fig. 2, median PMR: 120.40% vs 65.38%, P = .013, age-adjusted P = .013). In addition, no differences in the DNA methylation of HTR4 promoter were found between male and female controls (P > .05, age-adjusted P > .05, Fig. 2).

We observed a significant inverse correlation between age and the DNA methylation of HTR4 promoter in the ASD cases (Fig. 3, r = -0.283, P = .027). A gender-based subgroup analysis found that the significant association between age and the DNA methylation of HTR4 promoter only existed in male cases, but not in female cases (Fig. 3, male cases: r = -0.431, P = .002; female cases: r = -0.108, P = .752). There was a moderate inverse correlation between age and the DNA methylation of HTR4 promoter in the controls (r = -0.224, P = .071).

Subsequently, a dual luciferase reporter gene assay was performed to examine whether the HTR4 gene fragment (~657 bp to ~566 bp) was able to increase promoter activity. Our results showed that the transcriptional activity of the recombinant pGL3-Promoter-HTR4 plasmid was significantly higher than that of the pGL3-promoter plasmid (Fig. 4, fold change = 2.01, P = .0065). In addition, TCGA data (http://www.chiopor4tal.org/) also demonstrated an inverse correlation between HTR4 expression and HTR4 DNA methylation (Fig. 5, r = -0.215, P = .002).

Finally, we have checked the influence of the outliers on the statistics, including four points in Figure 2 and 2 points in Figure 3, and have found that these samples did not make big differences to the results (all P < .05).

4. Discussion

This study demonstrated that the DNA hypomethylation of HTR4 promoter was significantly associated with ASD. Our results also showed that the DNA methylation of HTR4 promoter was significantly lower in male patients with ASD than in healthy male controls, and that the DNA methylation of HTR4 promoter was significantly lower in male ASD patients than in female ASD patients. In addition, a significant inverse correlation between age and DNA methylation was found in ASD cases, especially in male cases.

HTR4 encodes a serotonin receptor expressed in the central nervous system. HTR4 is involved in learning and memory, depression, anxiety, and feeding behavior. HTR4 is known to be involved in the regulation of neurotransmitter release, and irregular neurotransmission of serotonin has been shown to be involved in a variety of neuropsychiatric disorders. Although there is little research between HTR4 and ASD, HTR4 is associated with other neurological diseases. ASD, schizophrenia spectrum disorders and symptoms of bipolar disorder may overlap with biopsychosocial risk factors. Previous studies found that HTR4 polymorphism may contribute to bipolar disorder and ADHD.

The number of ASD boys diagnosed in all ethnic, and socioeconomic groups is 4 to 5 times higher than that of girls, but the underlying mechanisms for gender differences have not been established. Early studies have found that females have higher levels of serotonin in their brains compared to males, which can be observed on the second day of neonatal early days and persist throughout adulthood. The serum serotonin concentration in patients with ASD was significantly lower, suggesting that men with a lower serum serotonin concentration are more susceptible to ASD. Our work suggested that the DNA of HTR4 promoter was significantly hypomethylated in ASD men, and this finding may help elucidate the molecular mechanisms of ASD.

Studies have shown that folic acid can regulate DNA methylation and affect gene expression. In order to determine whether folate intake during pregnancy changes the gene expression of neonatal cerebral hemispheres, scientists examined HTR4 methylation in mice, and confirmed a downregulation of HTR4 in folate changes. A recent report shows that folic acid intake in Norwegian pregnant women from 4 weeks before pregnancy to 8 weeks after pregnancy helps reduce the risk of autism in newborns. Lack of pleasure may be associated with down-regulation of HTR4 expression in the hippocampus. In patients with pulmonary embolism, a negative feedback mechanism showed that the expression of HTR4 was downregulated. However, studies have also found that HTR4 is highly expressed in patients with functional dyspepsia. Increased expression of 5-HT4R in high Gleason grade tumours may be involved in tumor progression through 5-HT-induced increases in hormone or growth factor levels. Our dual luciferase reporter assay indicated that the HTR4 promoter-specific region contains potential regulatory elements. The HTR4 promoter
Figure 1. Target sequence on HTR4 CpG island (CGI) region. Two CpG sites are located on HTR4 gene promoter. F: forward primer; R: reverse primer. The genomic positions and function annotations of HTR4 were obtained from UCSC genome browser according to human 2013 (GRCh38/hg38). (B) Sequencing validation of the qMSP product. The first row of the sequences represents the original gene sequence, and the second row shows the converted sequence. The fragment length of qMSP product was 91 bp as expected. HTR4 = human serotonin receptor 4, qMSP = quantitative methylation-specific polymerase chain reaction, UCSC = University of California, Santa Cruz.
fragment may contain a transcription factor capable of upregulating promoter activity.

The current age threshold for diagnosing ASD is 2 years and the typical age for diagnosis is 4. Adaptive behavior includes important behaviors of independent living, including daily life skills, social skills, and communication skills. Children with ASD show adaptive behavioral disorders in all areas. Previous studies have found that patients with ASD have lower adaptive behavior scores.\(^{48}\) Age showed an inverse correlation with adaptive behavior of ASD.\(^{49}\) In young children, there is a moderate to strong association between ASD severity and adaptive behavior.\(^{50}\) In the current study, an inverse correlation between the DNA methylation of HTR4 promoter and age was found in male ASD cases. This may help explain the contribution of the DNA methylation of HTR4 promoter to ASD risk in different age groups.

To the best of our knowledge, this is the first report of the association between the DNA methylation of HTR4 promoter and ASD, but there are some limitations. First of all, our sample size is limited, especially for female samples. More female samples are needed in the future to investigate whether the DNA methylation of HTR4 promoter is associated with ASD in females. Second, since our work involves only Chinese people, it is necessary to confirm the correlation between the DNA methylation of HTR4 promoter and ASD in other ethnic groups in the future. Finally, although TCGA data analysis confirmed...
that HTR4 DNA methylation was negatively correlated with expression, we did not conduct experiments to assess the correlation between HTR4 DNA methylation and expression due to sample limitations, and future work needs to validate this association.

5. Conclusion
Taken together, our study suggested that hypomethylation of the HTR4 promoter is a potential biomarker for predicting the risk of male ASD. Our findings may provide new insights into the pathogenesis of ASD.

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