Significant association between *GPR50* hypomethylation and AD in males

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Abstract. Alzheimer's disease (AD) is a chronic neurodegenerative disease. G protein coupled receptor 50 (GPR50) is a candidate gene for AD. The present study was designed to determine the association between GPR50 methylation and AD. The methylation levels of the GPR50 promoter in 51 patients with AD and 61 healthy controls were determined by bisulfite pyrophosphate sequencing. All participants were Han Chinese, living in Ningbo. It was identified that the GPR50 promoter methylation level was significantly decreased in the male AD group compared with the male control group (9.15 vs. 16.67%, P=0.002). In addition, it was observed that the GPR50 methylation levels of the females was significantly increased compared with that of males in both the patients with AD and the healthy control group (AD patient group: 33.00 vs. 9.15%, P<0.0001; healthy control group: 29.41 vs. 16.67%, P<0.0001). This may be explained by the fact that GPR50 is located on the X chromosome. In addition, GPR50 methylation was positively correlated with plasma cholinesterase levels in female patients with AD (r=0.489, P=0.039). The present study demonstrated that hypomethylation of the GPR50 promoter in peripheral blood may be a potential biomarker for the diagnosis of AD in Chinese Han males.

Introduction

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disease with symptoms that are primarily characterized by progressive cognitive decline in patients (1). AD cases in the elderly population accounts for >60% of all types of dementia (2). At present, there are no effective interventions for AD, and the specific pathogenesis of AD is not clear. Therefore, studies investigating AD have attracted much attention and it has become a worldwide problem (3).

Epigenetic modification is the result of a combination of genetic and environmental factors (4,5). With the progress of studies in epigenetics, the association between epigenetic modification and AD has received extensive attention. DNA methylation is one of the most important epigenetic modifications; it occurs primarily in the CpG island of the promoter region and is involved in biological processes including X chromosome inactivation and cell differentiation (6,7). Previous data have highlighted the potential role of DNA methylation in AD. For example, increased methylation of bridge integron 1, associated with AD pathology, was identified in aging mice and transgenic AD mouse models (8). Similarly, ankyrin 1 was demonstrated to be more methylated in the olfactory cortex of patients with AD compared with in the control group (9). In addition, the protein products of certain genes (including amyloid beta precursor protein, microtubule associated protein Tau and beta-secretase 1) are directly involved in the aberrant DNA methylation in AD (10,11).

G protein-coupled receptors (GPCRs) are a general term for a class of cell surface receptors that bind and regulate G proteins. They participate in the maintenance of various biological functions by binding to different ligands, including neurotransmitters, peptides and lipids, to activate internal signal transduction pathways, such as regulating neuronal discharge, mediating intra- and extra-membrane ion transport, and controlling cell proliferation and differentiation. Disrupting these events may lead to diseases. One study confirmed that GPCRs are involved in the pathological processes of various diseases, including depression and neurodegenerative diseases (12), which makes GPCRs potential targets for the treatment of a number of diseases. Nearly 30% of all clinical drugs on the market are developed to target GPCRs (13). Previous studies have indicated that GPCRs serve an important role in the pathogenesis of AD (14).

G protein-coupled receptor 50 (GPR50) belongs to the family of melatonin receptor and is located on the X chromosome.

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GPR50 encodes a protein similar in chemical structure to the melatonin receptor (15). GPR50 is highly expressed in the hypothalamus, pituitary gland and blue plaques (16), and serves an important role in energy metabolism, thermoregulation and the stress response (17). Associated studies have suggested that functional changes in GPR50 may be associated with mental illness, and that it serves a key role in regulating stress and anxiety-associated diseases (18-20). Among Scottish females, GPR50 was identified as a risk gene for major depression and bipolar disorder (21). GPR50 is most closely associated with melatonin receptors. GPR50 and the melatonin receptors MT1 and MT2 have ~45% amino acid sequence identity; GPR50 is able to bind to MT1 and MT2 to form heterodimers, and GPR50 and MT1 binding inhibits the binding of melatonin to MT1, thereby inhibiting the melatonin signaling pathway (22). The melatonin signaling pathway serves an important role in the pathological progression of AD and studies have indicated that melatonin is able to alleviate the pathology of patients with AD (23). Based on the above results, we hypothesized that GRP50 may serve an important role in the pathogenesis of AD. The aim of the present study was to determine whether GPR50 methylation was associated with AD.

Materials and methods

Sample collection. A total of 51 patients with sporadic AD (27 males and 24 females, age range: 53-96) and 63 normal controls (39 males and 24 females, age range: 62-93) from Ningbo First Hospital (Ningbo, China) and Ningbo Kangning Hospital (Ningbo, China) we enrolled in the present study from September 2016 to September 2017. All participants were Han Chinese, and were living in Ningbo, Zhejiang province. All medical examinations, including neurological examinations, hematology studies, medical and family history collection, brain imaging examinations (computed tomography or magnetic resonance), neuropsychological examinations and cognitive screening examinations were performed based on the ICD-10 diagnostic criteria (24,25). Patients with sporadic AD were diagnosed by two experienced neurologists. The present study was approved by the Ethics Committee of Ningbo University and Ningbo First Hospital. Informed consent was provided by all participants or their guardians. The detection methods used for the biochemical parameters were performed as described previously (26) The concentration of blood metabolites, including triglycerides, total cholesterol, homocysteine (Hcy), blood glucose, high density lipoprotein, lipoprotein A, albumin,alanine aminotransferase and C-reactive protein (CRP), globulin, total bile acids, creatinine, uric acid, cholinesterase (ChE) and alkaline phosphatase were measured for each participant.

Bisulfite pyrosequencing assay. DNA was extracted from peripheral blood using a nucleic acid extraction analyzer (Lab-Aid® 820, Xiamen Zeesan Biotech Co., Ltd, (Xiamen, China) as previously described (26). DNA concentrations were determined by using the ultramicro nucleic acid ultraviolet tester (NanoDrop 2000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.). DNA was prepared by sodium bisulfite DNA transformation chemistry using a EZ DNA Methylation-Gold™ kit (Zymo Research Corp.) and polymerase chain reaction (PCR) amplification using the Pyromark PCR kit (Pyromark Gold Q24 Reagents; Qiagen Inc.). The recommended reaction condition was set according to the manufacturer's protocol (95˚C for 15 mins followed by 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec, and 70˚C for 30 sec). To detect GPR50 methylation levels, pyrophosphate sequencing analysis was performed using a Pyromark Q24 instrument. The PCR primers for methylation quantification were as follows: Forward primer: 5'-GGGGATTTAGAGAGG TTGTAAAG-3'; reverse primer: 5'-biotin-CCAACCTAT AAACCCAACTAACTACTCTAC-3'; and sequencing primer: 5'-GGGATTTTTTTAGTTGTTAGTTAT-3'. The results of pyrosequencing results are presented in Fig. 1.

Statistical analysis. All of the statistical analyses were performed by Statistical Program for Social Sciences (SPSS) software 16.0 (SPSS, Inc.) and a $P<0.05$ was considered to indicate a statistically significant difference. An independent t-test or Mann-Whitney U rank sum test were used to determine differences in baseline data between AD cases and controls. The association between GPR50 methylation and metabolic characteristics was assessed by Spearman correlation analysis.

Results

In the present study, GPR50 methylation in blood samples from patients with AD and controls was measured. The CpG island region of the GPR50 promoter (chrX: 150346251-150346464) was subjected to sodium bisulfite pyrosequencing. As demonstrated in Fig. 2, a total of 7 CpG sites were assessed. There was a significant correlation among the methylation of the 7 CpG sites (male: $r>0.754$, $P<0.001$, female: $r>0.887$, $P<0.001$, Fig. 2); therefore, the average DNA methylation of the 7 CpGs was used in subsequent analyses.

A total of 51 patients with AD and 61 controls were included in the present study. As shown in Table I, among the 19 clinical features, albumin, plasma levels of lipoprotein A, Hcy in the male groups and alanine aminotransferase, plasma levels of lipoprotein A, Hcy in the female AD groups were higher than in the control groups [$P=0.05$, $P=0.03$, $P=0.03$ (males), $P=0.04$, $P<0.01$, $P<0.01$ (females), respectively]. The increase in plasma Hcy level has long been considered a risk factor for AD, and recent studies have demonstrated that an increase in Hcy concentration increases total tau and phosphorylated tau and forms tau oligomers, thereby increasing AD risk (27,28). The levels of alanine aminotransferase and CRP in the AD group were lower than those in control group ($P=0.04$ and $P=0.02$). A previous study confirmed the decrease of plasma CRP levels in patients with moderate AD (29). In addition, lower CRP levels were previously hypothesized to be associated with increased rates of cognitive decline (30).

As GPR50 is located on the X chromosome, a stratified association analysis by sex was performed between the patients with AD and the control group (Table II). The results indicated a significantly decreased level of mean GPR50 methylation in the male AD group compared with in the male control group. ($P=0.002$). However, no significant differences were observed in the female AD group compared with the female control group.

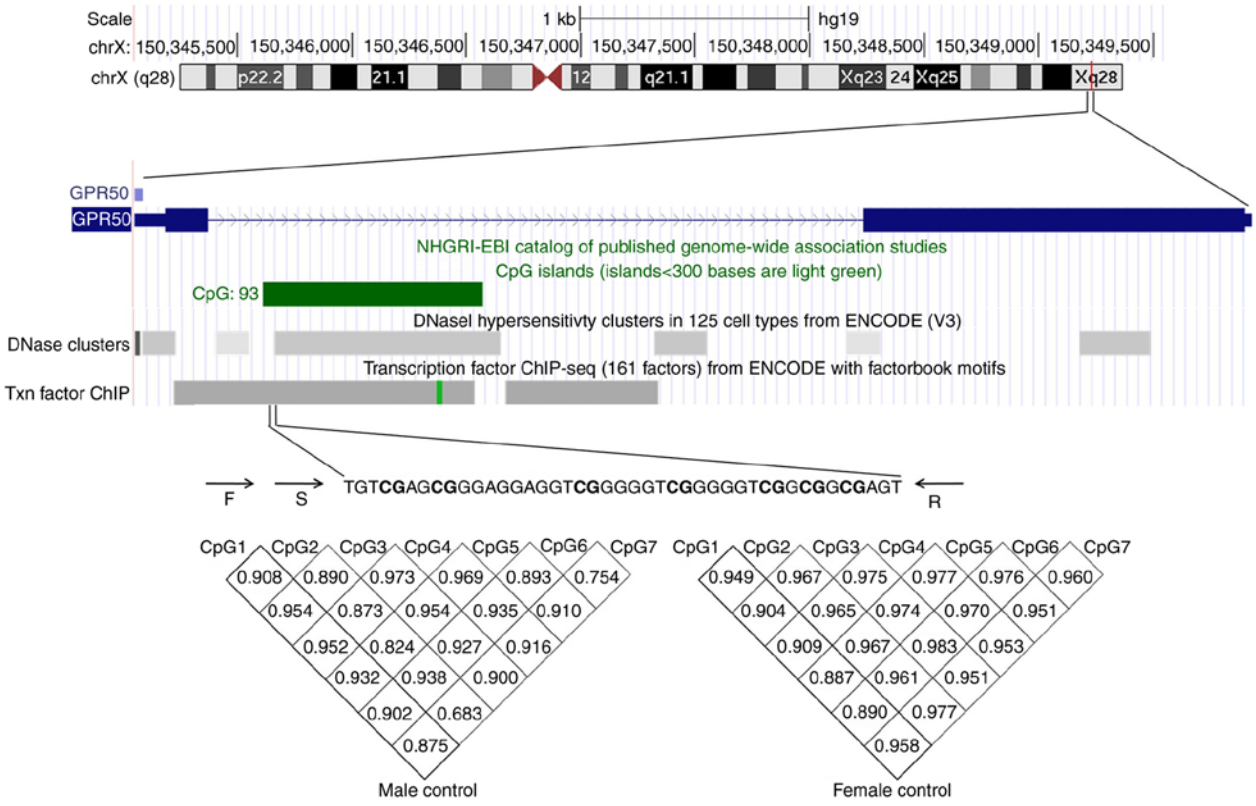

Figure 1. Bisulfite pyrosequencing of the amplified fragment from a case sample. The pyrogram (generated using the Qiagen PyroMark Q24 pyrosequencer) contains the 7 CpG sites (highlighted by blue shadowing). Proportions above each site in the graphs represent the corresponding proportions of DNA methylation. The grey arrows within the lower graph indicate individual CpG sites. CpG, 5'-C-phosphate-G-3'.

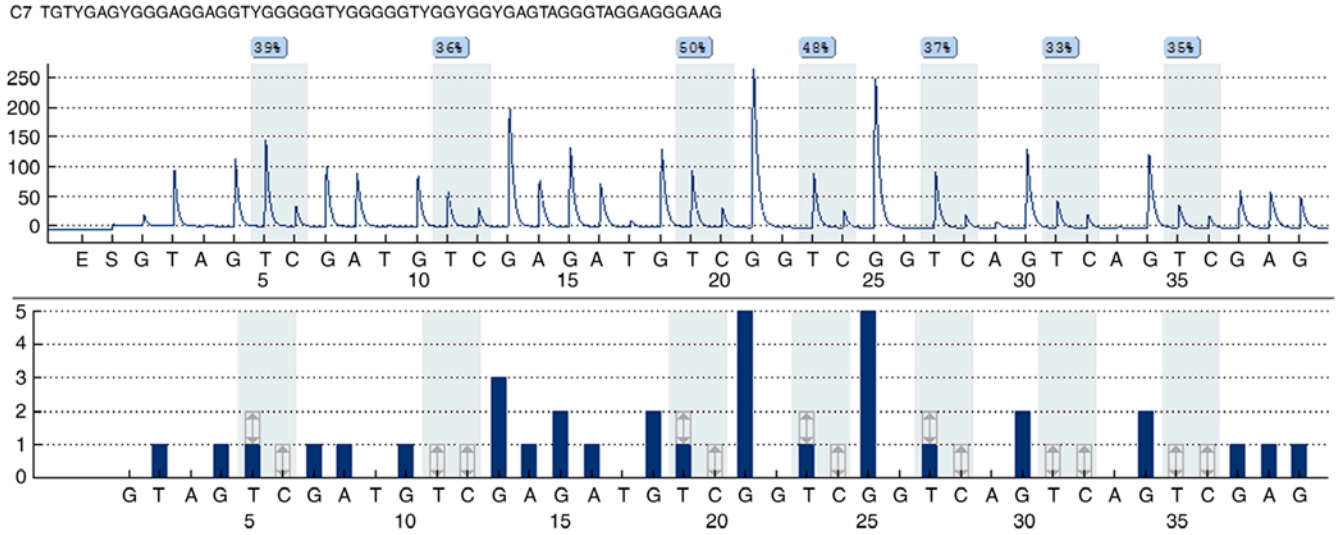

Figure 2. The target sequence is located in the CpG island region of GPR50. Correlations among 7 GPR50 promoter CpG sites. The target sequence was located in the CpG islands region (light green) of the GPR50 promoter. The F, R and S primer sequences are described in the Materials and methods section. The numbers in the lattice represent the correlation (correlation coefficient, r) between any two CpG sites. GPR50, G protein-coupled receptor 50; CpG, 5'-C-phosphate-G-3'; F, forward; R, reverse; S, sequencing; ChIP, chromatin immunoprecipitation.

To additionally confirm the differences in sex in GRP50 methylation, the levels of GPR50 methylation were compared between males and females (Table III). As expected, the GPR50 methylation levels in the female AD group were significantly increased compared with the levels in the male AD group ($P=2.3x10^{-9}$). Similarly, the GPR50 methylation levels in the female control group were also significantly increased compared with those in the male control group (P=0.0001). Subsequently, we determined the association between biochemical parameters and GPR50 methylation. As shown in Fig. 3, ChE was positively correlated with GPR50 methylation in female patients (r=0.489, P=0.039). No significant correlation was observed between GPR50 methylation and other parameters.

Table I. Characteristics of subjects from cases and controls.

A, Males

Characteristic	AD group	Control group	P-value
Age, years	85.00 (80.75,88.25)	83.00 (80.00,85.00)	0.14
BMI	20.96 (20.36,25.46)	22.99 (20.78,24.81)	0.52
ALB, g/l	38.06±2.50	35.55±3.66	0.05
GLB, g/l	29.60 (25.63,32.00)	36.30 (33.90,38.20)	0.72
ALT, U/l	10.00 (8.00,20.75)	12.00 (10.00,20.00)	0.27
ALP, U/l	69.00 (59.75,84.00)	77.00 (65.00,95.00)	0.22
TBA, μmol/l	6.75 (3.43,8.10)	5.10 (1.80,7.60)	0.24
Glu, mmol/l	4.59 (4.19,5.76)	4.79 (4.39,5.66)	0.32
TG, mmol/l	0.98 (0.64,1.51)	1.18 (0.72,1.76)	0.36
TC, mmol/l	4.06 (3.39,4.59)	3.63 (3.05,4.76)	0.21
HDL-C, mmol/l	1.00 (0.86,1.25)	0.94 (0.73,1.17)	0.13
ApoA, g/l	0.93 (0.78,1.13)	0.88 (0.77,1.02)	0.63
ApoB, g/l	0.60 (0.45,0.70)	0.56 (0.51,0.73)	0.67
Lp(a), g/l	1.76 (0.19,3.42)	0.28 (0.19,0.52)	0.03
ApoE, mg/l	23.80 (15.80,36.80)	32.60 (28.60,39.95)	0.53
CRE, μmol/l	87.50 (69.75,141.18)	87.00 (63.60,104.20)	0.11
UA, μmol/l	329.00 (280.25,433.50)	332.00 (256.00,379.00)	0.69
Hcy, μmol/l	19.00 (15.50,29.05)	14.90 (10.70,19.10)	0.03
CRP, mg/l	2.45 (0.38,13.61)	5.04 (1.53,17.81)	0.23

B, Females

Characteristic	AD group	Control group	P-value
Age, years	81.00 (70.25,85.00)	72.00 (68.00,82.50)	0.14
BMI	20.81 (19.99,26.27)	23.38 (20.43,25.38)	0.59
ALB, g/l	38.61±4.35	38.60±3.70	0.99
GLB, g/l	28.90 (26.80,33.25)	28.10 (25.50,33.10)	0.56
ALT, U/l	11.00 (10.00,14.00)	15.00 (11.00,25.50)	0.04
ALP, U/l	76.00 (61.00,102.00)	91.00 (68.00,123.50)	0.20
TBA, μmol/l	6.50 (3.20,9.93)	5.60 (1.80,9.40)	0.24
Glu, mmol/l	4.60 (4.26,5.35)	4.84 (4.40,5.49)	0.97
TG, mmol/l	1.25 (1.10,1.71)	1.06 (0.79,2.11)	0.27
TC, mmol/l	4.78 (4.21,5.65)	5.20 (4.40,6.29)	0.24
HDL-C, mmol/l	1.17 (1.02,1.38)	1.18 (0.98,1.42)	0.88
ApoA, g/l	1.12 (1.04,1.28)	1.00 (0.95,1.06)	0.13
ApoB, g/l	0.74 (0.59,0.83)	0.81 (0.76,1.07)	0.04
Lp(a), g/l	0.55 (0.26,1.31)	0.21 (0.10,0.60)	<0.01
ApoE, mg/l	43.80 (32.78,52.20)	35.90 (33.30,41.80)	0.28
CRE, μmol/l	64.00 (55.00,76.50)	53.00 (44.40,69.25)	0.11
UA, μmol/l	262.00 (215.50,354.50)	254.00 (205.50,306.50)	0.46
Hcy, μmol/l	16.20 (13.80,18.30)	13.40 (9.75,14.65)	<0.01
CRP, mg/l	2.05 (0.65,4.23)	3.06 (1.95,8.39)	0.10

Data are expressed as the median (quartile). AD, Alzheimer's disease; BMI, body mass index; ALB, albumin; GLB, serum globulin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TBA, total bile acid; Glu, blood glucose; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; Apo, apolipoprotein; Lp(a), lipoprotein(a); CRE, creatinine; UA, uric acid; Hcy, homocysteine; CRP, C-reactive protein.

Table II. Comparisons of *GPR50* methylation levels between cases and controls.

A, Males

Site	AD group	Control group	P-value
CpG1	7.12±6.48	14.85±10.06	**0.001**
CpG2	5.65±5.38	11.61±8.09	**0.002**
CpG3	14.46±9.49	22.76±12.60	**0.005**
CpG4	13.89±8.12	24.35±12.82	**0.0003**
CpG5	10.62±8.05	19.22±12.03	**0.003**
CpG6	5.62±5.13	11.80±7.93	**0.0002**
CpG7	6.44±5.63	12.57±8.63	**0.003**
mean	9.15±6.58	16.67±10.12	**0.002**

B, Females

Site	AD group	Control group	P-value
CpG1	31.54±4.71	26.23±10.35	0.123
CpG2	24.75±5.77	21.06±8.61	0.254
CpG3	44.33±7.15	39.35±13.42	0.441
CpG4	43.75±5.18	40.59±13.90	0.947
CpG5	37.46±5.34	33.94±12.04	0.652
CpG6	23.92±5.67	21.59±8.15	0.532
CpG7	24.92±6.39	22.94±9.47	0.482
mean	33.00±4.94	29.41±10.42	0.418

A two independent samples t-test was used. Bold font represents a significant difference between cases and controls.

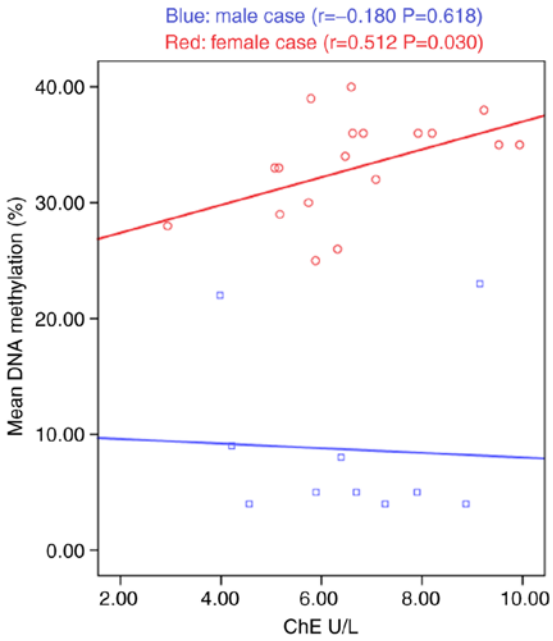

Figure 3. Correlation analysis between ChE and G protein-coupled receptor 50 promoter methylation in patients with AD. The degree of methylation in the female AD group was negatively correlated with ChE. Correlation analysis was performed using Pearson correlation analysis. ChE, cholinesterase.

Discussion

In the present study, pyrosequencing was used to analyze the promoter methylation level of GPR50 in patients with AD and control groups, to elucidate the association between GPR50 methylation and AD. The results indicated that the methylation level of the GPR50 promoter in the male AD group was significantly decreased compared with the control group. In addition, sex differences in GPR50 methylation were significantly different between the AD and the control groups. Correlation analysis suggested that ChE and GPR50 methylation were positively correlated in female patients with AD.

GPR50 knockdown may cause significant changes in the self-renewal of neurons and the inhibition of neuronal differentiation (31). Luciferase reporter experiments confirmed that GPR50 may regulate the self-renewal and neuronal differentiation of neural progenitor cells by regulating the Notch signaling and Wnt/β-catenin signaling pathways, suggesting that GPR50 was associated with mental illnesses including depression, affective disorder and AD (31). In addition, overexpression of GPR50 in neuronal cells increased axonal length, filamentous and lamellar lipid-like structures in differentiated neural sieve plate-1 cells, further indicating its potential role in AD (32). Another study demonstrated that GPR50 may affect neurite outgrowth through interacting with reticulon-4, and that the overexpression of reticulon-4 in the brain was associated with β-amyloid deposition in senile plaques (33). The analysis from the present study indicated that GPR50 methylation was decreased in the male AD group compared with the male control group, providing an important direction for future studies in AD, particularly in male AD.

It is well known that sex has an effect on the prevalence of AD, and females have a higher probability of developing AD compared with males (1,34). These differences may be affected by various factors; for example, estrogen in females is a neuroprotective factor, and females who have experienced menopause have an increased risk of developing AD (35,36). In addition, different exposure levels to smoking (37), stress (38) and various other genetic factors (39), may be responsible for this sex-based difference. The results from the present study indicated significant sex differences in the levels of GPR50 methylation. Inactivation of the X chromosome results in an increased level of methylation in females (40), which may be a reason for the relatively high level of GPR50 methylation in female subjects in the cohort from the present study.

Cholinergic system dysfunction has been confirmed by clinical studies to have an association with AD, and abnormal cholinergic signaling may trigger a decline in cognitive function (41-43). ChE inhibitors have also been commonly used in the treatment of AD (44,45). These data suggest a potential role for GPR50 in the treatment of AD. In the present study, it was observed that ChE levels were associated with GPR50 methylation in female AD, and this result suggests a target for ChE-associated therapy.

Although the data from the present study indicated that the male AD group exhibited a decreased level of GPR50 methylation compared with the control group, whether this would affect the expression level of GPR50 was not assessed. In addition, the analysis was performed with peripheral blood samples

Table III. Comparisons of *GPR50* methylation levels between males and females.

A, AD group

Site	Male Mean ± SD	Female Mean ± SD	P-value
AD group			
CpG1	7.12±6.48	31.54±4.71	**$1.7x10^{-9}$**
CpG2	5.65±5.38	24.75±5.77	**$3.5x10^{-9}$**
CpG3	14.46±9.49	44.33±7.15	**$1.0x10^{-8}$**
CpG4	13.89±8.12	43.75±5.18	**$1.7x10^{-9}$**
CpG5	10.62±8.05	37.46±5.34	**$2.4x10^{-9}$**
CpG6	5.62±5.13	23.92±5.67	**$4.0x10^{-9}$**
CpG7	6.44±5.63	24.92±6.39	**$6.7x10^{-9}$**
mean	9.15±6.58	33.00±4.94	**$2.3x10^{-9}$**

B, Control group

Site	Male Mean ± SD	Female Mean ± SD	P-value
CpG1	14.85±10.06	26.23±10.35	**$2.0x10^{-4}$**
CpG2	11.61±8.09	21.06±8.61	**$2.0x10^{-4}$**
CpG3	22.76±12.60	39.35±13.42	**$1.0x10^{-4}$**
CpG4	24.35±12.82	40.59±13.90	**$2.0x10^{-4}$**
CpG5	19.21±12.03	33.94±12.04	**$1.0x10^{-4}$**
CpG6	11.80±7.93	21.59±8.15	**$2.0x10^{-4}$**
CpG7	12.56±8.63	22.94±9.47	**$2.0x10^{-4}$**
mean	16.67±10.11	29.41±10.42	**$1.0x10^{-4}$**

A two independent samples t-test was used. Bold font represents a significant difference between males and females. SD, standard deviation.

only, and may not accurately reflect the conditions within the brain tissue. However, GPCR-mediated signal transduction was reported to serve an important role in cerebrospinal fluid (CSF); for example, in GPR157, an orphan GPCR identified in the primary cilia located in radial glial progenitor cells (RGPs) exposed to CSF (46). It has been confirmed that GPR157 binds to heterotrimer G protein and signals through Ca^{2+} cascade mediated by IP3. The activation of the GPR157-GQ signaling pathway promoted the neuronal differentiation of RGPS, while interference with the GPR157-GQ-IP3 cascade inhibited the neurogenesis of RGPS (46). In addition, the GPR157-GQ signal on the primary cilia of RGPS was activated by CSF and participated in neurogenesis (46). Finally, the present study was a case-control study, and additional analysis is required to investigate how GPR50 promoter methylation affects the development of AD.

In conclusion, the results of the present study indicated that GPR50 methylation was associated with male AD. Subsequent studies are required to clarify the specific mechanisms associated with GPR50 methylation in AD.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

WC and HJ performed the majority of the experiments, data collection, statistical analysis, data interpretation and wrote the manuscript. LL, CX and XZ performed sample collection, the biochemical tests and collated the data. TZ, WC and SX analyzed the data and revised the manuscript. SD and QW revised the manuscript critically for important intellectual

content, designed the overall study, supervised the experiments, analyzed the results and wrote the paper.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Ningbo University. Informed consent was provided by all participants or their guardians.

Patient consent for publication

Informed consent was provided by all participants or their guardians.

Competing interests

The authors declare that they have no competing interests.

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