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## Original Research Article

# Association between DNA Methylation of the *BDNF* Promoter Region and Clinical Presentation in Alzheimer's Disease

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## Key Words

Brain-derived neurotrophic factor · *BDNF* · DNA methylation · Alzheimer's disease · Epigenetics · Neurotrophic factors

## Abstract

**Background/Aims:** In the present study, we examined whether DNA methylation of the brain-derived neurotrophic factor (*BDNF*) promoter is associated with the manifestation and clinical presentation of Alzheimer's disease (AD). **Methods:** Of 20 patients with AD and 20 age-matched normal controls (NCs), the DNA methylation of the *BDNF* promoter (measured using peripheral blood samples) was completely analyzed in 12 patients with AD and 6 NCs. The resulting methylation levels were compared statistically. Next, we investigated the correlation between the DNA methylation levels and the clinical presentation of AD. **Results:** The total methylation ratio (in %) of the 20 CpG sites was significantly higher in the AD patients ( $5.08 \pm 5.52\%$ ) than in the NCs ( $2.09 \pm 0.81\%$ ;  $p < 0.05$ ). Of the 20 CpG sites, the methylation level at the CpG4 site was significantly higher in the AD subjects than in the NCs ( $p < 0.05$ ). Moreover, the methylation level was significantly and negatively correlated with some neuropsychological test subscores (registration, recall, and prehension behavior scores;  $p < 0.05$ ). **Conclusion:** These results suggest that the DNA methylation of the *BDNF* promoter may significantly influence the manifestation of AD and might be associated with its neurocognitive presentation.

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## Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is mainly characterized by memory disorder, visuospatial impairment, attentional impairment, and executive dysfunction as its core symptoms, leading to neuropsychiatric problems [1–3]. The deposition of causative proteins, such as amyloid-beta ( $A\beta$ ) and tau ( $\tau$ ), in the pathogenesis of AD has been reported previously and has been focused on as a possible therapeutic target [4, 5]. Since the 1990s, apolipoprotein E (*APOE*) has been reported as a strong candidate gene for sporadic AD, and this speculation has been verified in a large sample genome-wide association study [6, 7]. The pathophysiological mechanism responsible for the effect of *APOE* polymorphisms on degenerative alterations in AD patients has been shown to involve *APOE4*-mediated neurovascular vulnerability, leading to neural loss or dysfunction [8]. Moreover, the CpG methylation alterations of the amyloid precursor protein (*APP*), microtubule-associated protein tau (*MAPT*), and glycogen synthase kinase-3 $\beta$  (*GSK3B*) genes in neuronal cells have been reported as pathogenic mechanisms of sporadic AD [9]. Thus, among some epigenetic markers, such DNA methylations have been implicated as crucial biomarkers of AD [10].

Brain-derived neurotrophic factor (BDNF) promotes the development and survival or the maintenance function of neural cells [11]. From a neuroprotective viewpoint, BDNF secretion within the synapse cleft is modulated by an activity-dependent change and long-term potentiation, which influences cognitive function and protects against neural cell death caused by the aggregation of  $A\beta$  and  $\tau$  proteins in the AD pathology through the regulation of neural plasticity [12]. As a representative *BDNF* functional SNP, Val66Met influences intracellular BDNF trafficking and secretory regulation and is linked to cognitive functions or neuropsychiatric symptoms in AD patients [13–15]. This SNP (Val66Met) might also be a biomarker regulating or reflecting the clinical presentations and prognosis of AD patients.

In some previous studies, DNA methylation alterations of the *BDNF* promoter region (evaluated using postmortem or peripheral blood samples) have been reported in patients with neuropsychiatric disorders [16–18]. Hypermethylation of the *BDNF* promoter region in postmortem AD brain tissues has been described, supporting a reduction in the mRNA or protein levels in certain brain areas (hippocampus or frontal cortex) [16, 19, 20]. Such studies imply that *BDNF* methylation might be a useful surrogate marker of the neurodegenerative process that occurs in AD. However, the association between the *BDNF* DNA methylation levels and the clinical presentation of AD (e.g., cognitive function, neuropsychiatric symptoms, and plasma BDNF levels) has not been previously investigated.

In the present cross-sectional study, we hypothesized that DNA methylation in the *BDNF* promoter region might be a biomarker for the manifestation of AD or its relevant clinical presentations during the neurodegenerative course. Accordingly, we compared the DNA methylation levels of the exon I promoter region in *BDNF* between AD patients and age-matched normal controls (NCs). Then we investigated the correlation between the DNA methylation levels of *BDNF* and some clinical presentations using cognitive and neuropsychiatric test batteries.

## Patients and Methods

### Participants

Forty peripheral blood samples were provided by 3 institutions: 20 AD patients (8 males and 12 females; mean age:  $66.5 \pm 5.07$  years) were recruited out of consecutive memory clinic outpatients who visited the Jikei University Hospital (Tokyo) or the Jikei University Kashiwa Hospital (Kashiwa city), and 20 NCs (8 males and 12 females; mean age:  $66.5 \pm 5.12$  years)

were recruited from Juntendo University Hospital (Tokyo). Genomic DNA was obtained from mononuclear cells in the peripheral blood samples using the Qiagen DNA extraction kit [14]. All AD patients were diagnosed as having probable AD based on the National Institute of Neurology and Communicative Disorder and Stroke/Alzheimer Disease and Related Disorder Association (NINCDS/ADRDA) criteria [21]. The neuropsychiatric symptoms in the AD patients were assessed based on information obtained from a structured interview with each patient's caregiver by the same geriatric psychiatrists using the behavioral pathology in AD (Behave-AD) scale [22]. Two neuropsychological tests, the Frontal Assessment Battery (FAB) reflecting executive function and the Mini-Mental State Examination (MMSE) reflecting global cognitive function, were also administered to the AD patients by a clinical psychologist [23, 24]. NCs with a past history of treatment for any psychiatric disorder were excluded.

This study was approved by the Ethics Committee of the Jikei University School of Medicine (Tokyo and Kashiwa city) and the Juntendo University School of Medicine (Tokyo). Written informed consent was obtained from both the patients and their caregivers.

#### *Analysis and Measurement of DNA Methylation Levels in the BDNF Promoter Region*

According to the enrollment protocol, each DNA sample (1 µg) was subjected to bisulfite treatment with the MethylEasy™ Xceed Rapid DNA Bisulfite Modification Kit (Takara Bio Inc.). Using the bisulfite-treated DNA as a template, the *BDNF* promoter region was amplified by nested polymerase chain reaction (PCR) with TaKaRa EpiTaq HS (Takara Bio Inc.). The PCR products were purified using the QIAquick PCR purification kit (Qiagen), and the restriction sites *Pst*I and *Bgl*II were added to the second PCR product. The detailed conditions are described below: a total volume of 50 µl containing 0.25 µl of TaKaRa EpiTaq HS (5 U/µl), 5 µl of 10× EpiTaq PCR buffer (Mg<sup>+</sup> free), 5 µl of 25 mM MgCl<sub>2</sub>, 6 µl of dNTP mixture, 4 µl of template DNA, 0.4 µl of PCR forward primer (50 µM), 0.4 µl of PCR reverse primer (50 µM), and 28.95 µl of PCR-grade water. The primers used for the first PCR were as follows: 5'-GGGTT-GAGGTTGGGGTTGGA-3' (BDNF\_P\_F1 forward primer) and 5'-CTCCTCCAAACCTTCCCTCTC-TACTTC-3' (BDNF\_P\_R1 reverse primer). The thermal profile consisted of 30 cycles at 98°C for 10 s, at 55°C for 30 s, and at 72°C for 60 s. The primers used for the second PCR were as follows: 5'-ATCCTGCAGGTTTTAGGGGAGGAGGTGAGGATAGG-3' (Pst\_BDNF\_P\_F2 forward primer) and 5'-TCCAGATCTCCCCAAAATAACTCCAAATC-3' (BDNF\_P\_Bgl\_R2 reverse primer). The thermal profile was followed by 40 cycles at 98°C for 10 s, at 53°C for 30 s, and at 72°C for 60 s.

According to the standard technique, purified PCR products were digested with *Pst*I and *Bgl*II and inserted into the Litmus 28i vector (New England Biolabs). The plasmids were transformed into Stbl3 chemically competent *Escherichia coli* cells (Invitrogen), and colonies were obtained after overnight cultivation. Colony PCR was performed using KOD FX Neo (Toyobo) in a total volume of 50 µl containing 1 µl of KOD FX Neo (1 U/µl), 25 µl of 2× PCR buffer, 10 µl of 2 mM dNTP mixture, 0.2 µl of PCR forward primer (50 µM), 0.2 µl of PCR reverse primer (50 µM), and 13.6 µl of PCR-grade water. About 40 positive colonies were selected. The primers used for the colony PCR were as follows: 5'-CGCCAGGGTTTTCCAGTCACGAC-3' (M13 primer P7) and 5'-AGCGGATAACAATTTACACAGGAAAC-3' (M13 primer P8). The thermal profile was 94°C for 2 min, followed by 30 cycles at 98°C for 10 s and at 68°C for 30 s.

The final PCR product was treated using EXOSAP-IT (Affymetrix, Inc.), and direct nucleotide sequencing with an ABI 3730 DNA analyzer [sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems)] was performed to identify the DNA sequences. The primers used for the sequences were as follows: 5'-CGCCAGGGTTTTCCAGTCACGAC-3' (M13 primer P7), 5'-GTAAAACGACGGCCAGT-3' (M13 primer M3), 5'-AGCGGATAACAATTTACACAGGAAAC-3' (M13 primer P8), and 5'-CAGCTAT-GACCATGATTACG-3' (M13 primer RV2). All sequence analyses were performed using DNA

**Table 1.** Subject characteristics of the AD and NC groups

	NC (n = 6)	AD (n = 12)	t/ $\chi^2$	p value
Males/females	4/2	4/8	1.8 <sup>a</sup>	0.18
Age, years	64.33±6.53	65.67±5.18	0.473 <sup>b</sup>	0.642
Age at onset, years	–	61.90±6.11		
Duration of illness, months	–	44.25±26.06		
MMSE score	–	18.83±7.94		
FAB score	–	10.33±3.87		
Behave-AD score	–	5.25±3.98		
CDR SB	–	6.04±3.88		

All values are given as means ± SD except for the sex ratio.

<sup>a</sup>The sex ratio was analyzed using the  $\chi^2$  test. <sup>b</sup>Age was compared using the t test.

Sequencing Analysis software v5.3.1 (Applied Biosystems). The DNA methylation data were analyzed using the quantification tool for methylation analysis (QUMA; Riken, Japan; <http://quma.cdb.riken.jp/>).

#### Statistical Analysis

SPSS 19.0J for Windows (SPSS Japan Inc.) was used for all statistical analyses. First, we evaluated the data normality of each variable and the hypothesis that the distributions of all variables would be rejected by the Shapiro-Wilk test ( $p < 0.05$ ), except for age, when the AD and NC groups were compared. Thus, nonparametric statistics were used for most of the following data evaluations, except for the age comparison. We compared the DNA methylation levels (in %) of the *BDNF* promoter region between the AD and NC groups using the Mann-Whitney test. The sex ratio (female to male) between the AD and NC groups was assessed using the  $\chi^2$  test. Only the age comparison between the AD and NC groups was performed using the t test. Moreover, we examined the correlations between the methylation levels (in %) of significant sites, as detected based on comparisons between the groups, and the clinical presentations [age, duration of illness in months, MMSE total and subtest scores, FAB total and subtest scores, Behave-AD total score and each of the 7 subscale scores, Clinical Dementia Rating Scale sum of boxes (CDR SB), and the plasma BDNF concentration (in pg/ml)] using the Spearman rank correlation coefficient. A p value  $< 0.05$  was considered statistically significant.

## Results

#### Patient Characteristics

Of the 40 DNA samples available, the DNA methylation levels were completely analyzed in 18 subjects (12 AD patients and 6 NCs). Among the two groups, the sex and age ratios were not significantly different (table 1). All 12 AD patients had taken acetylcholine esterase inhibitors (AChEI) (11 patients had taken donepezil and 1 had taken galantamine) for over 6 months.

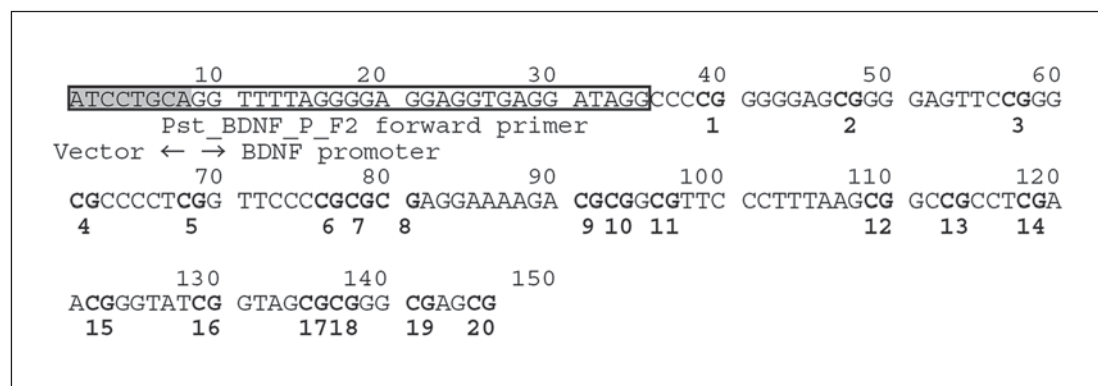
#### Comparison of DNA Methylation Levels in the *BDNF* Promoter Region

Within the promoter region of exon I, 20 CpG sites with sequences that had been verified by an analysis of the complementary strand were targeted for statistical comparison. The targeted regions, consisting of about 120 bp, were positioned from the terminal point of the

**Table 2.** Comparison of CpG methylation (in %) among the AD and NC groups

	NC (n = 6)		AD (n = 12)		Mann-Whitney Z score	p value
	mean ± SD	median	mean ± SD	median		
Total methylation ratio	2.09±0.81	2.03	5.08±5.52	3.28	14	0.041*
CpG1	1.53±1.75	1.09	3.49±6.81	0.00	3.15	0.616
CpG2	1.11±2.72	0.00	2.59±6.02	0.00	32.5	0.653
CpG3	0.00±0.00	0.00	2.64±5.97	0.00	27	0.194
CpG4	0.00±0.00	0.00	4.85±6.91	3.35	12	0.014*
CpG5	0.00±0.00	0.00	2.10±5.73	0.00	27	0.194
CpG6	1.54±2.46	0.00	1.83±6.34	0.00	28	0.249
CpG7	1.75±1.97	1.39	1.16±1.76	0.00	30	0.523
CpG8	3.55±6.86	0.00	7.31±18.36	0.00	33	0.749
CpG9	4.84±3.19	5.26	4.23±7.28	0.00	24	0.239
CpG10	1.84±3.16	0.00	1.29±2.04	0.00	34	0.823
CpG11	1.80±2.02	1.39	13.02±29.16	1.52	33	0.764
CpG12	9.04±9.57	7.98	20.39±33.12	3.81	35.5	0.962
CpG13	1.79±3.14	0.00	8.29±24.30	0.00	36	1
CpG14	1.18±1.84	0.00	5.42±11.37	0.00	30	0.523
CpG15	2.00±3.11	0.00	0.68±1.67	0.00	28	0.304
CpG16	3.69±3.49	3.33	4.73±5.44	3.42	35	0.924
CpG17	2.27±5.57	0.00	3.83±3.23	4.10	22	0.161
CpG18	2.60±4.07	0.00	2.89±5.13	0.00	36	1
CpG19	0.58±1.41	0.00	3.30±4.35	0.00	24.5	0.199
CpG20	1.52±2.44	0.00	6.85±10.23	0.00	28	0.394

The CpG methylation levels in the *BDNF* promoter region were analyzed using the Mann-Whitney test. \* p < 0.05.



**Fig. 1.** *BDNF* promoter region examined in the DNA methylation analysis. The target region used for the DNA methylation analysis contains 20 CpG sites, shown in bold type. The vector region has been shaded. The Pst\_BDNF\_P\_F2 forward primer is shown in the box. The targeted regions, consisting of about 120 bp, were positioned from the terminal point of the forward primer (GenBank: AF411339; at position 53144) for direct nucleotide sequencing.



**Table 3.** Correlations between CpG4 methylation levels and patient characteristics in the 12 AD patients

	$\rho$	p value
Age	-0.384	0.218
Duration of illness	0.588	0.044*
MMSE score	-0.391	0.209
FAB score	-0.308	0.329
Behave-AD score	0.156	0.629
CDR SB	0.541	0.07
Plasma BDNF levels	0.055	0.880

$\rho$  = Spearman's correlation coefficient. \*  $p < 0.05$ .

**Table 4.** Correlations between CpG4 methylation levels and FAB subtest scores in the 12 AD patients

	$\rho$	p value
Similarities	0.182	0.571
Lexical fluency	-0.248	0.437
Motor series	-0.352	0.262
Conflicting instructions	-0.539	0.07
Go/no-go	-0.122	0.706
Prehension behavior	-0.642	0.024*

$\rho$  = Spearman's correlation coefficient. \*  $p < 0.05$ .

forward primer (GenBank: AF411339; at position 53144) for direct nucleotide sequencing (fig. 1). The total methylation ratio of the 20 CpG sites was calculated and compared between the AD and NC groups, and a significantly higher value was seen in the AD group ( $5.08 \pm 5.52\%$ ) than in the NCs ( $2.09 \pm 0.81\%$ ;  $p = 0.041$ ; table 2). Moreover, among the 20 CpG sites, the DNA methylation level at the CpG4 site (fig. 1) was significantly higher in the AD patients than in the NCs ( $p = 0.014$ ; table 2). However, no significant differences in the methylation levels at the other CpG sites were observed (table 2).

#### *Correlations between the CpG4 Methylation Level and the Characteristics of the AD Patients*

We examined the associations between the CpG4 methylation level and the patient characteristics (age, duration of illness, MMSE, FAB, Behave-AD, CDR SB, and the plasma BDNF level) using the Spearman rank correlation coefficient. A significant positive correlation was found between the CpG4 methylation level and the duration of illness ( $\rho = 0.588$ ;  $p = 0.044$ ), but no other correlations with the other characteristics were seen (table 3).

#### *Correlations between the CpG4 Methylation Level and the FAB or MMSE Subscale Scores in the AD Patients*

A significant negative correlation was found between the CpG4 methylation level and the prehension behavior score ( $\rho = -0.642$ ;  $p = 0.024$ ) among the 6 FAB subtests as well as between the registration score ( $\rho = -0.651$ ;  $p = 0.022$ ) and the recall score ( $\rho = -0.663$ ;  $p = 0.019$ ) among the 11 MMSE subtest scores (tables 4, 5).

#### *Correlations between the CpG4 Methylation Level and the Behave-AD Subscale Scores in the AD Patients*

No significant correlations between the CpG4 methylation level and any of the Behave-AD subscale scores were found (table 6).

**Table 5.** Correlations between CpG4 methylation levels and MMSE subtest scores in the 12 AD patients

	$\rho$	p value
Orientation to time	-0.123	0.703
Orientation to place	-0.424	0.169
Registration	-0.651	0.022*
Attention and calculation	-0.319	0.313
Recall	-0.663	0.019*
Naming	-0.400	0.197
Repetition	-0.443	0.149
3-stage command	0.117	0.717
Reading	-0.400	0.197
Writing	-0.539	0.070
Copying	-0.235	0.463

$\rho$  = Spearman's correlation coefficient. \*  $p < 0.05$ .

**Table 6.** Correlations between CpG4 methylation levels and Behave-AD subtest scores in the 12 AD patients

	$\rho$	p value
Delusions	-0.398	0.200
Hallucinations	-	
Activity disturbances	0.146	0.651
Aggressiveness	0.130	0.687
Diurnal rhythm disturbances	-	
Affective disturbances	-0.308	0.330
Anxiety/phobias	-0.313	0.322

$\rho$  = Spearman's correlation coefficient.

## Discussion

In the present study, we found that the DNA methylation levels of the *BDNF* promoter region were higher in AD patients than in NCs. Moreover, the methylation level at the CpG4 site was significantly correlated with some clinical presentations examined using cognitive and neuropsychiatric test batteries, such as illness and neuropsychological subtest scores. However, no other significant correlations between the CpG4 methylation levels and other factors (e.g., neuropsychiatric symptoms, plasma BDNF levels, and CDR SB) were observed.

Our results support those of previous studies showing that elevated levels of methylation in AD patients lead to reductions in the mRNA or protein expression levels of BDNF [16, 19]. Some other studies investigating methylation alterations in patients with major depressive disorder or bipolar disorder have shown higher methylation levels of the exon I promoter region in *BDNF* compared with the levels in NCs [16–18, 25, 26]. The present study also found a higher methylated average score (in %) for most of the CpG sites (16 of the 20 CpG sites) in the AD patients than in the NCs (table 2). Future studies involving a larger sample are needed to reach a definite conclusion.

Among the neuropsychological subscores, memory function (registration and recall) or sensitivity to environmental cues (prehension behavior) were significantly and negatively correlated with the CpG4 methylation level (tables 4, 5). Furthermore, the duration of illness was significantly and positively correlated with the CpG4 methylation level. The acceleration of the methylation of CpG islands in *BDNF* is caused by aging, inflammatory-associated, and psychogenic stress, which may attenuate its neuroprotective effect against the accumulation of A $\beta$  and  $\tau$  linking to synaptic or neuronal loss during the course of degeneration [12, 17, 27, 28]. Neurotoxic exposure following the accumulation of A $\beta$  can cause secondary epigenetic

alterations via oxidative stress [28–31]. Therefore, a longer duration of illness might have influenced the elevation in the methylation levels of *BDNF* in the AD patients. Some previous studies using AD animal models have demonstrated that *BDNF* mRNA expression and its methylation levels within the hippocampus influence memory function, and the present study also supports a significant association between a *BDNF* hypermethylated state and memory impairment in AD patients [12, 20, 29, 32]. The hypermethylation of *BDNF* and the reduction in the protein levels in the AD prefrontal cortex have been shown in previous reports [16, 19, 23], while the comparatively simple task of prehension behavior among the 6 FAB subtests was significantly and negatively correlated with the CpG4 methylation levels in the present study. Moreover, the duration of illness was significantly and negatively correlated with prehension behavior ( $\rho = -0.654$ ;  $p = 0.021$ ) but not registration ( $\rho = -0.538$ ;  $p = 0.071$ ) or recall ( $\rho = -0.346$ ;  $p = 0.270$ ). Memory impairment is the most distinct core symptom among several cognitive dysfunctions observed in AD patients and presents at the initial stage of the illness. Thus, a reduction in the registration and recall scores might appear irrespective of the duration of illness [1, 2]. On the other hand, CpG4 hypermethylation in the frontal cortex may reflect a longer duration of illness and might cause a reduction in prehension behavior. However, the correlations between other FAB subtest scores reflecting relatively complex executive functions, such as conceptualization, sensitivity to interference, and inhibitory control, and the *BDNF* methylation levels should be investigated in future studies.

The present study has some limitations. Firstly, no significant correlations between the *BDNF* methylation and plasma BDNF levels or the Behave-AD score, reflecting neuropsychiatric problems, were found. The peripheral BDNF levels are influenced by the secretion from platelets and other comparatively transient sources of neuropsychiatric stress [33, 34]. The evidence obtained in the present study cannot explain the direct neurobiological or chemical mechanisms by which *BDNF* DNA methylation influences neuropsychological presentations. Other intermediate phenotypic information, such as neuroimaging or neurophysiological data, is needed in future studies. Moreover, the methylation levels are influenced by psychotropic agents (antidepressants or mood stabilizers), and the serum BDNF levels are also associated with AChEi usage [35–37]. In the present study, AChEIs had been taken by all AD patients, and 3 AD patients had been treated with antidepressants (2 patients had taken paroxetine and 1 patient had taken fluvoxamine); these medications might have modified the elevation in DNA methylation. Secondly, the sample size in this study is comparatively small; therefore, some type II errors may exist. However, we found a significant association between the methylation levels and cognitive function or AD manifestations, and the effect sizes or  $p$  values were about the same as those presented in previous reports [16, 27]. Of the 40 DNA samples in the present study, only 18 samples were completely analyzed. Therefore, more stable experimental results are needed in the future. Thirdly, the present study used peripheral blood samples, not brain tissue, similar to previous reports that investigated peripheral blood samples and found significant methylation alterations [17, 18].

In conclusion, despite some limitations of the present cross-sectional study, we observed elevated methylation levels in the *BDNF* promoter region in AD patients, and these alterations were associated with some neurocognitive functions. In particular, an increase in *BDNF* methylation may influence memory impairment, which is a distinct core symptom of AD that leads to dementia. Such associations between details of the clinical presentation and *BDNF* methylation in AD patients have not been previously reported. Our results may contribute to the elucidation of neurodegenerative mechanisms and a better understanding of the actual roles of the methylation of the *BDNF* gene in the progression of AD and its possible role as a functional surrogate marker. Therefore, a larger sample should be added to the present study, and the data should be reevaluated in the future.



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