Elevated *DRD4* promoter methylation increases the risk of Alzheimer's disease in males

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Abstract. Aberrant promoter methylation of multiple genes is associated with various diseases, including Alzheimer's disease (AD). The goal of the present study was to determine whether dopamine receptor D4 *(DRD4)* promoter methylation is associated with AD. In the current study, the methylation levels of the *DRD4* promoter were measured in 46 AD patients and 61 controls using bisulfite pyrosequencing technology. The results of the present study demonstrated that *DRD4* promoter methylation was significantly higher in AD patients than in controls. A further breakdown analysis by gender revealed that there was a significant association of *DRD4* promoter methylation with AD in males (23 patients and 45 controls). In conclusion, the results of the present study demonstrated that elevated *DRD4* promoter methylation was associated with AD risk in males.

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Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease characterized by a progressive decline in cognitive and memory function (1). The prevalence of AD was 35.6 million globally in 2010, and this number is expected to double by 2030. AD has become one of the most common forms of dementia in the elderly worldwide (2), exerting a huge burden on families and society.

AD is a complex disease that is influenced by environmental and genetic factors (3). Heritability studies have shown that ~70% of AD risk may be attributed to genetic factors (4). Epigenetic modifications are thought to link environmental and genetic factors (5-7). DNA methylation, a type of epigenetic modification, has been shown to have a significant role in the etiology of several diseases, including leukemia (8), type 2 diabetes (9,10), essential hypertension (9), coronary heart disease (11,12), schizophrenia (13) and AD (14,15). DNA methylation often occurs in a CpG dinucleotide context (16). The CpG islands of a promoter are CpG-rich regions that are predominantly hypomethylated (17). Alterations in promoter methylation often affect gene expression (7,18-20).

Dopamine receptor D4 (*DRD4*) encodes the D4 subtype of the dopamine receptor (21). An increasing amount of evidence supports a link between *DRD4* and AD (22). *DRD4* polymorphism has been observed to be significantly associated with AD (22) and *DRD4* promoter methylation (23). *DRD4* gene hypermethylation has been demonstrated to increase *DRD4* gene expression and the risk of schizophrenia in males (13). A hypermethylated *DRD4* promoter has also been identified in patients with alcohol addiction (24).

Although DNA methylation levels vary among tissues, independent studies have revealed that the DNA methylation patterns of multiple loci in peripheral blood were similar to those in brain tissues (25-28). In light of these previous findings,

the goal of the present study was to evaluate the contribution of *DRD4* promoter methylation to AD using peripheral blood as a surrogate of brain tissue.

Materials and methods

Sample collection. A total of 46 sporadic AD patients and 61 matched controls were selected from Ningbo No. 1 Hospital (Ningbo, China) and Ningbo Kangning Hospital (Ningbo, China). AD was diagnosed by two professional neurological physicians (CZ and ZQ) according to National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria from ICD-10 (29), on the basis of medical and family histories, neurological examination, blood studies, brain imaging studies, neuropsychological testing and cognitive screening tests. All controls were free of any type of physical or mental disorder. In the current study, two kinds of drugs (Exelon and Aricept) were used for AD patients. Exelon (Novartis Farmaceutica S.A, Spain) was administered at 1.5 mg twice per day with morning and evening meals. Following a minimum of two weeks of treatment, if the initial dose was well-tolerated, it was increased to 3 mg twice per day. Initial treatment with Aricept (Eisai China Inc., Jiangsu, China) was 5 mg per day at bedtime. The dose was increased to 10 mg per day after 4 to 6 weeks if the response was not adequate. All individuals in the present study were Han Chinese originating from Ningbo city in Eastern China. Peripheral blood samples were collected in 3.2% sodium citrate-treated tubes (Jiangsu Kailijian Medical Device Co., Ltd., Jiangsu, China) and then stored at -80˚C. The study protocol was approved by the Ethical Committee of Ningbo University (Ningbo, China), Ningbo No.1 Hospital and Ningbo Kangning Hospital. Written informed consent was obtained from all subjects or their guardians.

Detection of biochemical factors. The serum concentrations of total protein (TP) and albumin (ALB) were detected using the biuret (30) and bromocresol green methods (31), respectively. Globulin (GLB) was calculated as TP minus ALB. The concentrations of glutamic-pyruvic transaminase, alkaline phosphatase and glutamic oxalacetic transaminase were determined using the velocity method (32,33). The levels of total bile acid and homocysteine (Hcy) were measured using the cycling enzymatic method (34,35). Plasma concentrations of blood glucose, triglyceride, total cholesterol, carbamide (UREA), creatinine (CRE) and uric acid were determined using the enzymatic methods (36-41). The high-density lipoprotein cholesterol level was determined using the one-step detection method (42). The proportions of apolipoprotein-A (ApoA) and apolipoprotein-B were measured by the turbidimetry method (43,44). The concentrations of lipoprotein A (Lp (a)) and C Reactive Protein (CRP) were detected using the endpoint method (45) and latex agglutination assay (46), respectively. The apolipoprotein E levels were detected using the immunoturbidimetric assay (47).

Bisulfite pyrosequencing assay. DNA extraction and consequent bisulfite pyrosequencing assays were performed as described in our previous studies (8,9,12,13,48,49). PCR primers were designed using PyroMark Assay Design software version 2 (Qiagen China Co., Ltd., Shanghai, China). Primer sequences were 5'-biotin-GGGAGGTTTTGTTAGATATTA GGT-3' for the forward primer; 5'-CCACCCTAAACCCAA TATTTACTCATCTTA-3' for the reverse primer; and 5'-ACC AAACCAAACCCT-3' for the sequencing primer.

Statistical analyses. Statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA), and a $P<0.05$ was considered to indicate a statistically significant difference. The two independent samples *t* test was used to compare the differences in the mean values of continuous variables between AD patients and controls. Pearson's correlation test was used to assess the associations between *DRD4* methylation and the metabolic characteristics of the AD subjects. Bonferroni correction was used to adjust the results. Power analysis was estimated with the Power and Sample Size Calculation software version 3.043 (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). In the present study, according to the online power calculator, α was the type I error probability for a two-sided test; n was the sample number of AD patients; δ was the difference in population means, which was equal to the mean methylation levels in AD patients minus those in normal controls; σ was the within-group standard deviation; and m was the ratio of control to experimental patients.

Results

The selected promoter fragment in the current methylation assay. The bisulfite pyrosequencing assay was performed on the CpG island region (chr11:636877-637167) of the *DRD4* promoter. As shown in Fig. 1, a total of four CpG sites were measured. As there was a significant correlation among the methylation levels of the four CpG sites (Fig. 1; $r=0.442$, $P<0.001$), the mean DNA methylation of the four CpGs was also evaluated in the subsequent analyses.

Association tests between clinical phenotypes with AD. The present study involved a total of 46 AD patients and 61 controls. As shown in Table I, among the 22 phenotypes, the plasma levels of ApoA, Lp (a), Hcy and CRP were observed to significantly differ between AD patients and controls (Table I; ApoA, $P=0.011$; Lp (a), $P<0.001$; Hcy, $P=0.046$; CRP, $P=0.016$). A significant male-specific association was identified between the mean *DRD4* methylation and ApoA and level (Fig. 2; ApoA, $P=0.042$). A significant female-specific association was observed between the average *DRD4* methylation and several phenotypes, including ApoA and CRE levels (Fig. 2; ApoA, $P<0.001$; CRE, $P=0.045$). Age is a well-known risk factor for AD, therefore the association between age and *DRD4* methylation was tested. The results revealed an association between age and *DRD4* methylation (Fig. 2; males: $r=0.281$, $P=0.021$; females: $r=0.222$, $P=0.169$).

Association tests of DRD4 methylation with AD. Significantly increased *DRD4* methylation levels were observed in AD patients compared with controls (Table II; CpG1, $P=0.001$; CpG2, $P=0.013$; CpG3, $P=0.001$; CpG4, $P<0.001$; mean CpG1-4 methylation, $P<0.001$). Among 23 male patients and

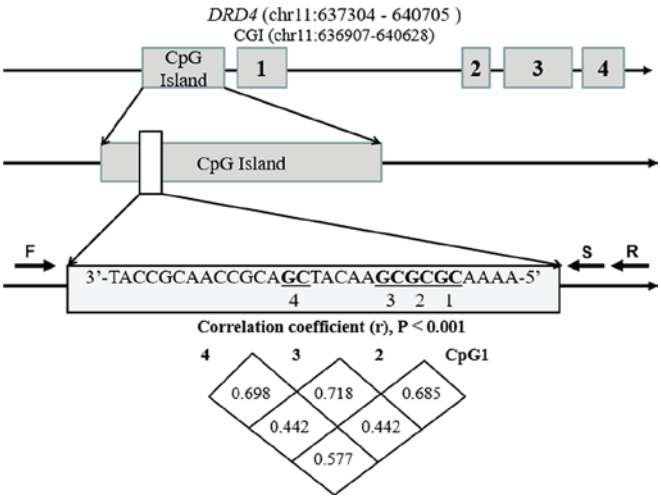

Figure 1. Significant correlation among the four CpGs of the *DRD4* promoter. *DRD4* (chr11: 637304-640705) encodes dopamine receptor D4. CGI (chr11: 636907-640628) represents CpG island, which is enriched with CpG sites; Four CpG sites from the CpG island of the upstream *DRD4* gene were selected and tested by pyrosequencing. Significant associations were observed among these sites (P<0.001). A total of three primers were used in the present study. F, forward primer; R, reverse primer; S, sequencing primer.

45 male controls, elevated methylation levels of four CpG sites of the *DRD4* promoter were observed following breakdown analysis by gender (Table II; CpG1, P=0.013; CpG2, P=0.012; CpG3, P<0.001; CpG4, P<0.001; mean CpG1-4 methylation, P<0.001). As shown in Table II, the power was sufficient in the overall (power=0.978) and male-based subgroup (power=0.976) case-control comparisons; however, the power was only 0.405 in the female-based subgroup analysis, suggesting that the negative association in the female subgroup may be due to a lack of power. A consequent breakdown analysis by gender revealed that the above significant association of *DRD4* methylation with AD existed only in males (Fig. 3A; males, P<0.001; females, P=0.080).

DRD4 methylation levels in AD patients with various treatments. Exelon and Aricept are commonly used acetylcholinesterase inhibitors in the treatment of AD (48). Both drugs aim to enhance cholinergic neurotransmission in specific parts of the brain and to improve the clinical symptoms of AD (50). The results of the present study revealed that the patients using Exelon-treated patients had significantly higher CpG3 methylation levels compared with Aricept-treated patients Aricept (Fig. 3B; P=0.044).

Discussion

The present study evaluated the levels of *DRD4* promoter methylation in AD patients and matched controls to clarify the contribution of *DRD4* promoter methylation to AD risk. It was observed that the *DRD4* promoter methylation levels in AD patients were significantly higher than those in controls. In a breakdown analysis by gender, there were varying associations of methylation status in males and females. Positive results were identified for all four CpG sites observed in males. Positive correlations between *DRD4* methylation and age, as well as *DRD4* methylation and ApoA level, were also observed in males. In addition, clear positive correlations were observed between *DRD4* methylation and ApoA and between *DRD4* methylation and CRE in females. Furthermore, varying methylation levels were observed in patients who used Exelon and those who used Aricept.

Anomalies in dopaminergic transmission may lead to the disturbance of synaptic plasticity and advanced cognitive behavior (22). A male-specific association between *DRD4* methylation and schizophrenia has been reported in Han Chinese (13). As a significant receptor of dopamine, *DRD4* is pivotal to the development of AD (51). In the present study, significantly hypermethylated *DRD4* promoters were observed in AD patients compared with controls. These results indicated that *DRD4* may be involved in the progression of AD. Understanding the DNA methylation changes in the *DRD4* promoter may aid in understanding the pathological mechanisms of AD. This information may also provide insight into the function of AD-associated genes, including *DRD4*, and help identify novel targets for therapeutic strategies to reverse the promoter methylation of *DRD4*.

Gender differences in AD have been widely documented. Females have a higher risk of AD at all ages, and the age-adjusted odds ratio for females has been shown to be 3.1 between AD patients and controls (52). Gender-specific DNA methylation exists in mice (53) and humans (12,54). *DRD4* methylation research in another nervous system disorder, schizophrenia, has also identified a male-specific significant association (13). Significant differences were reported in all CpG sites observed in the present study in males, but a significant difference was observed in only CpG1 in females. These phenomena of varying methylation statuses in the *DRD4* promoter also provide insight into gender differences in AD. The results of the present study support the idea that gender differences should be considered when establishing a clinical treatment plan for AD.

Furthermore, a significant association between DNA methylation and age in males but not in females was observed in the present study. Age is considered a major risk factor for AD (54). A previous study reported that DNA methylation is dynamically regulated in the human cerebral cortex throughout life, involves differentiated neurons and affects a substantial proportion of genes predominantly by an age-associated increase (55). Detailed descriptions of associations between DNA methylation and age in various gender subgroups require additional study.

A total of 22 phenotypes were analyzed in the present study. It is well-known that ApoA1, an increase in which leads to an increased risk of AD, is the major apolipoprotein constituent of high-density lipoprotein, and Apo A1 has been observed to affect brain cholesterol metabolism and angiogenesis (56). In an earlier study, serum ApoA concentration was shown to have a high correlation with the severity of AD (57). Significantly increased levels of ApoA1 were observed in AD patients compared with controls in the present study. Based on previous findings, it was speculated that Lp (a) may participate in the progression of dementia (58) and AD (59). The present study showed that AD patients had higher levels of Lp (a), which was similar to the results of a previous cross-sectional study (59). Hcy levels, another AD factor that may induce amyloid β accumulation, synaptic dysfunction and memory impairment, were significantly different between AD patients

Table I. Characteristics of the 107 subjects.

Characteristic	Patients (n=46), mean ± SD	Controls (n=61), mean ± SD	P-value
Age, years	80.67±9.20	79.54±7.87	0.495
TP, g/l	68.79±6.91	65.48±9.45	0.099
ALB, g/l	38.32±3.83	36.86±3.61	0.090
GLB, g/l	30.46±5.32	29.55±4.58	0.418
A/G	1.29±0.22	1.28±0.20	0.768
ALT, U/l	13.87±10.68	18.20±13.46	0.193[a]
ALP, U/l	78.00±24.30	96.82±63.34	0.113[a]
TBA, μmol/l	6.91±3.84	6.07±5.86	0.503
AST, U/l	20.52±7.22	23.58±11.70	0.258[a]
Glu, mmol/l	5.23±1.58	5.53±2.71	0.444[b]
TG, mmol/l	1.35±0.76	1.42±0.98	0.896[b]
TC, mmol/l	4.48±1.04	4.28±1.22	0.378
HDL-C, mmol/l	1.12±0.27	1.04±0.30	0.118
ApoA, g/l	1.06±0.21	0.94±0.18	0.011[c]
ApoB, g/l	0.66±0.19	0.73±0.25	0.194
Lp(a), g/l	184.86±233.63	34.86±27.32	<0.001[a,c]
ApoE, mg/l	37.73±17.44	36.69±10.37	0.800
UREA, mmol/l	7.77±10.00	6.45±3.45	0.804[b]
CRE, μmol/l	82.73±47.25	78.52±30.04	0.626
UA, μmol/l	309.93±106.30	308.88±112.75	0.967
Hcy, μmol/l	19.76±10.82	17.67±20.84	0.046[a,c]
CRP, mg/l	6.20±11.72	15.00±26.21	0.016[a,c]

[a]Log-transformation was used. [b]Nonparametric rank test was applied. [c]Significant difference between patients and controls. TP, total protein; ALB, serum albumin; GLB, serum globulin; A/G, ALB/GLB; ALT, glutamic-pyruvic transaminase; ALP, alkaline phosphatase; TBA, total bile acid; AST, glutamic oxalacetic transaminase; Glu, blood glucose; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoA, apolipoprotein A; ApoB, apolipoprotein B; Lp(a), lipoprotein A; ApoE, apolipoprotein E; UREA, carbamide; CRE, creatinine; UA, uric acid; Hcy, homocysteine; CRP, C reactive protein; SD, standard deviation.

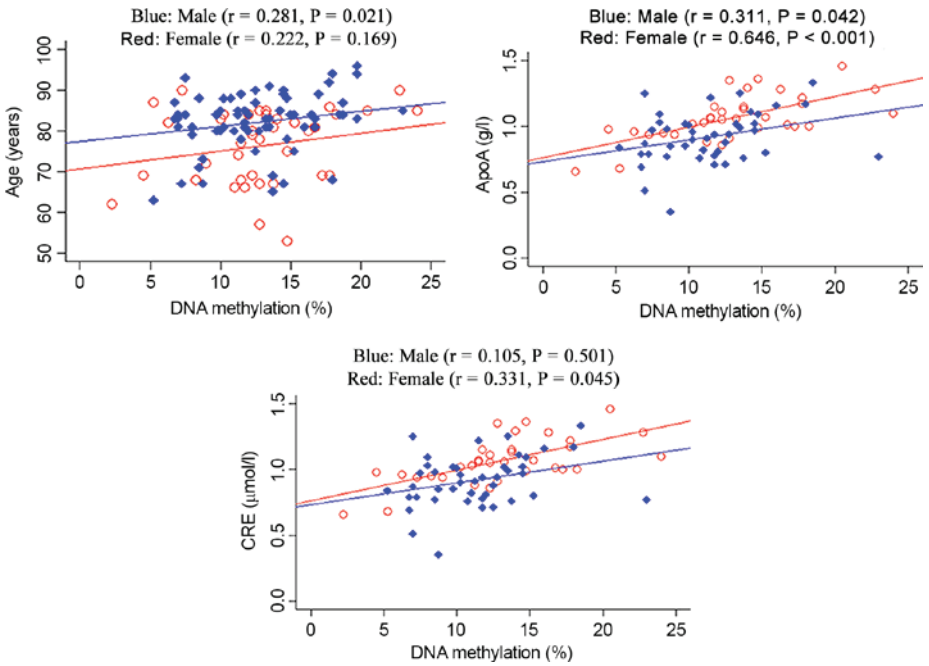

Figure 2. Correlation tests between *DRD4* methylation and three phenotypes. Pearson correlation analyses were used between *DRD4* methylation levels and phenotypes in two genders. Positive associations between *DRD4* methylation with age and APOA were observed in males (Age, P=0.021; ApoA, P=0.042). Positive associations between the average *DRD4* methylation with ApoA and CRE levels were observed in females (ApoA, P<0.001; CRE, P=0.045). *DRD4*, dopamine receptor D4; APOA, apolipoprotein A; CRE, creatinine.

Table II. Comparison of *DRD4* DNA methylation levels between AD patients and controls.

A, All patients

Characteristic, %	Patients, mean ± SD (n=46)	Controls, mean ± SD (n=62)	P-value	Power
CpG1	16.50±5.14	13.13±4.67	0.001[a,b]	0.916
CpG2	12.26±4.84	10.13±3.92	0.013[a,b]	0.610
CpG3	14.98±5.27	11.59±4.59	0.001[a,b]	0.906
CpG4	14.96±4.22	10.92±4.90	<0.001[a,b]	0.987
Mean methylation	14.67±4.12	11.44±3.72	<0.001[a,b]	0.978

B, Male patients

Characteristic, %	Patients, mean ± SD (n=46)	Controls, mean ± SD (n=62)	P-value	Power
CpG1	16.74±4.92	13.68±4.55	0.013[a,b]	0.666
CpG2	12.87±3.94	10.39±3.62	0.012[a,b]	0.676
CpG3	15.61±4.35	11.16±4.27	<0.001[a,b]	0.975
CpG4	15.65±4.60	10.36±4.42	<0.001[a,b]	0.992
Mean methylation	15.22±3.72	11.40±3.47	<0.001[a,b]	0.976

C, Female patients

Characteristic, %	Patients, mean ± SD (n=46)	Controls, mean ± SD (n=62)	P-value	Power
CpG1	16.26±5.45	11.71±4.82	0.009[a,b]	0.720
CpG2	11.65±5.63	9.47±4.67	0.201	0.212
CpG3	14.35±6.10	12.71±5.31	0.380	0.125
CpG4	14.26±3.78	12.35±5.88	0.220	0.162
Mean methylation	14.13±4.51	11.56±4.40	0.080	0.405

[a]Significant difference between patients and controls. [b]P-values remained significant after multiple tests. SD, standard deviation.

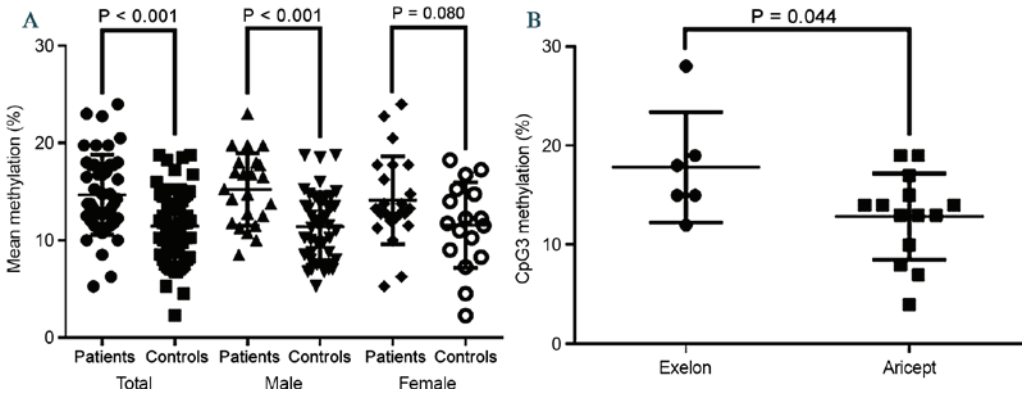

Figure 3. *DRD4* methylation comparisons between AD patients and controls and between Exelon-treated and Aricept-treated patients. (A) Comparison of *DRD4* methylation levels between patients and controls. *DRD4* methylation levels were significantly higher in male AD patients than male controls. (B) Comparison of CpG3 methylation levels between Exelon-treated and Aricept-treated patients. The Aricept-treated patients had significantly higher CpG3 methylation levels compared with Aricept-treated patients. *DRD4*, dopamine receptor D4; AUC, area under curve.

and matched controls in the present study (60-63). It has been reported that CRP participates in the systemic response to inflammation (64). Additional neuropathological studies have shown that CRP is associated with neurofibrillary tangles (65) and senile plaques (66) in AD brain tissue. The subjects with AD had significantly lower levels of plasma CRP than control subjects in the present study, which was consistent with the results of a previous study (64).

Aricept is an acetylcholinesterase inhibitor used for the symptomatic treatment of AD (67). Furthermore, Aricept

is a high-affinity sigma-1 receptor antagonist, which has been investigated as a potential disease-modifying agent for several CNS disorders (64). Exelon is an oral drug approved by the US Food and Drug Administration for the treatment of AD (67). Significantly higher levels of DNA methylation were reported at the CpG3 site in the patients who were treated with Exelon compared with those treated with Aricept. This finding indicates that varying DNA methylation effects were produced by Exelon and Aricept. However, additional research is required to uncover the detailed association between DNA methylation, Exelon and Aricept.

The current study has a number of limitations. Firstly, the sample size was small, which may have influenced the results, particularly for the gender-stratified association test of *DRD4* methylation with AD. In addition, the levels of DNA methylation of the four CpGs that were tested cannot represent the entire influence of *DRD4*. Additional studies investigating the *DRD4* promoter or gene body regions are required. Furthermore, samples were mainly from the elderly, who may have underlying diseases. In other words, there may be certain unknown or potential risk factors for AD present in this study. Also, the DNA methylation level of *DRD4* was measured in peripheral blood only, which may not be an accurate reflection of the situation in the brain tissue. Additional comprehensive studies on the association of *DRD4* methylation within brain tissues and peripheral blood are required. Finally, four CpG positions were assessed per pyrosequencing read. Certain P-values may not retain their significance after being corrected for this number of CpG sites. The possibility that the present positive findings arose by chance cannot be excluded.

In conclusion, the present study supports *DRD4* promoter hypermethylation as a risk factor for AD in males. Additionally, positive associations between *DRD4* methylation and age, as well as *DRD4* methylation and ApoA levels, were observed in males. Disparate *DRD4* methylation levels were reported for patients taking various drugs: Patients taking Exelon appear to have higher levels than those taking Aricept. The results of the present study may contribute to an improved understanding of the molecular mechanisms underlying the pathophysiology of AD.

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References

1. Wilson RS, Barnes LL, Mendes de Leon CF, Aggarwal NT, Schneider JS, Bach J, Pilat J, Beckett LA, Arnold SE, Evans DA and Bennett DA: Depressive symptoms, cognitive decline, and risk of AD in older persons. Neurology 59: 364-370, 2002.
2. Querfurth HW and LaFerla FM: Alzheimer's disease. N Engl J Med 362: 329-344, 2010.
3. Kubota T: Epigenome: What we learned from Rett syndrome, a neurological disease caused by mutation of a methyl-CpG binding protein. Rinsho Shinkeigaku 53: 1339-1341, 2013 (In Japanese).
4. Alonso Vilatela ME, Lopez-Lopez M and Yescas-Gomez P: Genetics of Alzheimer's disease. Genetics of Alzheimer's disease 43: 622-631, 2012.
5. Tang LL, Liu Q, Bu SZ, Xu LT, Wang QW, Mai YF and Duan SW: The effect of environmental factors and DNA methylation on type 2 diabetes mellitus. Yi Chuan 35: 1143-1152, 2013 (In Chinese).
6. Zhang W, Zheng Y and Hou L: Pharmacogenomic discovery delineating the genetic basis of drug response. Curr Genet Med Rep 1: 143-149, 2013.
7. Wang T, Garcia JG and Zhang W: Epigenetic regulation in particulate matter-mediated cardiopulmonary toxicities: A systems biology perspective. Curr Pharmacogenomics Person Med 10: 314-321, 2012.
8. Jiang D, Hong Q, Shen Y, Xu Y, Zhu H, Li Y, Xu C, Ouyang G and Duan S: The diagnostic value of DNA methylation in leukemia: A systematic review and meta-analysis. PloS One 9: e96822, 2014.
9. Cheng J, Wang L, Xu L, Wang H, Liu P, Bu S, Ye M, Zhang L, Wang Q and Duan S: Gender-dependent miR-375 promoter methylation and the risk of type 2 diabetes. Exp Ther Med 5: 1687-1692, 2013.
10. Tang LL, Liu Q, Bu SZ, Xu LT, Wang QW, Mai YF and Duan SW: The effect of environmental factors and DNA methylation on type 2 diabetes mellitus. Yi Chuan 35: 1143-1152, 2013 (In Chinese).
11. Xu L, Zheng D, Wang L, Jiang D, Liu H, Xu L, Liao Q, Zhang L, Liu P, Shi X, *et al*: GCK gene-body hypomethylation is associated with the risk of coronary heart disease. Biomed Res Int 2014: 151723, 2014.
12. Jiang D, Zheng D, Wang L, Huang Y, Liu H, Xu L, Liao Q, Liu P, Shi X, Wang Z, *et al*: Elevated PLA2G7 gene promoter methylation as a gender-specific marker of aging increases the risk of coronary heart disease in females. PloS One 8: e59752, 2013.
13. Cheng J, Wang Y, Zhou K, Wang L, Li J, Zhuang Q, Xu X, Xu L, Zhang K, Dai D, *et al*: Male-specific association between dopamine receptor D4 gene methylation and schizophrenia. PloS One 9: e89128, 2014.
14. Hodgson N, Trivedi M, Muratore C, Li S and Deth R: Soluble oligomers of amyloid-b cause changes in redox state, DNA methylation and gene transcription by inhibiting EAAT3 mediated cysteine uptake. J Alzheimers Dis 36: 197-209, 2013.
15. Huihui Ji, Xiaohui Zhou, Zhongming Chen, Ying Li, Dongsheng Zhou, Renjie Zhuo, Shiwei Duan and Qinwen Wang: Research progress of DNA methylation in Alzheimer's disease. Chi J Cell Biol 36: 1551-1559, 2014.
16. Al Akeel R: Role of epigenetic reprogramming of host genes in bacterial pathogenesis. Saudi J Biol Sci 20: 305-309, 2013.
17. Fatemi M, Pao MM, Jeong S, Gal-Yam EN, Egger G, Weisenberger DJ and Jones PA: Footprinting of mammalian promoters: Use of a CpG DNA methyltransferase revealing nucleosome positions at a single molecule level. Nucleic Acids Res 33: e176, 2005.
18. Yu Z, Kong Q and Kone BC: Aldosterone reprograms promoter methylation to regulate αENaC transcription in the collecting duct. Am J Physiol Renal Physiol 305: F1006-F1013, 2013.
19. Moen EL, Zhang X, Mu W, Delaney SM, Wing C, McQuade J, Myers J, Godley LA, Dolan ME and Zhang W: Genome-wide variation of cytosine modifications between European and African populations and the implications for complex traits. Genetics 194: 987-996, 2013.
20. Moen EL, Stark AL, Zhang W, Dolan ME and Godley LA: The role of gene body cytosine modifications in MGMT expression and sensitivity to temozolomide. Mol Cancer Ther 13: 1334-1344, 2014.
21. Kirley A, Hawi Z, Daly G, McCarron M, Mullins C, Millar N, Waldman I, Fitzgerald M and Gill M: Dopaminergic system genes in ADHD: Toward a biological hypothesis. Neuropsychopharmacology 27: 607-619, 2002.
22. Lin WY, Wu BT, Lee CC, Sheu JJ, Liu SH, Wang WF, Tsai CH, Liu HP and Tsai FJ: Association analysis of dopaminergic gene variants (Comt, Drd4 And Dat1) with Alzheimer s disease. J Biol Regul Homeost Agents 26: 401-410, 2012.
23. Docherty SJ, Davis OS, Haworth CM, Plomin R, D'Souza U and Mill J: A genetic association study of DNA methylation levels in the DRD4 gene region finds associations with nearby SNPs. Behav Brain Funct 8: 31, 2012.

24. Zhang H, Herman AI, Kranzler HR, Anton RF, Zhao H, Zheng W and Gelernter J: Array-based profiling of DNA methylation changes associated with alcohol dependence. Alcohol Clin Exp Res 37 (Suppl 1): E108-E115, 2013.
25. Mill J and Heijmans BT: From promises to practical strategies in epigenetic epidemiology. Nat Rev Genet 14: 585-594, 2013.
26. Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TW, Mercer KB, Mayberg HS, Bradley B, *et al*: Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nature Neurosci 16: 33-41, 2013.
27. Provencal N, Suderman MJ, Guillemin C, Massart R, Ruggiero A, Wang D, Bennett AJ, Pierre PJ, Friedman DP, Côté SM, *et al*: The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. J Neurosci 32: 15626-15642, 2012.
28. Liu X, Zheng Y, Zhang W, Zhang X, Lioyd-Jones DM, Baccarelli AA, Ning H, Fornage M, He K, Liu K and Hou L: Blood methylomics in response to arsenic exposure in a low-exposed US population. J Expo Sci Environ Epidemiol 24: 145-149, 2014.
29. Fiedler U, Wiltfang J, Peters N and Benninghoff J: Advances in the diagnostics of Alzheimer's disease. Nervenarzt 83: 661-673, 2012 (In German).
30. Doumas BT: Standards for total serum protein assays-a collaborative study. Clin Chem 21: 1159-1166, 1975.
31. McPherson IG and Everard DW: Serum albumin estimation: Modification of the bromcresol green method. Clinic Chim Acta 37: 117-121, 1972.
32. Yang X, Liu B, Sang Y, Yuan Y, Pu J, Liu Y, Li Z, Feng J, Xie Y, Tang R, *et al*: Kinetic analysis of the lactate-dehydrogenase-coupled reaction process and measurement of alanine transaminase by an integration strategy. Anal Sci 26: 1193-1198, 2010.
33. Nath RL and Ghosh NK: A preliminary report on the determination of the normal values of serum alkaline phosphatase activity by velocity constant method. Bull Calcutta Sch Trop Med 10: 71-72, 1962.
34. Zhang GH, Cong AR, Xu GB, Li CB, Yang RF and Xia TA: An enzymatic cycling method for the determination of serum total bile acids with recombinant 3alpha-hydroxysteroid dehydrogenase. Biochem Biophys Res Commun 326: 87-92, 2005.
35. Roberts RF and Roberts WL: Performance characteristics of a recombinant enzymatic cycling assay for quantification of total homocysteine in serum or plasma. Clin Chim Acta 344: 95-99, 2004.
36. Jaynes PK, Feld RD and Johnson GF: An enzymic, reaction-rate assay for serum creatinine with a centrifugal analyzer. Clin Chem 28: 114-117, 1982.
37. Tabacco A, Meiattini F, Moda E and Tarli P: Simplified enzymic/colorimetric serum urea nitrogen determination. Clin Chem 25: 336-337, 1979.
38. Whitlow K and Gochman N: Continuous-flow enzymic method evaluated for measurement of serum triglycerides with use of an improved lipase reagent. Clin Chem 24: 2018-2019, 1978.
39. Knob M and Rosenmund H: Enzymic determination of total serum cholesterol with centrifugal analyzers (author's transl). Z Klin Chem Klin Biochem 13: 493-498, 1975 (In German).
40. Hunziker P and Keller H: A mechanized enzymic method for the determination of uric acid (author's transl). Z Klin Chem Klin Biochem 13: 89-96, 1975 (In German).
41. Asrow G: Semiautomated enzymic micro methods for blood glucose and lactic acid on a single filtrate. Anal Biochem 28: 130-138, 1969.
42. Egloff M, Léglise D, Duvillard L, Steinmetz J, Boyer MJ, Ruelland A, Agher R and Beucler I: Multicenter evaluation on different analyzers of three methods for direct HDL-cholesterol assay. Ann Biol Clin (Paris) 57: 561-572, 1999.
43. DaCol P and Kostner GM: Immunoquantification of total apolipoprotein B in serum by nephelometry: Influence of lipase treatment and detergents. Clin Chem 29: 1045-1050, 1983.
44. Girault A, Loiseau D and Girault M: Quantitative determination of apolipoprotein A in human serum by laser nephelometry. Ric Clin Lab 11 (Suppl 2): S19-S29, 1981.
45. Cazzolato G, Prakasch G, Green S and Kostner GM: The determination of lipoprotein Lp(a) by rate and endpoint nephelometry. Clin Chim Acta 135: 203-208, 1983.
46. Deyo RA, Pope RM and Persellin RH: Interference by rheumatoid factor with the detection of C-reactive protein by the latex agglutination method. J Rheumatol 7: 279-287, 1980.
47. Rifai N and Silverman LM: A simple immunotechnique for the determination of serum concentration of apolipoprotein E. Clin Chim Acta 163: 207-213, 1987.
48. Ma J, Cheng J, Wang L, Wang H, Xu L, Liu P, Bu S, Zhang L, Le Y, Ye M, *et al*: No association between IRS-1 promoter methylation and type 2 diabetes. Mol Med Rep 8: 949-953, 2013.
49. Zhang LN, Liu PP, Wang L, Yuan F, Xu L, Xin Y, Fei LJ, Zhong QL, Huang Y, Xu L, *et al*: Lower ADD1 gene promoter DNA methylation increases the risk of essential hypertension. PloS One 8: e63455, 2013.
50. Zemek F, Drtinova L, Nepovimova E, Sepsova V, Korabecny J, Klimes J and Kuca K: Outcomes of Alzheimer's disease therapy with acetylcholinesterase inhibitors and memantine. Expert Opin Drug Saf 13: 759-774, 2014.
51. Pritchard AL, Ratcliffe L, Sorour E, Haque S, Holder R, Bentham P and Lendon CL: Investigation of dopamine receptors in susceptibility to behavioural and psychological symptoms in Alzheimer's disease. Int J Geriatr Psychiatry 24: 1020-1025, 2009.
52. Fratiglioni L, Viitanen M, von Strauss E, Tontodonati V, Herlitz A and Winblad B: Very old women at highest risk of dementia and Alzheimer's disease: Incidence data from the Kungsholmen Project, Stockholm. Neurology 48: 132-138, 1997.
53. Penaloza CG, Estevez B, Han DM, Norouzi M, Lockshin RA and Zakeri Z: Sex-dependent regulation of cytochrome P450 family members Cyp1a1, Cyp2e1, and Cyp7b1 by methylation of DNA. FASEB J 28: 966-977, 2014.
54. Piferrer F: Epigenetics of sex determination and gonadogenesis. Dev Dyn 242: 360-370, 2013.
55. Siegmund KD, Connor CM, Campan M, Long TI, Weisenberger DJ, Biniszkiewicz D, Jaenisch R, Laird PW and Akbarian S: DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons. PloS One 2: e895, 2007.
56. Paula-Lima AC, Tricerri MA, Brito-Moreira J, Bomfim TR, Oliveira FF, Magdesian MH, Grinberg LT, Panizzutti R and Ferreira ST: Human apolipoprotein A-I binds amyloid-beta and prevents Abeta-induced neurotoxicity. Int J Biochem Cell Biol 41: 1361-1370, 2009.
57. Fagan AM, Younkin LH, Morris JC, Fryer JD, Cole TG, Younkin SG and Holtzman DM: Differences in the Abeta40/Abeta42 ratio associated with cerebrospinal fluid lipoproteins as a function of apolipoprotein E genotype. Ann Neurol 48: 201-210, 2000.
58. Emanuele E, Peros E, Tomaino C, Feudatari E, Bernardi L, Binetti G, Maletta R, Micieli G, Bruni AC and Geroldi D: Relation of apolipoprotein(a) size to alzheimer's disease and vascular dementia. Dement Geriatr Cogn Disord 18: 189-196, 2004.
59. Solfrizzi V, Panza F, D'Introno A, Colacicco AM, Capurso C, Basile AM and Capurso A: Lipoprotein(a), apolipoprotein E genotype, and risk of Alzheimer's disease. J Neurol Neurosurg Psychiatry 72: 732-736, 2002.
60. Suszynska-Zajczyk J, Luczak M, Marczak L and Jakubowski H: Inactivation of the paraoxonase 1 gene affects the expression of mouse brain proteins involved in neurodegeneration. J Alzheimers Dis 42: 247-260, 2014.
61. Nazef K, Khelil M, Chelouti H, Kacimi G, Bendini M, Tazir M, Belarbi S, El Hadi Cherifi M and Djerdjouri B: Hyperhomocysteinemia is a risk factor for Alzheimer's disease in an Algerian population. Arch Med Res 45: 247-250, 2014.
62. Hosseinzadeh S, Dabidi Roshan V and Pourasghar M: Effects of intermittent aerobic training on passive avoidance test (shuttle box) and stress markers in the dorsal hippocampus of wistar rats exposed to administration of homocysteine. Iran J Psychiatry Behav Sci 7: 37-44, 2013.
63. Lu YQ, Luo Y, He ZF, Chen J, Yan BL, Wang Y and Yu Q: Hydroxysafflor yellow A ameliorates homocysteine-induced Alzheimer-like pathologic dysfunction and memory/synaptic disorder. Rejuvenation Res 16: 446-452, 2013.
64. Yarchoan M, Louneva N, Xie SX, Swenson FJ, Hu W, Soares H, Trojanowski JQ, Lee VM, Kling MA, Shaw LM, *et al*: Association of plasma C-reactive protein levels with the diagnosis of Alzheimer's disease. J Neurol Sci 333: 9-12, 2013.
65. Duong T, Nikolaeva M and Acton PJ: C-reactive protein-like immunoreactivity in the neurofibrillary tangles of Alzheimer's disease. Brain Res 749: 152-156, 1997.
66. Iwamoto N, Nishiyama E, Ohwada J and Arai H: Demonstration of CRP immunoreactivity in brains of Alzheimer's disease: Immunohistochemical study using formic acid pretreatment of tissue sections. Neurosci Lett 177: 23-26, 1994.
67. Ponnayyan Sulochana S, Sharma K, Mullangi R and Sukumaran SK: Review of the validated HPLC and LC-MS/MS methods for determination of drugs used in clinical practice for Alzheimer's disease. Biomed Chromatogr 28: 1431-1490, 2014.