Elevated methylation of *OPRM1* and *OPRL1* genes in Alzheimer's disease

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Abstract. Previous studies have suggested that increased opioid receptor κ1 (*OPRK1*) and opioid receptor δ1 (*OPRD1*) methylation levels are involved in Alzheimer's disease (AD). In the present study, the methylation levels of two opioid receptor genes, opioid receptor μ1 (*OPRM1*) and opioid related nociceptin receptor 1 (*OPRL1*), were analyzed for their association with AD. Gene methylation levels were measured using bisulfite pyrosequencing in DNA samples derived from blood samples of 51 AD patients and 63 controls. The results indicated that there were significantly elevated promoter methylation levels of *OPRM1* and *OPRL1* in AD (*OPRM1*: P=0.007; *OPRL1*: P=2.987x10^{-6}). Dual-luciferase reporter gene assays demonstrated that the promoter fragments of these two genes were able to promote gene expression (*OPRM1*: Fold-change=2.616, P=0.003; *OPRL1*: Fold change=11.395, P=0.007). In addition, receiver operating characteristic analyses further indicated that a methylation panel of four opioid receptor genes (area under the curve=0.848, sensitivity=0.723, and specificity=0.879) performed well in the prediction of AD. These results suggested that opioid receptor genes may be used as potential methylation biomarkers for the diagnosis of AD.

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Introduction

Alzheimer's disease [AD, (MIM:104300)] is a complex neurodegenerative disorder, affecting approximately 5% of the population worldwide. The prevalence of AD increases markedly after the age of 65 (1), and it accounts for 65-75% of total dementia cases (2). The development of AD is a complex process involving genetic (3,4), physiological, and environmental factors. AD is thought to result from the deposition of β-amyloid (Aβ) peptides and intraneuronal neurofibrillary tangles (NFTs) in the brain (5,6). The alterations in DNA methylation that result from both genetic and environmental causes, have become an emerging topic of interest in AD research (7-9).

The opioid receptor family includes three major opioid receptors (κ-, δ- and μ-opioid receptors), and nociceptin, an opioid receptor-like 1 (*OPRL1*) receptor. The previous study conducted by our group demonstrated that increased methylation in *OPRK1* (κ-opioid receptor gene) and *OPRD1* (δ-opioid receptor gene) was associated with AD (9). Furthermore, the mu-opioid receptor, encoded by opioid receptor μ1 (*OPRM1*), is a major molecular target of morphine and the primary target for its neuropharmacological effects (10). *OPRM1* was also found to be linked with morphine-induced immunosuppression (11). It is interesting to note that the mu-opioid receptor was shown to attenuate Aβ oligomer-induced neurotoxicity by a recent study (12). The opioid related nociceptin receptor 1 (*OPRL1*) is widely expressed in the central nervous system, including the hippocampal dentate gyrus, cortical areas, striatum, thalamus, and hypothalamus (13-15). *OPRL1* has been found to play an essential role in cognition (16), and to modulate inflammation and immune responses (17). In light of these findings, the present study investigated the association of *OPRM1* and *OPRL1* methylation with AD.

Materials and methods

Sample collection. A total of 51 sporadic AD patients and 63 normal subjects were recruited from Ningbo No. 1 Hospital and Ningbo Kangning Hospital, based on the ICD-10

diagnostic criteria (18). All participants were Han Chinese, and were living in Ningbo city of the Zhejiang province. Their detailed information was collected as described previously (7). The present study was approved by the Ethics Committees of Ningbo No. 1 Hospital and Ningbo Kangning Hospital, and all participants signed the informed consent for their participation in the study. Each patient was independently diagnosed by two experienced neurologists. The details of patient screening and diagnosis, and the preparation of samples were performed as described previously (7,9). The concentration of blood metabolites, including triglycerides (TG), total cholesterol (TC), homocysteine (Hcy), blood glucose (Glu), high density lipoprotein (HDL), lipoprotein A (Lp(a)), and alkaline phosphatase (ALP), were measured from each participant. The biochemical parameters of the blood samples were detected using previously described methods (7,19). Bisulfite pyrosequencing assay.

DNA extraction from peripheral blood and subsequent bisulfite conversion were performed as previously described (7,9). DNA methylation was measured by pyrosequencing technology (Pyromark Gold Q24 Reagents; Qiagen, Dulce, Germany). The primer sequences were as follows: 5'-biotin-TAGTTAGGATTGGTTTTTGTAAGAAATAG-3' for the *OPRM1* forward primer, 5'-ATACCCCAAAACATC AATACAATTACTAAC-3' for the *OPRM1* reverse primer, 5'-CTATACCAAATAACCAAAAACAC-3' for the *OPRM1* sequencing primer, 5'-biotin-GTTTGTTTAGTTTGGGAA AGAGG-3' for the *OPRD1* forward primer, 5'-ACACAAAAA TCTCCCCCTTC-3' for the *OPRD1* reverse primer, and 5'-ACCCCCCACAACACA-3' for the *OPRD1* sequencing primer.

Dual-luciferase assays. *OPRM1* and *OPRL1* fragments (*OPRM1*: From +123 to +677 bp; *OPRL1*: From +110 to +500 bp) were synthesized and cloned into the pGL3-basic vector (Sirui Biotech, Ningbo, China). HEK-293T cell culture and seeding were conducted as previously described (9,20). Transient transfections with pRL-SV40 vectors for transfection efficiency control of both constructs were conducted according to TransLipid® HL Transfection Reagent manufacturer protocols (TransGen Biotech, Beijing, China). Following 18 to 72 h of transfection, the activities of *Renilla* and firefly luciferase were measured using the Dual-Luciferase® Reporter Assay Systems (Promega, Madison, WI, USA). The luminescence was quantified with a SpectraMax 190 (Sunnyvale, CA, USA). The pGL3 Promoter Vector (Promega Corporation, Madison, WI, USA), which comprised an SV40 promoter upstream of the luciferase gene, was used as positive control. Three independent transfections were conducted for each construct in triplicate.

Statistical analyses. SPSS software version 16.0 (SPSS, Inc, Chicago, IL, USA) was used for all statistical calculations. The two tailed independent samples t-test and one-way analysis of variance followed by a Bonferroni post hoc test were used to assess differences between continuous variables. Receiver operating characteristic (ROC) analysis was employed to evaluate the diagnostic value of gene methylation for AD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

As shown in Fig. 1, significant pairwise correlations between CpG sites of both genes were identified ($P<0.001$). Therefore, the mean methylation of each gene was used in the subsequent analyses. To evaluate the potential role of *OPRM1* and *OPRL1* methylation in the risk of AD, we carefully selected AD patient and control groups. No significant differences in biological and/or physiological characteristics were noted between the two groups, with the exception of the parameter smoking status ($P=0.025$; Table I) (21). The case-control comparisons revealed significantly higher methylation levels of *OPRM1* and *OPRL1* in AD patients compared with healthy controls (smoking-adjusted $P<0.05$, Table I and Fig. 2). Subgroup analyses by gender indicated that the methylation levels of *OPRM1* and *OPRL1* were significantly and/or moderately increased in AD patients compared with control subjects, in both male and female participants (Table I and Fig. 2). In addition, no significant correlation between *OPRM1* and *OPRL1* methylation was noted ($P>0.05$, data not shown).

Functional activities of the target regions of *OPRM1* and *OPRL1* were assessed with dual-luciferase reporter gene assays. Both gene promoter fragments were able to significantly increase luciferase expression levels (Fig. 3, *OPRM1*: Fold-change=2.616, $P=0.003$, *OPRL1*: Fold-change=11.395, $P=0.007$).

Taken together, the data indicated that the four opioid receptor genes (*OPRK1*, *OPRM1*, *OPRD1* and *OPRL1*) had higher methylation levels in AD patients compared with healthy subjects. These conclusions were consistent with our previous studies. ROC analyses were conducted for each gene and their combined index was used to evaluate their diagnostic values for AD. As expected, the combined index of methylation of all four genes was more informative than that of each single gene (combined index: AUC=0.843, *OPRL1*: AUC=0.765, *OPRM1*: AUC=0.652, *OPRK1*: AUC=0.660, *OPRD1*: AUC=0.800, Fig. 4).

Discussion

The present study provides substantial evidence the methylation levels of *OPRM1* and *OPRL1* are associated with AD. Using a dual-luciferase assay, we demonstrated that the promoter fragments with the tested CG sites could enhance gene transcription, and that their hypermethylation could influence their expression levels. Moreover, ROC analyses indicated a significant contribution of opioid receptor gene methylation to the prediction of AD risk.

Opioid receptors with their ligands comprise an important signaling pathway in the central nervous system (20,22,23). Considerable evidence supports their involvements in tau hyperphosphorylation, Aβ production, and neuro-inflammation (24), suggesting a connection with AD pathogenesis. Promoter DNA methylation is well-known for its function in gene expression, and aberrant regulation of opioid receptors may play a role in disease pathologies, as shown by previous studies conducted on methylation of opioid receptor genes (25). For example, hypermethylated *OPRM1* was shown to inhibit the analgesic effect of morphine in a mouse model of neuropathic pain, while its demethylation restored morphine function (26).

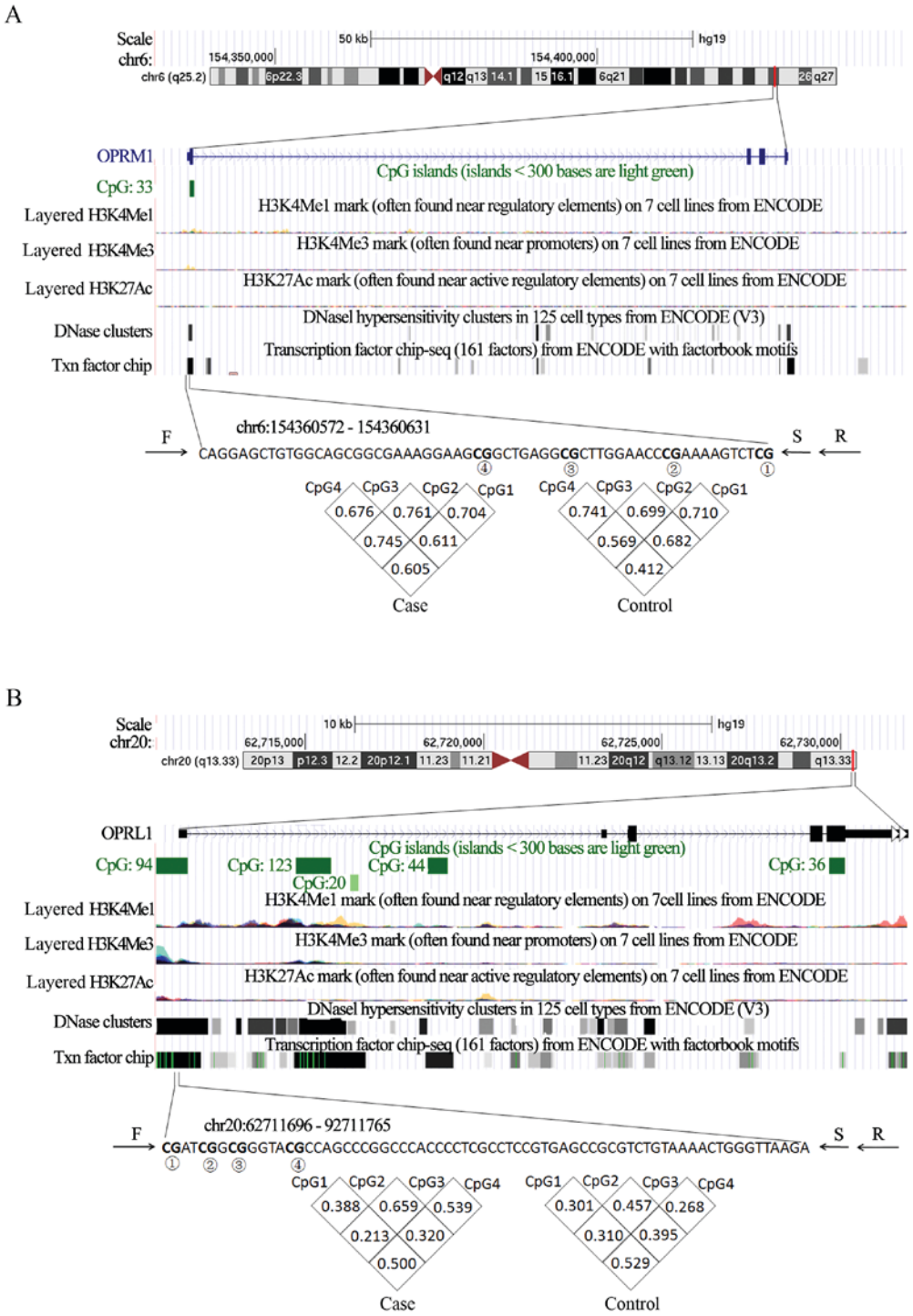

Figure 1. Correlation among four CpG sites in *OPRM1* and *OPRL1*. Schematic representations of correlations of (A) *OPRM1* and (B) *OPRL1* methylation in control subjects, indicating pairwise correlations among CpG sites in the two genes. Fragments in CpG islands overlap with histone marker sites and DNase I hypersensitivity sites. *OPRM1*, opioid receptor μ1; *OPRL1*, opioid related nociceptin receptor 1.

Similarly, *OPRM1* was hypermethylated in oral cancer, and demethylation of *OPRM1* provided an analgesic effect similar to that noted by demethylating drugs (27). Furthermore, opiate addicts indicated higher *OPRM1* methylation levels compared with control subjects (28). Previous results have further revealed increased methylation levels for *OPRL1* in patients with alcohol dependence as well as in control subjects who experienced childhood adversity (29). In the present study, hypermethylation of both *OPRM1* and *OPRL1* genes was shown to be associated with AD. The results suggest that a low density of opioid receptors is noted in AD. Moreover, previous evidence indicated altered densities of opioid receptors in various AD brain regions (24,30). However, the precise mechanism involved in these alterations and its association with methylation requires further research. It is important to note that the, present data suggest a possible diagnostic application for AD by a combinatorial approach. Opioid receptors have shown potential as therapeutic targets in neuropsychiatric

Table I. Comparison of the mean methylation levels of *OPRM1* and *OPRL1* between AD cases and control subjects.

Gene	Cases (n=51)	Controls (n=63)	P-value	Adjusted P-value[a]
OPRM1 methylation (%), total	13.86±4.56	11.67±3.88	0.007	0.004
OPRM1 methylation (%), in males	14.30±5.23	11.85±3.93	0.028	0.024
OPRM1 methylation (%), in females	13.50±3.84	11.18±3.84	0.064	0.064
OPRM1 methylation (%), total	4.08±0.85	3.37±0.70	2.987×10^{-6}	7.35×10^{-5}
OPRM1 methylation (%), in males	3.96±0.83	3.27±0.53	4.679×10^{-5}	0.001
OPRM1 methylation (%), in females	4.26±0.85	3.65±1.00	0.041	0.041

[a]P-values that were adjusted by history of smoking. *OPRM1*, opioid receptor μ1; *OPRL1*, opioid related nociceptin receptor 1; AD, Alzheimer's disease.

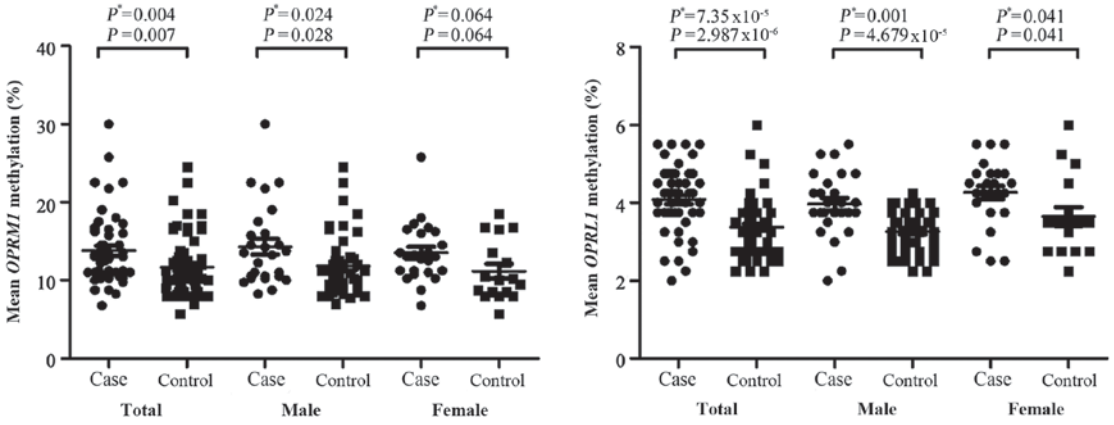

Figure 2. Comparison of mean methylation of *OPRM1* and *OPRL1* between Alzheimer's disease cases and control subjects. *Denotes a P-value that has been adjusted by smoking status. *OPRM1,* opioid receptor μ1; *OPRL1,* opioid related nociceptin receptor 1.

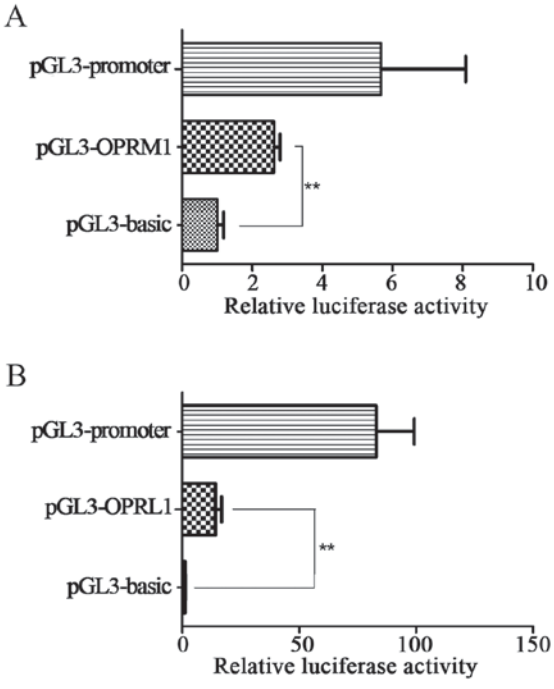

Figure 3. Dual luciferase assays of *OPRM1* and *OPRL1* fragments. Potential promoter-binding activity of fragments from the two opioid receptors was assessed using dual luciferase assays. Significant differences of (A) *OPRM1* (P=0.003) and (B) *OPRL1* (P=0.007) were denoted by **P<0.01, as indicated. *OPRM1,* opioid receptor μ1; *OPRL1,* opioid related nociceptin receptor 1.

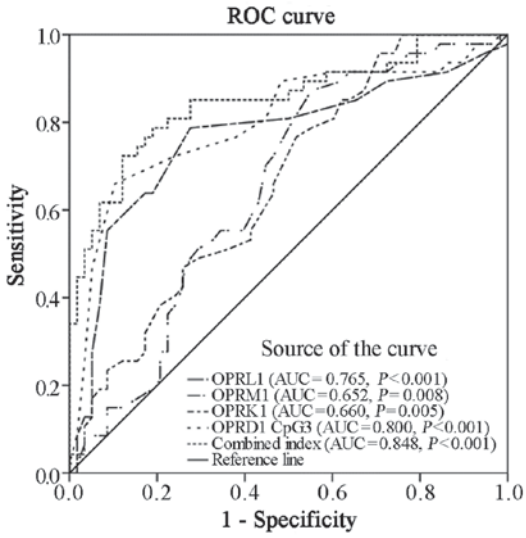

Figure 4. ROC analyses of the methylation levels of opioid receptor genes as diagnostic biomarkers for Alzheimer's disease. ROC analyses were conducted to classify the health status of individuals (*OPRL1*: AUC=0.765, sensitivity=0.787, specificity=0.724, P<0.001; *OPRM1*: AUC=0.652, sensitivity=0.872, specificity=0.448, P=0.008; *OPRK1*: AUC=0.660, sensitivity=0.957, specificity=0.293, P=0.005; *OPRD1* CpG3: AUC=0.800, sensitivity=0.660, specificity=0.897, P<0.001; Combined index: AUC=0.848, sensitivity=0.723, specificity=0.879, P<0.001). The combined index represented the composite methylation of 4 opioid receptor genes. ROC, receiver operating characteristic; *OPRM1,* opioid receptor μ1; *OPRL1,* opioid related nociceptin receptor 1; *OPRK1*, opioid receptor κ1; *OPRD1*, opioid receptor δ1.

diseases as demonstrated by previous studies (31,32). Given the wide distribution of opioid receptor expression in the brain, and the absence of previous blood-based studies on the association between opioid receptor genes methylation and AD, further work is necessary to confirm our findings and test the present hypothesis.

A few limitations are evident in the present study. Firstly, the sample size was small, and additional, larger studies will be necessary to confirm these findings. Secondly, the *OPRM1* and *OPRL1* DNA fragments analyzed may not fully represent the overall level of DNA methylation in both genes, and thus their total contribution to AD may not be accurately represented. In addition, since AD is a neurological disease, future studies using brain tissue are required to confirm our results.

In summary, the present findings on *OPRM1* and *OPRL1* methylation together with our previous study on *OPRK1* and *OPRD1*, suggest an epigenetic involvement of the opioid system in AD, and a potential diagnostic value of opioid receptor methylation for AD.

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Availability of data and materials

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

Authors' contributions

CX, GL and HJ performed the majority of the experiments, data collection, statistical analysis, data interpretation and wrote the manuscript. WHC, DD, ZC, DZ and LX performed sample collection, the biochemical tests and collated the data. HH, WC, LC, QZ and LL 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 Committees of Ningbo No. 1 Hospital and Ningbo Kangning Hospital (Zhejiang, China), and all participants provided written informed consent for their participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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