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Short Communication

Lack of Genetic Association Between *TREM2* and Late-Onset Alzheimer's Disease in a Japanese Population

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Abstract. Rare non-synonymous variants of *TREM2* have recently been shown to be associated with Alzheimer's disease (AD) in Caucasians. We here conducted a replication study using a well-characterized Japanese sample set, comprising 2,190 late-onset AD (LOAD) cases and 2,498 controls. We genotyped 10 non-synonymous variants (Q33X, Y38C, R47H, T66M, N68K, D87N, T96K, R98W, H157Y, and L211P) of *TREM2* reported by Guerreiro *et al.* (2013) by means of the TaqMan and dideoxy sequencing methods. Only three variants, R47H, H157Y, and L211P, were polymorphic (range of minor allele frequency [MAF], 0.0002–0.0059); however, no significant association with LOAD was observed in these variants. Considering low MAF of variants examined and our study sample size, further genetic analysis with a larger sample set is needed to firmly evaluate whether or not *TREM2* is associated with LOAD in Japanese.

Keywords: Alzheimer's disease, Japanese, rare variants, SNP, *TREM2*

INTRODUCTION

Alzheimer's disease (AD) is the main cause of dementia in the elderly. AD is thought to be caused by complex interactions between genetic and environmental factors. A twin study demonstrated that the heritability of late-onset AD (LOAD) is approximately 60~80% [1]. It is also assumed that multiple genes/loci contribute to LOAD development [2]. Rare non-synonymous mutations of *APP*, *PSEN1*, and *PSEN2* are well known to cause familial cases of early-onset AD (EOAD) [3], which accounts for several percent of AD. Concerning LOAD, genome-wide association studies with large numbers of subjects have been conducted, based on the common diseases-common variants hypothesis. As a result, over a dozen genes other than *APOE* have been to be associated with the susceptibility to LOAD [4–10].

TREM2 was recently identified as a novel susceptibility gene for LOAD in Caucasians by two independent study groups [11, 12], both studies being performed on the basis of the common diseases-rare variants hypothesis. A noteworthy fact is that the most significant non-synonymous variant, R47H

(rs75932628: CGC⟶CAC; and minor allele frequency [MAF] < about 1%), located within exon 2 of *TREM2*, shows an odds ratio (OR) range of 2.0–5.0 [11, 12], which is almost equal to the risk magnitude for the *APOE*-ε4 allele [13, 14]. The association of this variant with LOAD [15–19] as well as EOAD [20] has been reproducibly confirmed in multiple Caucasian populations. As to Asians, at present there has only been one genetic association study on *TREM2* variants and LOAD, a northern Han Chinese population being involved [21]. In that study, it was demonstrated that no *TREM2* variants, including R47H, examined show significant association with LOAD [21]. It is assumed that *TREM2* may be a Caucasian-specific susceptibility gene for AD. Therefore, in this study we attempted to replicate the association of *TREM2* with LOAD utilizing a Japanese sample set, comprising 4,688 subjects in total.

SUBJECTS AND METHODS

Subjects

This study was approved by the Institutional Review Board of Niigata University and by all participating institutes. All subjects were Japanese and anonymously genotyped.

We prepared a Japanese sample set, comprising 2,190 LOAD cases (clinically-verified, $n = 1{,}977$; and neuropathologically-characterized, $n = 213$) and 2,498 controls (clinically-verified, $n = 2{,}128$; and neuropathologically-characterized, $n = 370$) (Table 1). From power analysis on the basis of Guerreiro et al.'s study with Caucasians [11], this sample set was estimated to be large enough to detect risk alleles with an OR of 1.1–2.5 (range of risk allele frequency = 0.01–0.99, $\alpha = 0.05$, power = 80%) [29]. A large proportion of the clinically-verified subjects were the same (74.8%) as those in the overall sample set used in our previous genetic study on *GAB2* [22]. The LOAD patients met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association for a diagnosis of probable AD [23]. Non-dementia controls were recruited from among elderly people living in an unassisted manner in the local community. Mini-Mental State Examination [24], Clinical Dementia Rating [25], and/or Function Assessment Staging [26] were applied to assess the severity of the cognitive impairment. All neuropathologically-characterized subjects were utilized in our recent genetic study on *SORL1* [27].

Extraction and quantification of genomic DNA, and *APOE* genotyping are described elsewhere [27, 28]. The *APOE* alleles exhibited strong association with LOAD, as expected: $p_{\text{allele}} = 6.71\text{E-}171$ with χ^2 test (χ^2 value = 783.7, degree of freedom = 2), and $OR_{\varepsilon 4/\varepsilon 3}$ (95% confidence interval [CI]) = 4.81 (4.26–5.42) and $OR_{\varepsilon 2/\varepsilon 3}$ (95% CI) = 0.59 (0.46–0.76).

TREM2 variants and genotyping

To determine whether or not *TREM2* is associated with LOAD in Japanese, we focused on 12 non-synonymous variants of this gene, which were examined in Guerreiro *et al.*'s study with Caucasians [11]: Q33X (rs104894002), Y38C (rs ID, not available), R47H (rs75932628), R62H (rs143332484), T66M (rs201258663), N68K (rs ID, not available), D87N (rs142232675), T96K (rs2234253), R98W (rs147564421), R136Q (rs149622783), H157Y (rs2234255), and L211P (rs2234256). However, two variants, R62H and R136Q, were excluded since one (R62H) did not satisfy the design criteria for the TaqMan® genotyping assay and the other (R136Q) did not work well on TaqMan® genotyping. Consequently, we determined the genotypes of the remaining ten *TREM2* variants using the TaqMan® method (Table 2, Supplementary Table 1). Heterozygotes were further evaluated by means of dideoxy DNA sequencing. Information on sequencing primers is available on request.

Statistical analysis

To detect genotyping errors, a Hardy-Weinberg equilibrium (HWE) test based on Fisher's exact test was conducted. From a 2 × 2 contingency table (case-control status and genotype [MM and Mm]), we computed genotypic p (p_{genotype}) based on Fisher's exact test and OR with 95% CI as the relative risk of disease for each polymorphic variant. We further performed multiple variant analysis as one of gene-based case-control association studies: distribution of minor-allele carriers (Mm) and non-carriers (MM) as to three polymorphic variants, R47H, H157Y and L211P, was compared between cases and controls on the basis of χ^2 test from a 2 × 2 contingency table. Subjects with undetermined genotype data in these variants were omitted for this analysis, with 4,582 subjects remaining. We used SNPAlyze software (DYNACOM, Japan; http://www.dynacom.co.jp/) for these statistical analyses, as described in detail elsewhere [35].

The statistical significance was set at $p < 0.05$.

Table 1
Demographics of the study sample set

	No. of subjects	Age		*APOE* allele frequency		
	(Female %)	Mean (SD)	Range	ε2	ε3	ε4
Cases	2,190 (70.1)	75.2 (6.2)	57–102	0.02	0.67	0.31
Controls	2,498 (54.9)	76.3 (6.6)	65–105	0.05	0.87	0.08

SD, standard deviation.

RESULTS AND DISCUSSION

We attempted to replicate the association of *TREM2* with LOAD in a Japanese sample set, comprising 4,688 subjects in total: cases, $n=2,190$; and controls, $n=2,498$ (Table 1). Three variants, R47H, H157Y, and L211P, were found to be polymorphic; however, the remaining seven, Q33X, Y38C, T66M, N68K, D87N, T96K, and R98W, did not show polymorphisms (Table 2, Supplementary Table 1). The MAF of the variants, R47H, H157Y, and L211P, were less than 0.01 (Supplementary Table 1). Concerning variant R47H [11, 12], three heterozygous subjects were observed: one clinically-verified case (female, age at onset of 76 years old, and *APOE*-ε3*3) and two neuropathologically-characterized controls (one female, age at death of 99 years old, and *APOE*-ε3*3; and one male, age at death of 79 years old, and *APOE*-ε3*3). Variant L211P exhibited the highest MAF among them: 0.0041 in cases and 0.0059 in controls (Supplementary Table 1). Variants R47H, H157Y, and L211P were all in HWE (Supplementary Table 1). In both single and multiple variant analyses, we observed no significant association of *TREM2* with LOAD (Table 2).

TREM2 is mainly expressed in microglia in the brain [30]. This protein directly interacts with a type I transmembrane adapter protein, DAP12 [30]. Recent whole transcriptome analysis of microglia, purified from mouse brains by means of flow cytometry, revealed that *TREM2* belongs to a DAP12-centered protein network, in which multiple microglial marker proteins such as Cd68 are included [31]. A *TREM2*-DAP12 signaling pathway is involved in innate immune responses as well as the differentiation of myeloid progenitor cells into mature microglia [30, 32]. Microglia play an important role in the clearance of amyloid-β protein in the brain [33]. Thus, it is likely that genomic variants of not only *TREM2* but also other genes involved in the *TREM2*-DAP12 signaling pathway may accelerate amyloid plaque deposition through microglial dysfunction [34]. Although none of the rare non-synonymous *TREM2* variants investigated here exhibited association with LOAD in our sample sets (Table 2), we could not rule out the possibility that *TREM2* is one of the crucial proteins for AD from the point of view of biological functions of this protein.

In conclusion, we were not able to detect the significant association of *TREM2* variants examined with LOAD in Japanese, which is consistent with a recent study involving Chinese [21]. On the other hand, *TREM2* has been reproducibly shown to be strongly associated with both LOAD [15–19] and EOAD [20] in multiple Caucasian sample sets. Given these data, *TREM2* may contribute to the susceptibility of LOAD only in Caucasians, i.e., not or only weakly in Asians. However, considering the very low MAF of variants investigated (Table 2, Supplementary Table 1) and our study sample size (Table 1), a large-scale meta-analysis is further needed to comprehensively evaluate whether or not *TREM2* is associated with LOAD in Asians.

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Table 2
Genotypic distribution of three polymorphic variants, R47H, H157Y, and L211P, on *TREM2* in Japanese

Single variant analysis		Allele		Cases (frequency)			Controls (frequency)			$P_{genotype}$ [a]	OR_{Mm} (95% CI)[b]
Variant	dbSNP	M	m	MM	Mm	mm	MM	Mm	mm		
R47H	rs75932628	G	a	2,171 (0.9995)	1 (0.0005)	0 (0.0)	2,477 (0.9992)	2 (0.0008)	0 (0.0)	1.00E+00	0.57 (0.05–6.30)
H157Y	rs2234255	C	t	2,147 (0.9972)	6 (0.0028)	0 (0.0)	2,474 (0.9984)	4 (0.0016)	0 (0.0)	5.29E-01	1.73 (0.49–6.13)
L211P	rs2234256	T	c	2,161 (0.9917)	18 (0.0083)	0 (0.0)	2,461 (0.9884)	29 (0.0116)	0 (0.0)	3.04E-01	0.71 (0.39–1.28)
Multiple variant analysis		Combind genotype		Cases (frequency)			Controls (frequency)			$P_{genotype}$ [c]	OR_{CG-2} (95% CI)[d]
Combind variant	Combind dbSNP	CG-1	CG-2	CG-1	CG-2	others	CG-1	CG-2	others		
R47H-H157Y-L211P	rs75932628-rs2234255-rs2234256	GG-CC-TT	Ga-CC-TT, GG-Ct-TT, GG-CC-Tc	2,104 (0.9883)	25 (0.0117)	0 (0.0)	2,419 (0.9861)	34 (0.0139)	0 (0.0)	5.26E-01	0.85 (0.50–1.42)

In single variant analysis, only three variants, L211P, H157Y, and R47H, are shown here since heterozygotes (Mm) were observed. M, major allele; m, minor allele; MM, major genotype; Mm, heterozygous genotype; mm, minor genotype; CG, combined genotype. [a]Fisher's exact test; [b]OR_{Mm} (95% CI) for the heterozygote (Mm); [c]chi-squared test (degree of freedom = 1); [d]OR_{CG-2} (95% CI) for CG-2 (Ga-CC-TT, GG-Ct-TT, and GG-CC-Tc).

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SUPPLEMENTARY MATERIAL

The supplementary table is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-140225.

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Relative Ratio and Level of Amyloid-β 42 Surrogate in Cerebrospinal Fluid of Familial Alzheimer Disease Patients with Presenilin 1 Mutations

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Key Words

Alzheimer disease · Amyloid-β peptide · Amyloid-β-like peptide · Amyloid precursor-like protein-1-derived Aβ-like peptide · Presenilin · γ-Secretase · γ-Secretase inhibitor

Abstract

Background: Presenilin 1 (PS1) mutations associated with familial Alzheimer disease (FAD) generally increase the amyloid-β 42 (Aβ42) to Aβ40 ratio secreted in cultured cells. Some of these mutants reduce the secretion of Aβ40 rather than increase that of Aβ42. Since it has been difficult to estimate Aβ42 secretion in brains of PS1-FAD patients due to substantial Aβ42 accumulation, it remains unknown whether the enhanced Aβ42 to Aβ40 ratio in brains of FAD patients is caused by elevated Aβ42 secretion or by reduced secretion of Aβ40. ***Objective/Methods:*** Cerebrospinal fluids (CSF) of PS1-FAD patients and neurological control patients (controls) were collected. Levels of CSF amyloid precursor-like protein-1-derived Aβ-like peptide (APL1β), including APL1β28, an Aβ42 surrogate marker, were quantified by liquid chromatography tandem mass spectrometry, and Aβ42 secretion in the brain was estimated. ***Results:*** The relative ratio of CSF APL1β28 to total APL1β was higher in PS1-FAD patients than in controls. Importantly, CSF APL1β28 was not significantly higher. However, C-terminally shorter CSF APL1β25 and APL1β27 were significantly lower in PS1-FAD patients and, as expected, so were CSF Aβ40 and Aβ42. ***Conclusion:*** A higher relative ratio of the CSF Aβ42 surrogate in PS1-FAD patients is not due to its increase in CSF, suggesting that massive Aβ42 accumulation in the PS1-FAD brain occurs without an apparent increase in Aβ42 secretion.

Introduction

The relative ratio of amyloid-β 42 peptide (Aβ42) to total Aβ (the Aβ42 ratio) in secreted Aβ is elevated in cultured media of cells expressing presenilin 1 (PS1) familial Alzheimer disease (FAD) mutations [1]. An in vitro assay demonstrated that purified mutant PS1/γ-secretases also increase the Aβ42 ratio in de novo Aβ [2]. Since substantial amounts of Aβ42 accumulate in brains of FAD patients with PS1 mutations (PS1-FAD patients), it is easy to

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Table 1. Clinical information and levels of CSF APL1β and Aβ peptides of FAD patients and controls

Mutation	Diagnosis	Age at onset, years	APL1β25, pM	APL1β27, pM	APL1β28, pM	Total APL1β, pM	APL1β28 ratio	Aβ40, pM	Aβ42, pM	Aβ42 ratio
PS1 L85P	early-onset AD with spastic paraplegia (variant type AD)	26	806.5	1,009	821.8	2,638	0.3116	396	6.163	0.01531
PS1 G206V	early-onset AD with psychosis	37	886.4	727	868.2	2,481	0.3499	282	12.65	0.04299
PS1 L286V	early-onset AD	40	1,202	809	1,006	3,017	0.3335	253	11.53	0.04361
PS1 L381V	early-onset AD with spastic paraplegia (variant type AD)	29	935.2	1,057	814.9	2,807	0.2903	123	8.919	0.06759
None	neurological control patients	n.r.	1,953± 683.8	1,649± 683.8	948.4± 402.3	4,551± 1,721	0.2080± 0.033	1,009± 456.3	90.91± 41.93	0.0840± 0.016

The values of the controls represent the means ± SD. n.r. = Not recorded.

assume that the accumulation is caused by increased Aβ42 secretion in the brain. However, some PS1 mutations reportedly affect the activity of PS/γ-secretase to secrete Aβ [3–5]. This finding suggests that exogenous expressions of PS1 mutants in cultured cells can increase the Aβ42 ratio in secreted Aβ without elevating the level of Aβ42 secretion; that is, the mutant-expressing cells reduce secretion of major Aβ species such as Aβ40. If this is also the case in mutant-bearing brains, no increase in the level of Aβ42 secretion but an increase in the Aβ42 ratio plays an essential role in the promotion of brain Aβ42 accumulation.

Although this interpretation seems mysterious [3–5], the findings of some studies do not contradict this notion. First, a small increase in the Aβ42 ratio is more crucial for neurotoxicity than the absolute amounts of Aβ42 [6]. Second, amyloid pathology is exacerbated in the brain of knock-in mice with an artificial PS1 mutation which reduces the level of Aβ40 but not of Aβ42 in an in vitro γ-secretase assay [7]. Third, additional Aβ40 expression in the Tg2576 mouse brain was found to diminish Aβ42 accumulation [8].

The Aβ42 level in cerebrospinal fluid (CSF) may be the best indicator to determine whether Aβ42 secretion is elevated in FAD brains. However, it is quite common that both the ratio and the level of CSF Aβ42 are lowered in FAD patients [9]. Although it is still controversial [10], it may be the result of brain Aβ42 accumulation [11].

Previously, we reported that amyloid precursor-like protein-1-derived Aβ-like peptide 28 (APL1β28) in CSF is a non-amyloidogenic surrogate marker for brain Aβ42 production. The relative ratio of CSF APL1β28 to total APL1β (the APL1β28 ratio) is higher in PS1-FAD cases than in non-AD cases [12]. In this study, we analyzed CSF APL1β levels, including those of APL1β28, in PS1-FAD patients and in neurological control patients and tried to estimate whether the absolute values of newly generated Aβ42 levels are comparatively elevated in the brains of the PS1-FAD patients.

Results and Discussion

The CSF Aβ40 Level Is Lower in PS1-FAD Patients

CSF samples of 4 FAD patients with a PS1 mutation (PS1 L85P, L286V, L381V and G206V; PS1-FAD patients) and 22 neurological control patients (controls) were used (table 1) [12]. CSF samples of the 2 other FAD patients with a PS1 mutation (PS1 H163R and M233L) were excluded from this study, because these two mutations do not affect γ-cleavage of amyloid precursor-like protein 1 [12].

First, we quantified CSF Aβ40 and Aβ42 by means of ELISA. As expected, both Aβ40 and Aβ42 levels in the PS1-FAD patients were significantly lower than those in the controls ($p < 0.0005$ for Aβ40 and $p < 0.0005$ for Aβ42; fig. 1a, b; table 1) [13]. Aβ40 is the most predominant Aβ species and much less amyloidogenic than Aβ42. Thus, a lowered Aβ40 level in CSF of PS1-FAD patients may to some extent reflect a reduction in the total Aβ secretion level in the brain.

In accordance with previously published results, the ratios of CSF Aβ42 to total Aβ of PS1-FAD patients were significantly lower than those of the controls ($p < 0.005$; fig. 1c; table 1) [9].

The CSF APL1β28 Level, a Surrogate Marker of Brain Aβ42 Production, Is Not Elevated in PS1-FAD Patients

We next quantified levels of CSF APL1β25, APL1β27 and APL1β28 by means of liquid chromatography tandem mass spectrometry (LC/MS/MS) [12]. Because all three APL1β species are non-amyloidogenic, the level of each APL1β species in CSF is considered to reflect the secretion level of the corresponding species in the brain [12]. Moreover, the APL1β28 and Aβ42 ratios change in parallel [12].

The APL1β28 ratios to total APL1β of the PS1-FAD patients were significantly higher than those of the control ($p < 0.0005$; fig. 2a; table 1) [12]. Importantly, CSF APL1β28 levels of the PS1-FAD patients were not higher than those of the control ($p = 0.8291$; fig. 2b; table 1). Moreover, levels of CSF APL1β25 and APL1β27 were lower in the PS1-FAD patients than in the controls ($p < 0.05$ for APL1β25 and $p < 0.05$ for APL1β27; fig. 2c, d; table 1). The summed levels of three peptides, that is, the total level of APL1β, were also lower in the PS1-FAD patients ($p < 0.05$; fig. 2e; table 1). These results suggest that the higher APL1β28 ratio in CSF of the PS1-FAD patients is not due to an increase in the absolute level of APL1β28 generated but to a decrease in the absolute levels of C-teminally shorter APL1β species generated in brains.

These data for the Aβ42 surrogate marker lead us to speculate that in the brains of the PS1-FAD patients (1) the Aβ42 ratio is elevated, (2) the absolute values of newly generated Aβ42 levels are not elevated, and (3) the absolute values of newly generated C-terminally shorter Aβ species such as Aβ40 are lowered. Thus, Aβ42 accumulation in brains of the PS1-FAD patients may be induced not by an increase in Aβ42 generation but by a reduction in the secretion of C-terminally shorter Aβ species.

How does such a change, i.e. a decrease in C-terminally shorter APL1β species without an accompanying increase or decrease in the longer APL1β28 in CSF of the PS1-FAD patients, occur? Recently, we found that PS/γ-secretase cleaves elongated Aβ and Aβ-like peptides into shorter species, i.e. Aβ42 into Aβ38, Aβ43 into Aβ40/38, and APL1β28 into APL1β25 [14]. Because mutant PS1/γ-secretases diminish Aβ42 cleavage [14], the ratios of longer Aβ and Aβ-like peptides to the corresponding shorter species inevitably increase. Thus, a reduction in cleavages of APL1β28 by the mutant PS/γ-secretases may at least in part account for the increase in the CSF APL1β28 ratio to total APL1β in the PS1-FAD patients.

The very small number of CSF samples analyzed is a limitation to our study. However, it should be noted that patients with several different PS1 mutations all showed the similar phenotype.

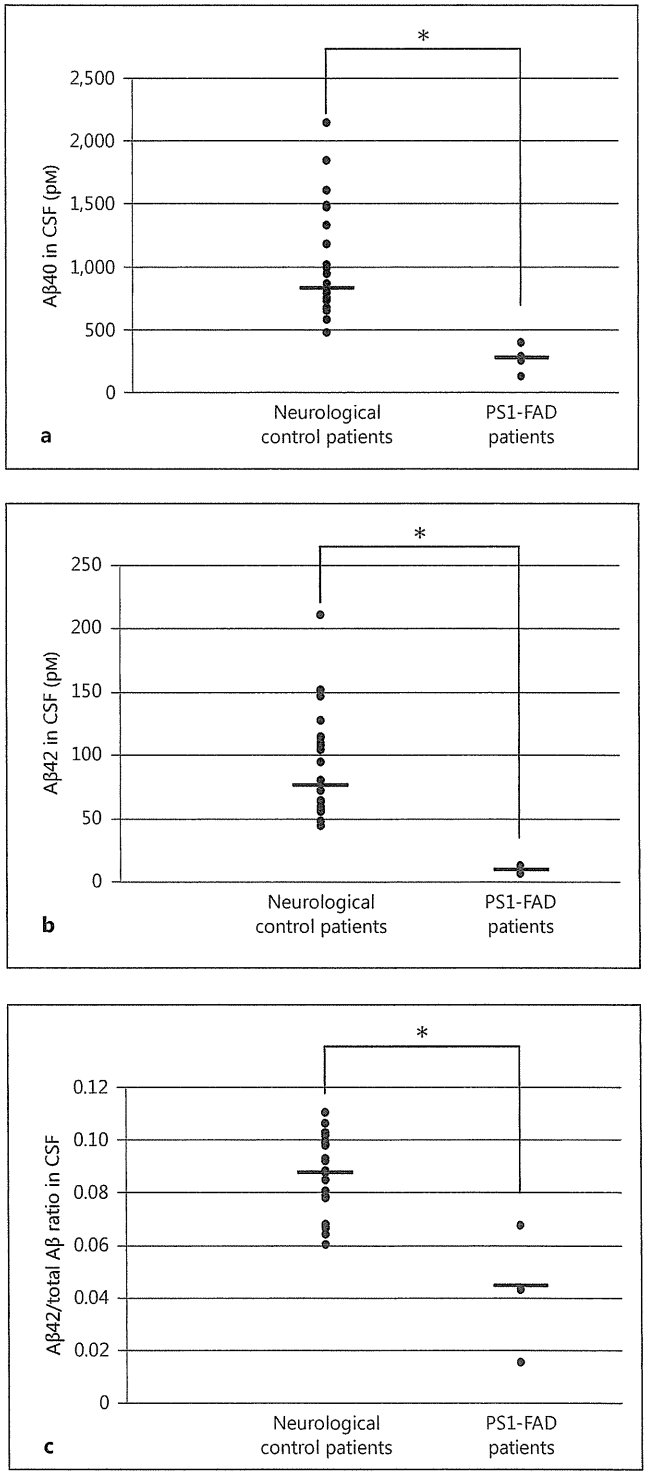

Fig. 1. Measurement of Aβ species levels in CSF. CSF Aβ40 levels (**a**), Aβ42 levels (**b**) and Aβ42 to total Aβ ratios (**c**) of the PS1-FAD patients and the controls. The bars represent median values. The asterisks indicate statistical significance (nonparametric statistical analysis).

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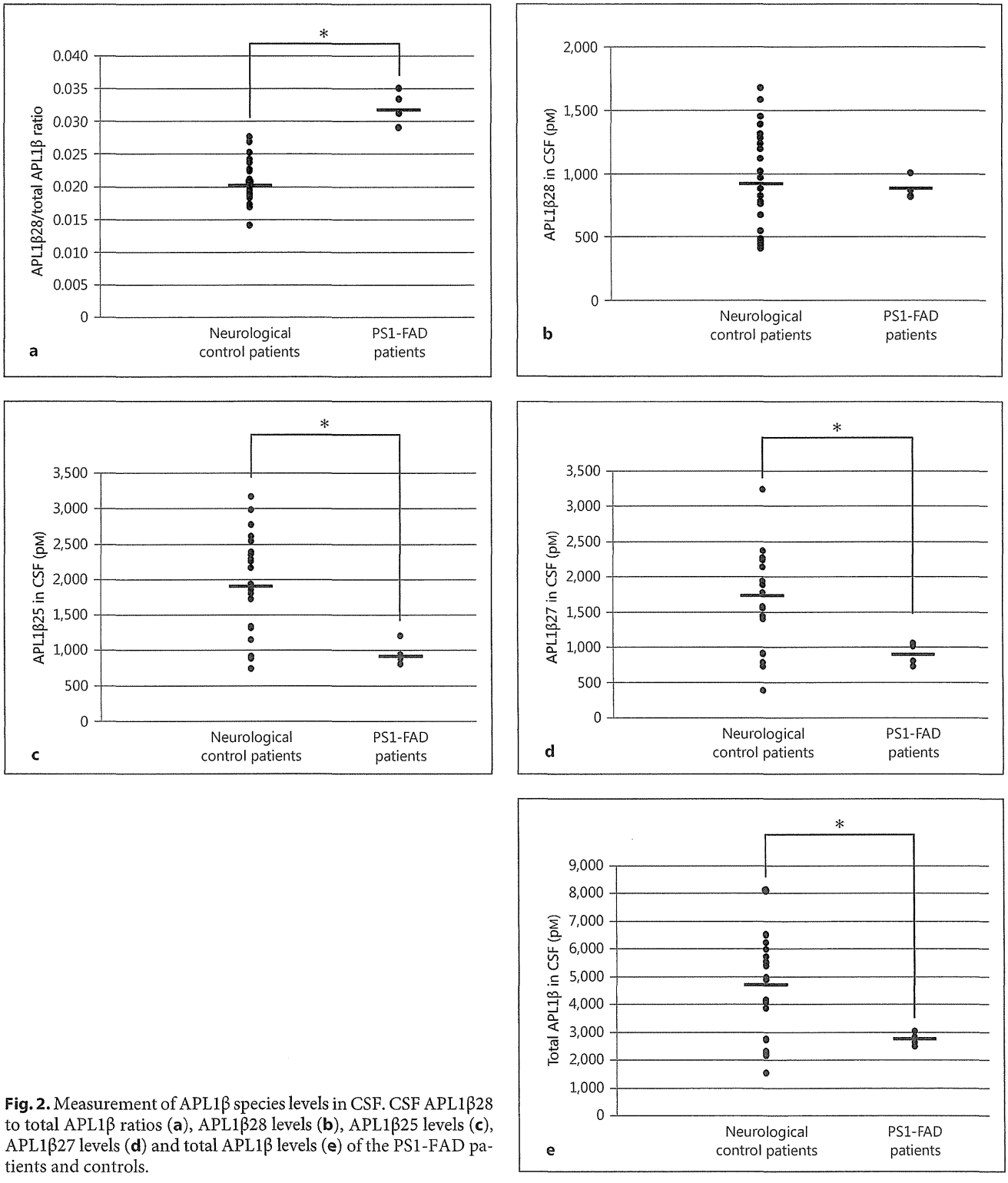

Fig. 2. Measurement of APL1β species levels in CSF. CSF APL1β28 to total APL1β ratios (**a**), APL1β28 levels (**b**), APL1β25 levels (**c**), APL1β27 levels (**d**) and total APL1β levels (**e**) of the PS1-FAD patients and controls.

γ-Secretase inhibitors (GSIs) are one of the main disease-modifying drugs being developed. Our findings point to the need for caution regarding GSI development because GSIs inhibit Aβ42 cleavage as well as total γ-secretase activity. Thus, GSI administration will inevitably increase the Aβ42 ratio unless it completely blocks Aβ production [15]. The Aβ42 ratio will thus remain higher with GSI administration, which leads to a risk of enhancing disease progression even if total Aβ secretion is reduced.

Experimental Procedures

Aβ ELISA

Aβ40 or Aβ42 levels in human CSF were quantified with commercial sandwich ELISA kits (Wako Pure Chemical Industries, Ltd.). Human CSF was appropriately diluted with the standard diluents provided in the kit.

LC/MS/MS Analysis

The levels of APL1β25, APL1β27 and APL1β28 were quantified by means of LC/MS/MS analysis as described previously [12].

Acknowledgements

All the experiments using CSF of FAD and employing CSF from non-demented patients were approved by the ethics committee of Osaka University Hospital (No. 07176-4). We are grateful for funding from KAKEN-C, the National Institute of Biomedical Innovation (05-26), the National Center for Geriatrics and Gerontology (23-5), and the Strategic Research Program for Brain Sciences, Japan.

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Altered CpG methylation in sporadic Alzheimer's disease is associated with APP and MAPT dysregulation

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The hallmark of Alzheimer's disease (AD) pathology is an accumulation of amyloid β (Aβ) and phosphorylated tau, which are encoded by the amyloid precursor protein (APP) and microtubule-associated protein tau (*MAPT*) genes, respectively. Less than 5% of all AD cases are familial in nature, i.e. caused by mutations in *APP*, *PSEN1* or *PSEN2*. Almost all mutations found in them are related to an overproduction of $A\beta_{1-42}$, which is prone to aggregation. While these genes are mutation free, their function, or those of related genes, could be compromised in sporadic AD as well. In this study, pyrosequencing analysis of post-mortem brains revealed aberrant CpG methylation in *APP*, *MAPT* and *GSK3B* genes of the AD brain. These changes were further evaluated by a newly developed *in vitro*-specific DNA methylation system, which in turn highlighted an enhanced expression of *APP* and *MAPT*. Cell nucleus sorting of post-mortem brains revealed that the methylation changes of *APP* and *MAPT* occurred in both neuronal and non-neuronal cells, whereas *GSK3B* was abnormally methylated in non-neuronal cells. Further analysis revealed an association between abnormal *APP* CpG methylation and apolipoprotein E ε4 allele (*APOE* ε4)-negative cases. The presence of a small number of highly methylated neurons among normal neurons contribute to the methylation difference in *APP* and *MAPT* CpGs, thus abnormally methylated cells could compromise the neural circuit and/or serve as 'seed cells' for abnormal protein propagation. Our results provide a link between familial AD genes and sporadic neuropathology, thus emphasizing an epigenetic pathomechanism for sporadic AD.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease and is pathologically characterized by an accumulation of amyloid β (Aβ) peptide and phosphorylated tau (1). Since the discovery of the gene mutations responsible for familial AD (FAD), namely *PSEN1*, *PSEN2* and *APP*, which encode presenilin 1, 2 and amyloid precursor protein (APP), respectively, huge advances have been made in our understanding of the disease pathomechanism. Pathologically, sporadic AD and FAD are almost identical in terms of abnormal Aβ and phosphorylated tau accumulation, which suggests that the same genes involved in FAD may also play a role in the pathogenesis of sporadic AD; however, no mutations in these genes have been noted in sporadic cases. Indeed, the etiology of sporadic AD, which accounts for >95% of all AD cases, remains largely unknown.

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Recently, it has been shown that an increase in *APP* gene dosage is a rare cause of FAD (2); in these cases, a 1.5-fold increase in the *APP* expression level resulted in early onset AD. In addition, patients with Down syndrome have been known to exhibit AD pathology in their fourth to fifth decades of life; this is noteworthy because those individuals have an extra copy of chromosome 21, where the *APP* gene is located (3). Thus, *APP* expression in Down syndrome patients is also 1.5-fold higher than in normal controls (NCs). These findings provide convincing evidence that AD can be caused by increased *APP* translation due to increased gene dosage; however, whether or not *APP* gene expression is increased in sporadic AD cases remains controversial (4–7). One of the main reasons for this discrepancy may be due to differences in the quality of post-mortem brain samples. For example, RNA can be compromised by a lengthy post-mortem interval and affected by long-term storage conditions. Alternatively, since the brain is a mixture of several different cell types, it may be difficult to extract subtle expression changes that are occurring in only a limited population of certain cells. We previously reported aberrant CpG demethylation associated with alpha-synuclein (*SNCA*) over-expression in the substantia nigra of patients with Parkinson's disease (8). In this study, we also found that the methylation status remained stable for 24 h post-mortem, which provides good rational for studying DNA methylation instead of RNA expression profiles in post-mortem brains.

Herein, we demonstrate that pyrosequencing analysis of post-mortem brains revealed epigenetic changes in *APP*, *MAPT* and *GSK3B* genes in sporadic cases of AD. Additionally, newly developed *in vitro* experiments confirmed the effect of altered methylation on gene expression. Moreover, the increased methylation observed in sporadic AD brains was more prominent in an apolipoprotein ε4 (APOE4)-negative population. Our results shed new light on sporadic AD pathogenesis by revealing a missing link between genes involved in FAD and proteins accumulated in sporadic AD.

RESULTS

We examined age-matched samples from three institutes in Japan (Table 1). The cerebellum, anterior parietal lobe and inferior temporal lobe cortices were analyzed since those areas were available for the majority of cases (Table 2); also, they are important regions for AD neuropathological diagnosis (9,10). We then selected genes of interest related to sporadic AD or FAD (11), including *ACE*, *APOE*, *APP*, *BACE1*, *GSK3B*, *MAPT* and *PSEN1*. CpG islands were located within those genes using a software program (12). Multiple CpGs for each gene were selected, and primer sets were designed for pyrosequencing (Supplementary Material, Table S1). After precise primer calibration (Supplementary Material, Fig. S2) and selection of validated primer sets, small-scale analyses were performed using 15–20 samples from NC and AD temporal lobe samples (Figs 1A–D and 2A–C). Student's *t*-tests revealed several CpGs of interest (Fig. 2D–E), after which we proceeded with a full investigation of those CpGs using all the available samples; this revealed 15 CpGs among 3 different genes that were differentially methylated in AD brains compared with NC brains (Table 3, Fig. 3). Interestingly, statistical significance was mainly observed in temporal lobe samples, while patterns of methylation difference in parietal and cerebellum samples showed at best some resemblance. To test whether these observed differences were specific for AD, we also assessed temporal lobe samples from 50 patients with dementia with Lewy bodies (DLB); this produced similar results to NC, thus confirming that the higher *APP* 60–63 methylation level is an AD-specific phenomenon (Supplementary Material, Fig. S3).

Table 1. Demographics of the postmortem cases analyzed in this study

	NC	AD
Age at death (year old)	76.59 ± 4.506	78.68 ± 7.987
Male%	58	46.5
Brain weight (g)	1262	1185
Post mortem interval (h)	12	14.5
APOE4 (%)	18.84	53.5

AD, Alzheimer's disease; NC, normal control. There were no statistical differences with age at death.

Table 2. Number of samples per regions used in this study

Region	Cerebellum	Parietal	Temporal
NC	71	76	74
AD	45	59	56
Total	126	135	130

Our initial analysis was performed by bulk DNA samples from the cortices, which was comprised of several different cell types, including neuronal, glial and vascular cells. Thus observed finding might be due to alteration of cellular composition, due to selective loss of neurons in the AD brains. To address this, we utilized an established fluorescence-activated cell sorting (FACS) technique (13) in order to enrich neuronal and non-neuronal nucleus separately. Six AD samples and nine NC samples that were representative of high or low methylation status, as determined by previous analyses, were subjected to this procedure. Average NeuN+ events/NeuN- events ratio was 0.593 ± 0.096 in NC and 0.495 ± 0.047 in AD ($P = 0.4493$ by Student's *t*-test). After successful purification of neuronal and non-neuronal nuclei, DNA was extracted. Subsequent pyrosequencing revealed that for *APP* and *MAPT* CpGs, the difference was due to both neurons and non-neuronal cells. Conversely, the difference in *GSK3B* methylation was mainly observed in non-neuronal cells (Fig. 4). These results suggest that aberrant CpG methylation among these genes could play a role in sporadic AD pathology.

Epigenetic alteration without transcriptional change is of little pathomechanistic interest. However, transcriptome analyses using post-mortem brains have inevitable RNA degradation problems that can compromise the result. Thus, we aimed to obtain *in vitro* experimental data that could thoroughly determine the effect of aberrant methylation. In cultured cells, the methylation status of the four regions identified in this study and the expression levels of corresponding genes showed some correlations, but they were not conclusive (Supplementary Material, Fig. S4), possibly because these cell lines are polyploids with huge numbers of chromosomal rearrangements. To overcome this issue, we

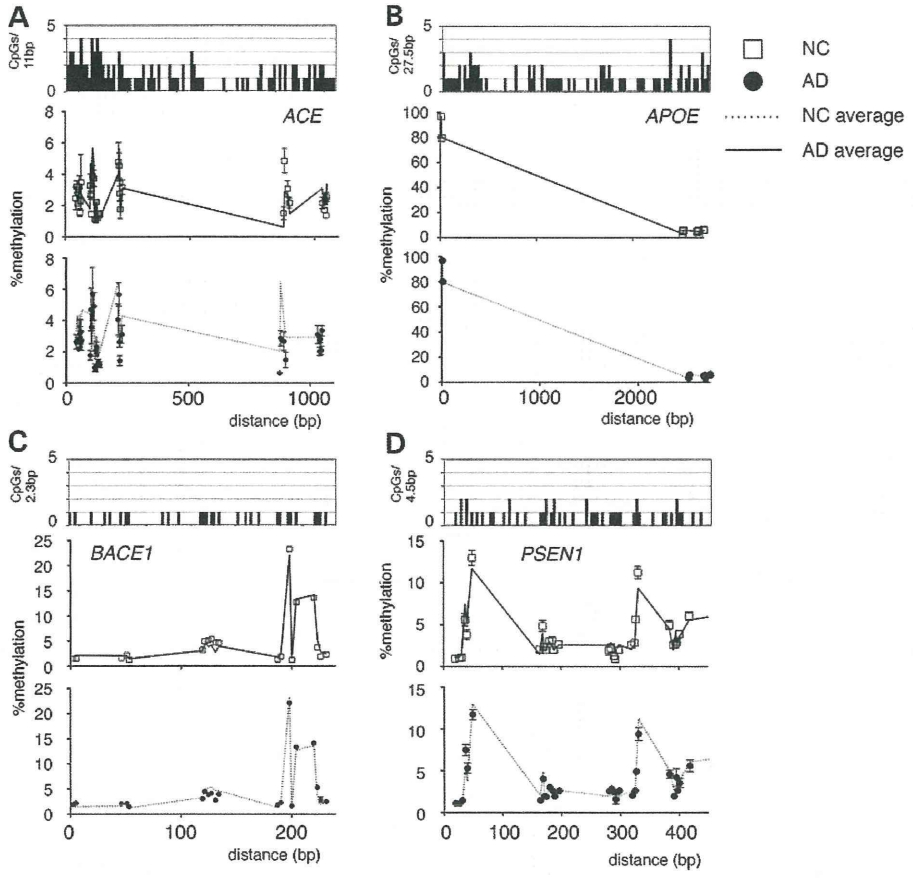


Figure 1. Overview of the methylation status of *ACE*, *APOE*, *BACE1* and *PSEN1* in a small sample group which were obtained before approval of choline esterase inhibitors in Japan. CpG density is shown at the top of the graphs, and the methylation status at the analyzed positions is plotted below. Upper graph panels show normal control (NC) plotted on Alzheimer's disease (AD) average background and the lower panels vice versa. No statistically significant differences were found. (**A**) *ACE*, (**B**) *APOE*, (**C**) *BACE1*, (**D**) *PSEN1*. Open squares: NC with SEM, closed circles: AD with SEM, dotted lines: connecting line of NC average, straight lines: connecting line of AD average. Number of samples used for each groups were 15 in *APP*, *MAPT*, *GSK3B* and 20 in ACE, *APOE*, *BACE1* and *PSEN1*.

established an *in vitro* sequence-specific methylation system using a TAL (transcription activator-like) effector construct fused to the DNA methylase domain of DNMT3a. TALs can be designed to bind specific DNA sequences according to their protein subsequences (14–16). As a control, we generated a methylation-defective DNMT3a mutant V777G construct (17). Among several TAL sequences tested, we found two *APP* CpG 60–63-specific sequences and one *MAPT* 58–62-specific sequence that were effective in altering the methylation level of those two regions. There were no effective TAL sequences for *APP* 88 and *GSKB* 78–82 despite rigorous screening. Although the TAL binding effectiveness was relatively low and the fold methylation change was at most four times compared with the control vector when analyzed by the whole cultured cell population, expression levels of APP and MAPT, as measured by qPCR, were successfully altered along with specific CpG methylation (Fig. 5A–C) and actual methylation level was similar to the values obtained from human samples (Fig. 5D). This result clearly shows that increased *APP* CpG 60–63 methylation was associated with APP expression enhancement, whereas increased *MAPT* 58–62 methylation was associated with MAPT expression suppression, thus leading to the conclusion that epigenetic changes in AD brains, as observed in our study, are associated with an increased expression of both APP and MAPT.

To understand the role of altered methylation in AD pathogenesis, we next tried to correlate other clinical information with CpG methylation. We found that increased methylation of the first half of the *APP* 60–63 CpG region was more prominently observed in *APOE* ε4-negative AD cases (Fig. 6). Moreover, there was some correlation between the methylation status and the *APOE* ε4 gene dosage at *APP* CpGs 60 and 61, although this was not statistically significant due to the small number of *APOE ε4* homozygotes (Supplementary Material, Fig. S6). Other clinical information such as age at death or sex had no correlation with the methylation level of *APP*, *MAPT* and *GSK3B* (Supplementary Material, Figs S6 and S7).

DISCUSSION

We have previously demonstrated that the methylation level was conserved within 24 h of the post-mortem period (8,18). In

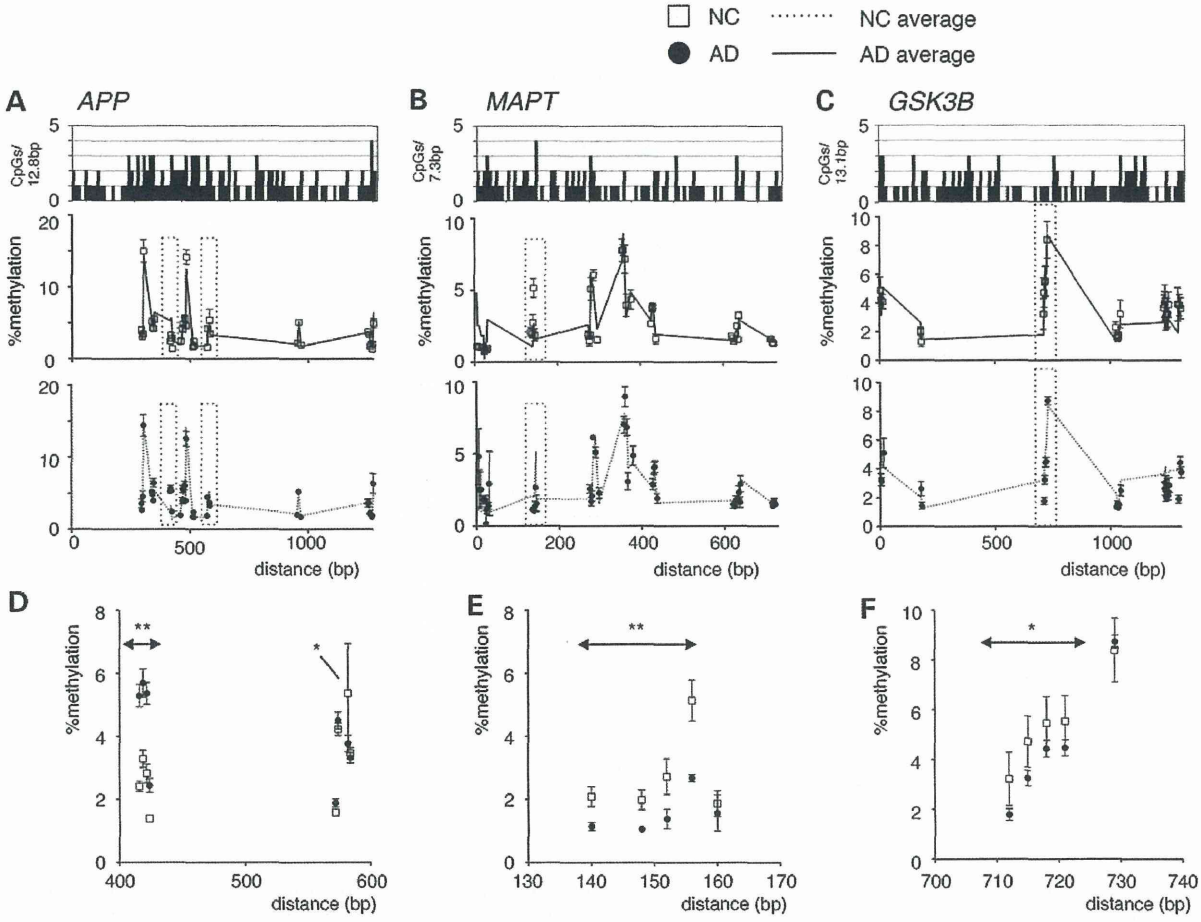


Figure 2. Overview of methylation status of *APP*, *MAPT* and *GSK3B* in a small sample group. (**A**–**C**) CpG density is shown at the top of the graphs, and methylation status at analyzed positions is plotted below. Upper graph panels show NC plotted on AD average background and the lower panels vice versa. Open squares: NC with SEM, closed circles: AD with SEM, dotted lines: connecting line of NC average, straight lines: connecting line of AD average. In each plots, regions of interests that showed statistically significant differences between AD and NC are shown with dotted lines and are magnified in (**D**–**F**). (**A** and **D**) *APP*; (**B** and **E**) *MAPT*; (**C** and **F**) *GSK3B*. $^{*}P < 0.05$, $^{**}P < 0.01$

Table 3. Analyzed genes and the number of CpG sites tested. Statistical analysis revealed five CpG sites in three genes

Gene	Tested CpGs	NC/AD significant CpGs	CpG position
ACE	35	0	
APOE	11	0	
APP	35	5	60–63, 88
BACE1	20	0	
GSK3B	26	5	78–82
MAPT	43	5	58–62
PSEN1	33	0	

addition, since DNA is more stable then RNA, they could reflect the disease process more precisely than transcriptome analysis that can be affected by other factors such as end-stage complications. Thus, our rationale for employing epigenome rather than transcriptome analysis of the post-mortem brain was to avoid the possibility of post-mortem mRNA degradation and transcriptome alterations induced at the agonal stage. Aberrant CpG methylation in AD has been reported; however, there has been no direct link to the pathogenesis of the disease (19). We chose to analyze CpG methylation by pyrosequencing rather than microarray analysis. This is because commercially available microarrays do not cover every single CpG on the genome, and we were concerned with missing CpGs that were of significance. Indeed, past reports on epigenome analysis in either APP CpG island or in AD brains failed to detect significant alteration in AD brains (18,20). In addition, we decided not to employ TA cloning and bisulfite sequencing for large-scale analysis due to its low throughput and cloning bias problems (21,22). However, there were CpGs that could not be assessed in the regions depicted in Figures 1 and 2 due to faulty pyrosequencing primer calibration, there is still a chance that we missed other CpGs of importance.

The analyzed samples were age-matched (Table 1), and the methylation level did not show any correlation with age at death (Supplementary Material, Fig. S7). As usually observed in the AD population, our AD cases were female dominant (Table 1); however, the methylation levels were not affected by sex (Supplementary Material, Fig. S8). Thus, we concluded

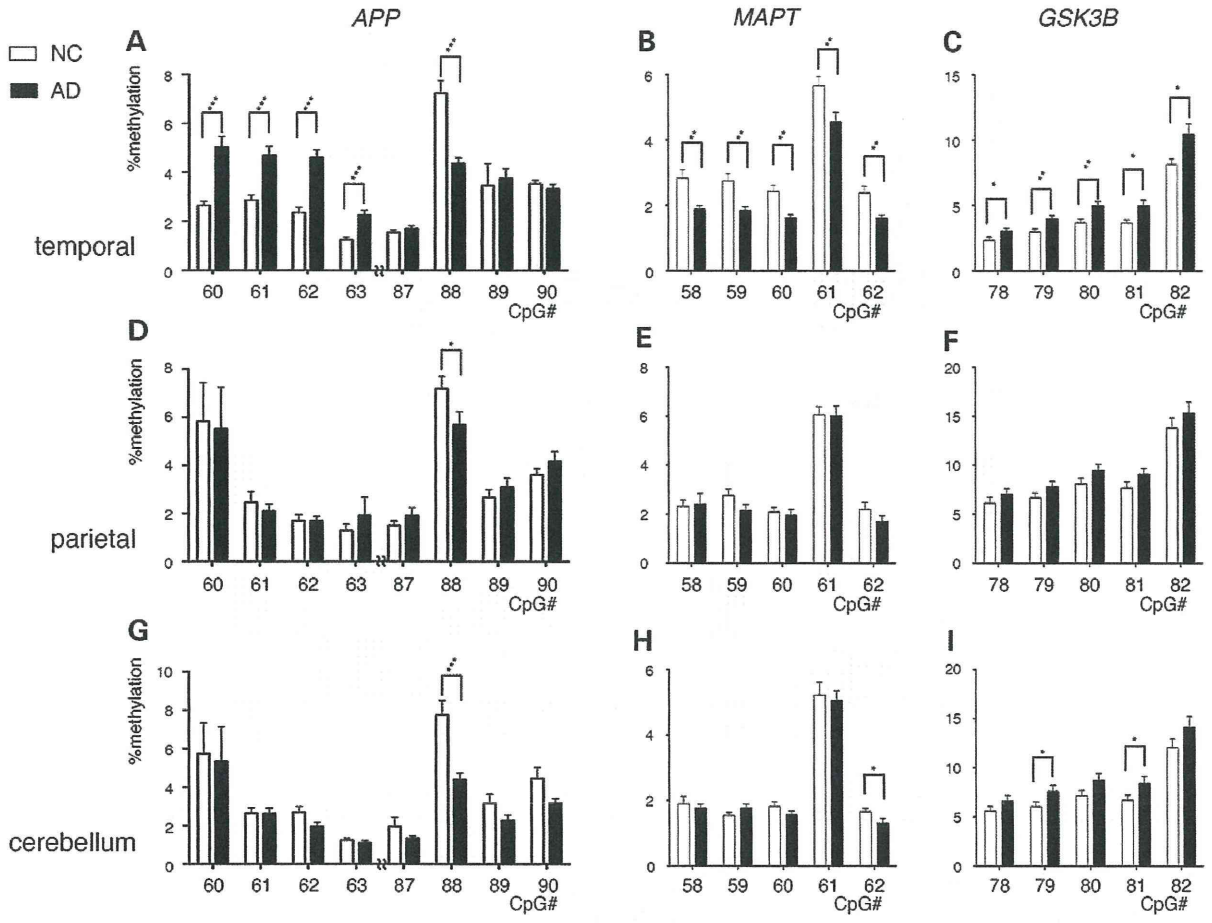


Figure 3. Large-scale analysis of CpG sites of *APP*, *MAPT* and *GSK3B* in three different brain regions. All the samples described in Table 2 was used for analyses. NC, open bars; AD, closed bars. (**A**, **D** and **G**) *APP*; (**B**, **E** and **H**) *MAPT*; (**C**, **F** and **I**) *GSK3B*. (**A**–**C**) Temporal lobe; (**D**–**F**) parietal lobe; (**G**–**I**) cerebellum. Bar = SEM. Two-way ANOVA and Bonferroni's multiple comparison tests revealed statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

that the results were not biased by age or sex. Direct genome sequencing excluded any single nucleotide polymorphisms in the analyzed regions. Since large numbers of AD patients take choline esterase inhibitors (ChEI), it raises the possibility that such drugs could affect the results. However, our initial screening process (shown in Figs 1 and 2), which was carried out on samples obtained before the approval of donepezil, the first ChEI, in October 1999 in Japan, eliminates this possibility. Thus, we concluded that the CpG alterations observed in AD brains are indeed reflecting the underlying pathological process.

CpGs identified in the analysis were located at different position relative to exons and transcription initiation sites (Fig. 7). CpG methylation at the 5′ promoter region is associated with low transcription factor binding that reduces transcription, whereas CpG methylation in other regions could be associated with enhanced transcriptional activity (23–25). Our *in vitro* experiment data showed higher methylation results had differential effects on gene expression, which is in accordance with these previous findings. Regardless of the CpG methylation alteration, we found all methylation changes in AD brains were associated with an increased expression of *APP* and *MAPT*. Furthermore, our FACS experiment clearly demonstrates that those changes resulted in expression occur in both neuronal and non-neuronal cells. We were initially concerned that significant neuronal loss in AD brains could bias the result. However, comparison of FACS event did not show significant difference in the NeuN+/NeuN− ratio between the NC and AD group, indicating that the neuronal loss did not contribute to epigenetic alteration observed in bulk derived DNA.

Our present finding is of particular interest since increased APP production and MAPT can be directly linked to AD pathogenesis. As for *GSK3B*, we could not determine the effect of hypermethylation in our *in vitro* experiments; however, considering the position of *GSK*3B 78–82 (Fig. 7C), we speculate that hypermethylation may act as a gene expression suppressor. Based on the FACS result, *GSK3B* down-regulation can occur mainly in non-neuronal cells, which in turn might provide some protection against abnormal tau phosphorylation compared with neuronal cells; this is compatible with neuropathological findings that neurofibrillary tangles (NFTs) are seldom found in glial cells of the AD brain while large number of neurons harbors NFTs (26).

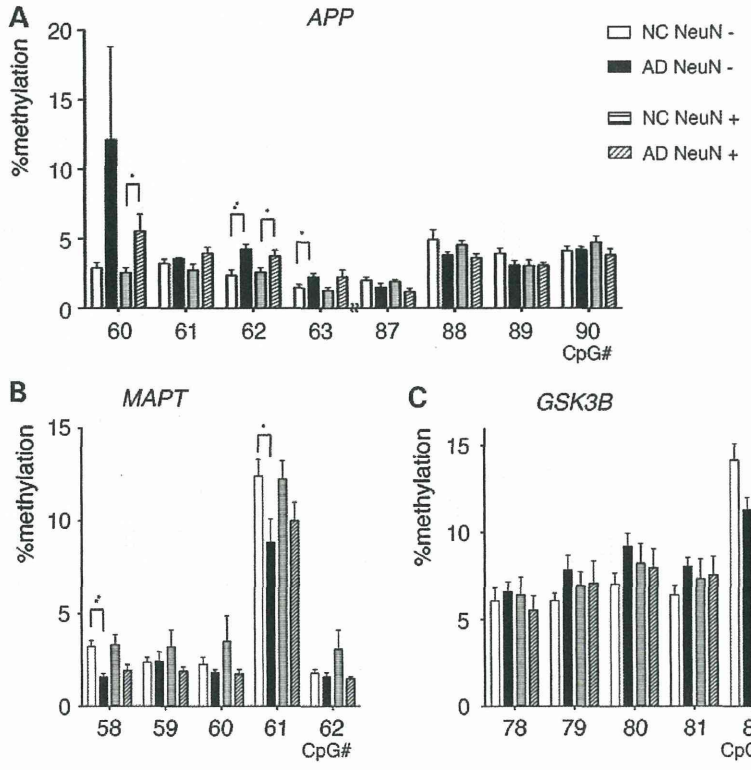
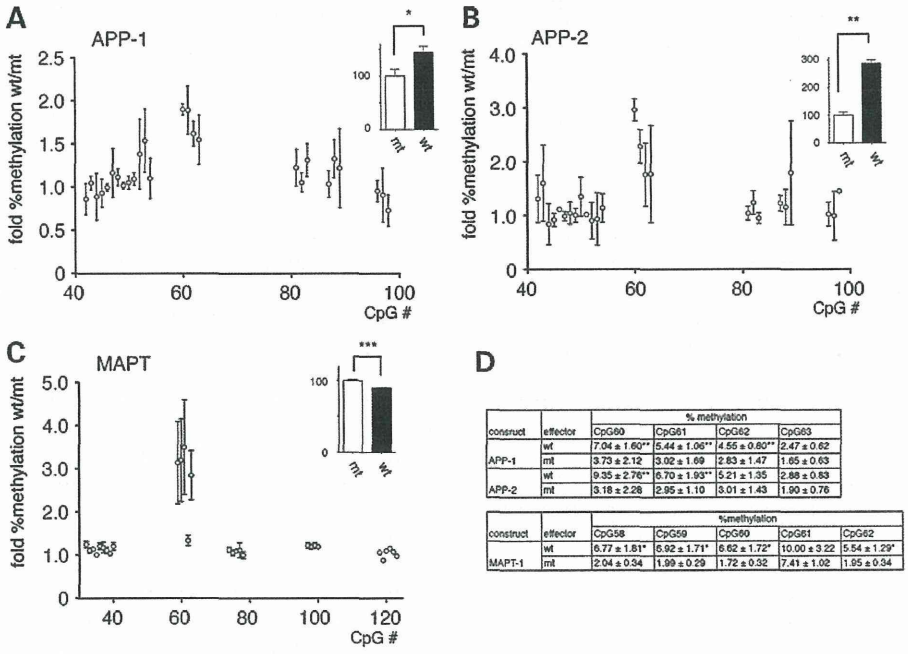


Figure 4. Results of FACS sorting and pyrosequencing analyses. NeuN-positive (+) are neuronal and NeuN-negative (−) are non-neuronal cells. (**A**) *APP*, (**B**) *MAPT*, (**C**) *GSK3B*. Two-way ANOVA and Bonferroni's multiple comparison tests revealed statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



consruct	effector	% methylation			
		CpG60	CpG61	CpG62	CpG63
APP-1	wt	7.04 ± 1.60**	5.44 ± 1.06**	4.55 ± 0.80**	2.47 ± 0.62
	mt	3.73 ± 2.12	3.02 ± 1.69	2.83 ± 1.47	1.65 ± 0.63
APP-2	wt	9.35 ± 2.76**	6.70 ± 1.93**	5.21 ± 1.35	2.88 ± 0.83
	mt	3.18 ± 2.28	2.95 ± 1.10	3.01 ± 1.43	1.90 ± 0.76

construct	effector	%methylation				
		CpG58	CpG59	CpG60	CpG61	CpG62
MAPT-1	wt	6.77 ± 1.81*	6.92 ± 1.71*	6.62 ± 1.72*	10.00 ± 3.22	5.54 ± 1.29*
	mt	2.04 ± 0.34	1.99 ± 0.29	1.72 ± 0.32	7.41 ± 1.02	1.95 ± 0.34

Figure 5. Results of TALE-DNMT3a construct transfection. Two different constructs coding *APP* CpGs and one against *MAPT* CpGs were transfected into 293 T cells, which were then incubated for 48 h. RNA and DNA were simultaneously extracted and subjected to qPCR and pyrosequencing. (**A** and **B**) TALE construct against *APP*. (**C**) TALE construct against *MAPT*. Fold % methylation was calculated as the relative value of methylation comparing the wild-type DNMT3a construct against the methylation-defective mutant. Average value from three independent experiments are shown (bar = SEM). Insets are qPCR expression assay results (DNMT V777G mutant = 100). $^{*}P = 0.001$, $^{**}P = 0.0020$, $^{***}P < 0.0001$. (**D**) Actual methylation measurement value (average and SD) of region of interest upon transfection of the constructs are shown. $^{*}P < 0.05$ versus mt, $^{**}P < 0.01$ versus mt.