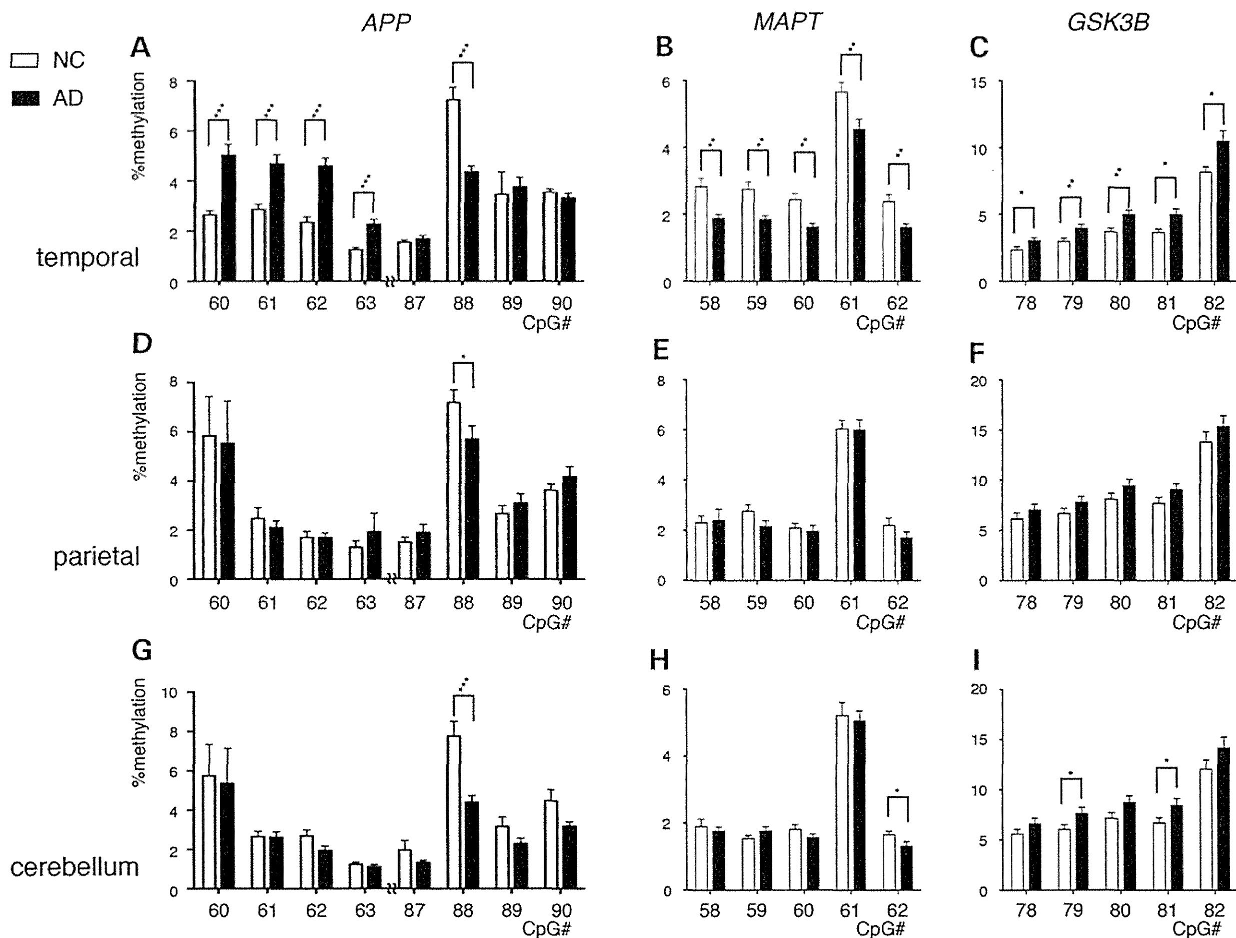


Figure 3. Large-scale analysis of CpG sites of *APP*, *MAPT* and *GSK3B* in three different brain regions. All the samples described in Table 2 was used for analyses. NC, open bars; AD, closed bars. (**A**, **D** and **G**) *APP*; (**B**, **E** and **H**) *MAPT*; (**C**, **F** and **I**) *GSK3B*. (**A**–**C**) 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).

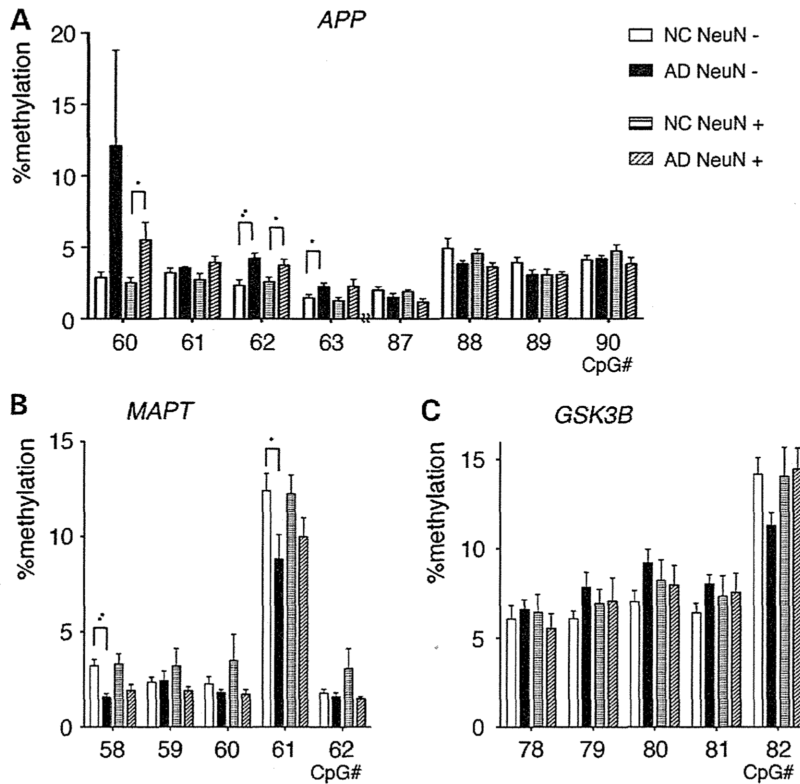
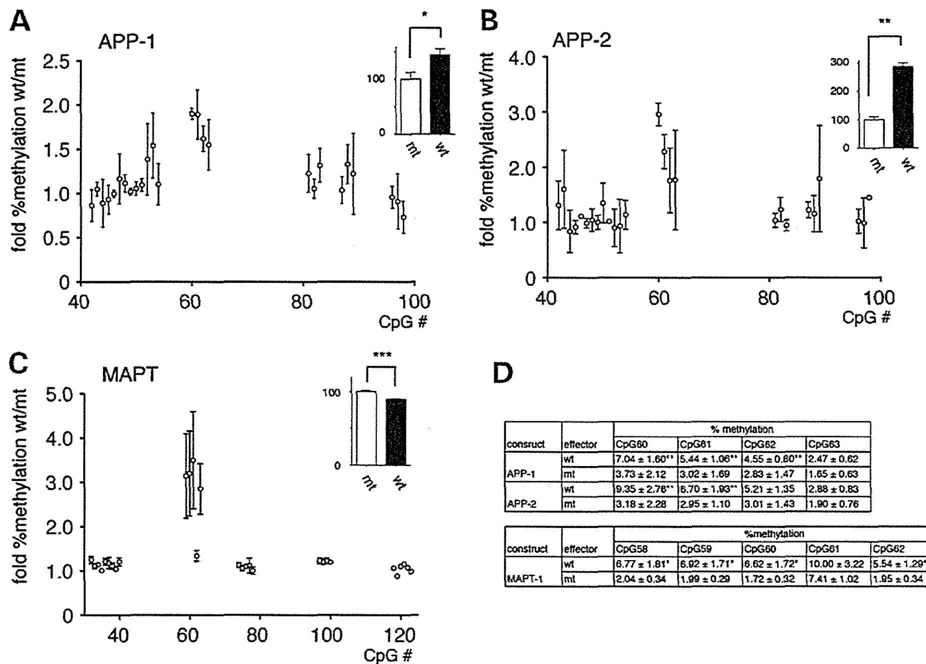


Figure 4. Results of FACS sorting and pyrosequencing analyses. NeuN-positive (+) are neuronal and NeuN-negative (−) are non-neuronal cells. (**A**) *APP*, (**B**) *MAPT*, (**C**) *GSK3B*. Two-way ANOVA and Bonferroni's multiple comparison tests revealed statistical significance. $*P < 0.05$, $**P < 0.01$,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



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.

Since our results are considering relatively low methylation level differences between AD and NC brains, it could raise the concern of pathological significance. For this reason, the results were further analyzed by bisulfite cloning and sequencing of *APP* and *MAPT* in a limited numbers of samples. This revealed some heavily methylated clones among fully unmethylated clones in the AD samples (Supplementary Material, Fig. S8), thus suggesting that a small percentage of abnormally methylated cells are located among normal cells in AD brains. This result supports the aggregation propagation hypothesis that proposes aggregation seed formed somewhere in the brain spreads to other areas (27), that these 'abnormally' methylated cells could serve as seed clones for aggregated protein production. Regional differences observed in this study that most of the methylation differences were observed only in the temporal lobe, where AD pathology usually begins, could also be supportive of the aggregation propagation hypothesis. Our result suggests that there are nearly 2–5% of abnormally methylated cells in the AD temporal cortex. Those

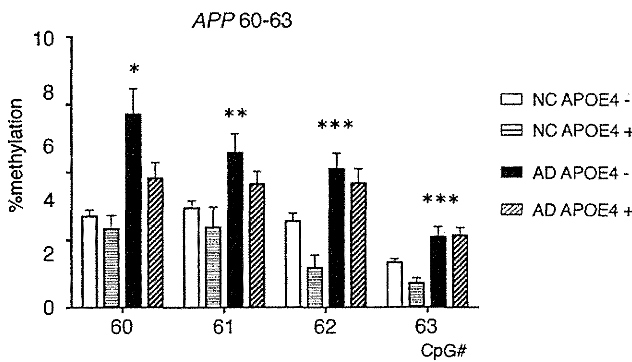


Figure 6. Subgroup analysis of the *APP* methylation status in temporal lobe samples by the presence or absence of APOE ε4 (APOE4). Overall significance was tested by two-way ANOVA and Bonferroni's multiple comparison tests, which revealed a statistically significant positive relationship $^{*}P < 0.0001$ versus NC APOE4−, $P = 0.0333$ versus AD APOE4+, $^{**}P = 0.0015$ versus NC APOE4−, $^{***}P < 0.005$ versus NC APOE4−. We analyzed 64 NC APOE4− 10 NC APOE4+, 27 AD APOE4− and 29 AD APOE4+ cases.

cells overproduce APP and MAPT, which could aggregate locally and further spread to adjacent areas of the brain where abnormal seed cells are less abundant. This is further supported by the data shown in Figure 5D that even increase in <10% methylation level can associate with expression alteration, which is due to low transfection and expression efficiency resulting in similar situation observed in the brain that a few abnormally methylated cells are present among normal cells.

Several genes are considered risk factors for AD; *APOE*, especially the ε4 genotype, confers the strongest risk. This has been shown to affect the disease pathogenesis by impairing Aβ clearance. Approximately 60% of patients with sporadic AD have this allele (28); however, possession of the ε4 allele does not guarantee that an individual will develop AD. Similarly, a significant portion of patients with AD has ε3 alleles, which does not increase the risk of dementia (29). Thus, it is of great interest to identify AD risk factors for the APOE ε4-negative population. Our results suggest a potential role of epigenetic alterations in the disease pathogenesis, especially in the APOE ε4-negative AD population. APOE is a protein related to Aβ clearance, while the E4 protein is reported to be less effective at this task (30); for this reason, it is thought to play a major role in Aβ accumulation in APOE ε4 cases. Thus, in APOE ε4-negative individuals, it may be increased APP production rather than less effective APOE that is related to the disease pathogenesis.

AD is the most prevalent neurodegenerative disease among the elderly and is characterized by the slow progressive decline in memory and executive function, both of which impair the patient's quality of life. As a result of the growing aging population in both developed and developing countries, the number of AD patients will increase dramatically by the year 2050, and the subsequent impact of this on the world economy will be disastrous (31). Existing symptomatic treatments do not change the underlying disease process or halt symptomatic progression (32). Sporadic AD pathogenesis is still unclear, but it is assumed to be somewhat similar to the FAD disease process. Here, we report a novel epigenetic alteration that specifically occurs in sporadic AD patient brains. This result pathomechanistically links FAD and sporadic AD. We hope this finding improves our

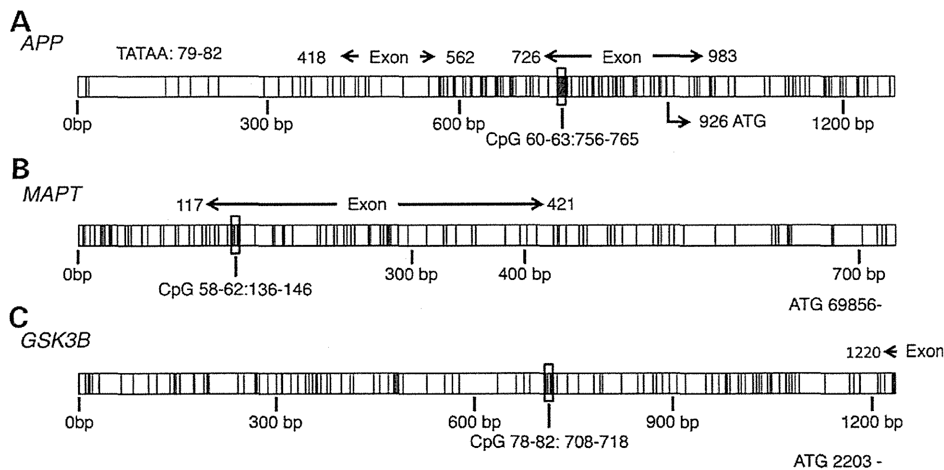


Figure 7. Structures of CpG islands analyzed in this study. Each vertical bar represents a CpG. Regions translated to mRNAs are shown as 'exon', and the first ATG positions are shown. Detected CpG regions are located below the sequences. (**A**) *APP*, (**B**) *MAPT*, (**C**) *GSK*3B.

understanding of AD and can lead to better therapies for this debilitating disease.

MATERIALS AND METHODS

Sample preparation and pyrosequencing

Post-mortem brains were obtained with written consent from patient families, and frozen at −80°C until use. Fifty NC, AD and DLB subjects were obtained from Tokyo Metropolitan Geriatric Hospital brain bank, 16 NC and 10 AD were from University of Tsukuba and 30 NC and 2 AD were from the University of Tokyo. The research was approved by the ethics committee of the University of Tokyo (#2183-6). Unless otherwise noted, gray matter from the inferior temporal lobe, the superior parietal lobe and the cerebellum were excised, and DNA was extracted using the DNeasy Blood and tissue kit (Qiagen, Hilden, Germany), as according to the manufacturer's protocol. After extraction, DNA concentration was measured using a Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA). Next, 500 ng genomic DNA was subjected to the Epitect Bisulfite Kit (Qiagen) and eluted with 40 μl buffer. Next, 0.5 μl of the post-bisulfite reaction eluate was amplified via polymerase chain reaction (PCR) with a Pyromark PCR Kit (Qiagen), subjected to pyrosequencing with a Pyromark Q24 analyzer (Qiagen), and the result was analyzed with the Pyromark Q24 software (Qiagen). The list of PCR primers, sequencing primers and analysis settings are shown in Supplementary Material, Table S1. Primer sets for pyrosequencing were designed by the Pyromark Assay Design 2.0 software (Qiagen). EpiTect PCR Control DNA set (Qiagen) was used for primer calibration.

Statistical analyses

Statistical analyses were performed using the Graphpad Prism software (Graphpad Software, La Jolla, CA, USA). Statistical significance was tested by *t*-test and two-way ANOVA with Bonferroni's multiple comparison tests. Correlation analysis was tested by Pearson product-moment correlation coefficient analysis.

Neuropathological diagnosis

According to established criteria by Braak and McKeith (33–35), trained neuropathologists made diagnosis of AD, DLB or NC using hematoxylin–eosin, Nissl and silver staining, as well as immunostainings. Diagnosis of AD was based on Braak stage $\geq$3 and amyloid stage $\geq$B. DLB samples were at Lewy body score $\geq$4, Braak stage $\leq$3 and amyloid stage $\leq$B.

CpG island detection

CpG islands were detected using the CpG island searcher software (www.uscnorris.com/cpgislands/) (12).

Quantitative PCR

Cells were cultured under 5% CO_2 and 95% air, and kept at 37°C in ATCC recommended medium conditions. Cultured cells included 293, 293T, BE-(2)-C, H4, HeLa, HeLa-S3, IMR-32, SH-SY5Y and SK-SN which were used in Supplementary Material, Figure S5 experiments. Cells were treated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract RNA and DNA. A total of 1 μg total RNA per sample was reverse transcribed with Rever-Tra-ACE (Toyobo, Osaka, Japan) and analyzed by a Taqman assay using Hs00902194_m1 (*MAPT*), Hs01552283_m1 (*APP*), Hs01047719_m1 (*GSK*) and Hu GAPDH probe sets (Applied Biosystems, Foster City, CA, USA) in the 7900HT Fast Real-time PCR system (Applied Biosystems). Each individual experiments were assayed in quadruplicate and average values were used for further statistical analysis.

APOE genotyping

APOE genotyping was performed with a Taqman assay using probes C_3084793_20 and C_904973_10 (Applied Biosystems).

FACS nucleus sorting

FACS sorting was performed according to a published protocol (13). One hundred to 200 mg of brain tissue were processed to obtain 100 000–2 000 000 events following NeuN antibody staining.

TALE construct

TALE constructs were made with the TALE toolbox kit (Addgene, Cambridge, MA, USA). The target sequences for *APP* were 5′-TGCCGAGCGGGGTGGGCCGG-3′ and 5′-TGG GCCGGATCAGCTGACTC-3′. The target sequence for *MAPT* was 5′-TTCTCCTCCGGCCACTAGTG-3′. The TALE effector sequence was confirmed by direct sequencing. DNMT3a cDNA (FXC03883) was purchased from Kazusa DNA Research Institute (Kisarazu, Ciba, Japan). The V777G mutation was introduced by PCR. Transfection was performed by Lipofectamine2000 (Lifetechnologies, Carlsbad, CA, USA) following manufacturer's protocol.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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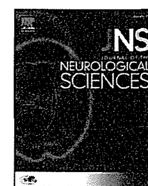

Sudden death in Parkinson's disease: A retrospective autopsy study

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ABSTRACT

The aim of this paper is to reveal the causes of death and to verify sudden death of Parkinson's disease (PD) in an autopsy study. We reviewed the clinical data and the causes of death in 16 PD patients who had postmortem examinations. Prior to autopsy, nine patients died of known causes: five patients died of aspiration pneumonia, two of myocardial infarction, one of asphyxia, and one of dilated cardiomyopathy. Autopsy confirmed that the putative causes of death were compatible with the pathological ones. The remaining seven patients died suddenly of unknown causes. Autopsy revealed that the causes of death were asphyxia in two patients and perforation of a duodenal ulcer in one patient. Autopsy did not determine the causes of unknown death in the remaining four patients. Consequently, autopsy revealed that eight patients died of swallowing problems such as aspiration pneumonia and asphyxia, four of sudden death, three of cardiac problems, and one of a gastrointestinal problem. Although there was a bias that all patients had a postmortem examination, our study revealed that several PD patients died of sudden death without any satisfactory causes of death determined even by autopsy. Therefore, we propose that a non-negligible number of PD patients die of sudden death.

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1. Introduction

The most common cause of death in Parkinson's disease (PD) is aspiration pneumonia [1–9]. Most PD patients die of swallowing problems such as aspiration pneumonia and asphyxia. Several papers have also described sudden death in PD [10–12]. In particular, Rajput and Rozdilsky (1976) reported that their necropsy study revealed that one out of six PD patients died suddenly without any satisfactory causes of death [10]. Sato et al. (2006) reported that 10 out of 131 PD patients died of sudden death (7.6%) [12,13]. However, autopsies were not conducted to investigate the true causes of death in their study [12, 13]. Therefore, there are few pathological studies on sudden death in PD patients.

Here, to reveal the causes of death and to verify sudden death of PD, we reviewed the clinical data and the causes of death in 16 PD patients who had postmortem examinations. On the basis of the results, we propose that a non-negligible number of PD patients die of sudden death.

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2. Materials and methods

We reviewed 451 serial autopsy cases from 1991 to 2006 in Yokohama Rosai Hospital to extract definite PD cases that were confirmed by the pathological findings [14]. This autopsy study was conducted only if the patient's family agreed with our recommendation on autopsy. All the patients fulfilled the criteria of idiopathic PD according to the British Parkinson's Disease Society Brain Bank Criteria [15]. The patients who fulfilled the criteria of dementia with Lewy bodies were excluded [16]. The brain and spinal cord were removed from the body and the tissue was fixed in 20% buffered formalin. After gross inspection, the appropriate areas were processed for deparaffinized and 6-μm-thick sections were stained with hematoxylin-eosin (HE) and Klüver–Barrera methods. The pathological diagnosis of PD was based on finding a clear depletion of brainstem pigmented neurons with Lewy bodies [15]. In the 16 total PD patients (12 males and 4 females), a diagnosis of PD was confirmed by both clinical and pathological findings. The clinical characteristics of the 16 PD patients are shown in Table 1. The mean ± standard deviation (SD) age of death in PD patients was 72.8 ± 8.4 years (range: 48–84 years) and that of onset was 63.6 ± 10.9 years (range: 41–77 years). The duration of disease was 10.2 ± 6.1 years (range: 1–22 years). Hoehn and Yahr stage ranged from 3 to 5 (stage 3: 2 patients, stage 3.5: 2 patients, stage 4: 2 patients, and stage 5: 10 patients). All patients had taken anti-Parkinsonian drugs. Written informed consent to conduct an autopsy study was

Table 1
Clinical characteristics and causes of death in 16 patients with PD proven by autopsy.

Case	Age of death (years)	Gender	Age of onset (years)	Disease duration (years)	Hoehn & Yahr	Tremor (resting)	Rigidity	Akinesia	Laterality of onset	Postural instability	Dementia (years)	Duration to dementia	Autonomic dysfunction	Response to levodopa	Cause of death (clinical)	Cause of death (pathological)
1	84	M	76	9	5	+	N.D.	N.D.	N.D.	N.D.	+	9	N.D.	good	Sudden death	Unknown
2	75	M	73	3	5	N.D.	N.D.	+	N.D.	N.D.	+	2	+	poor	Sudden death	Asphyxia
3	77	M	77	1	4	N.D.	+	+	R	N.D.	N.D.	N.D.	N.D.	poor	Aspiration pneumonia	Aspiration pneumonia
4	77	M	69	9	4	+	+	+	R	+	+	2	+	good	Sudden death	Unknown
5	48	F	41	8	3	+	+	N.D.	L	N.D.	−	−	−	good	Dilated cardiomyopathy	Dilated cardiomyopathy
6	71	M	52	20	3.5	+	+	+	R	+	−	−	+	good	Sudden death	Duodenal ulcer
7	68	F	59	10	5	+	+	+	R	N.D.	+	9	N.D.	good	Sudden death	Unknown
8	72	M	63	10	5	−	+	+	L	+	+	5	+	good	Aspiration pneumonia	Aspiration pneumonia
9	68	F	50	19	5	+	+	+	R	N.D.	+	11	N.D.	good	Asphyxia	Asphyxia
10	71	M	64	8	3.5	+	+	+	L	+	−	−	N.D.	poor	Sudden death	Asphyxia
11	78	M	76	3	5	−	+	+	R	+	+	2	+	poor	Aspiration pneumonia	Aspiration pneumonia
12	81	F	68	14	5	+	+	+	R	N.D.	+	14	+	good	Myocardial infarction	Myocardial infarction
13	78	M	66	13	5	+	+	+	R	N.D.	+	12	+	good	Aspiration pneumonia	Aspiration pneumonia
14	80	M	74	7	5	−	+	+	N.D.	+	+	5	+	poor	Sudden death	Unknown
15	66	M	60	7	5	+	+	+	R	N.D.	+	6	+	poor	Aspiration pneumonia	Aspiration pneumonia
16	71	M	50	22	3	+	+	+	R	+	−	−	+	good	Myocardial infarction	Myocardial infarction
	72.8 ± 8.4	M 12/F 4	63.6 ± 10.9	10.2 ± 6.1		11/14	14/14	14/14		7/7	11/15	7.0 ± 4.3	10/11	10/16		

M: male, F: female, N.D.: not described, R: right, L: left.

obtained from all families of the patients. The present study was conducted in accordance with the ethical standards of the Declaration of Helsinki.

To identify the cause of death in PD patients, all of the major organs in the body were retrieved for the pathological examination. We defined the cause of death as 'sudden death' when no satisfactory causes of death were revealed even by autopsy. We also retrospectively investigated the clinical features by reviewing the charts of inpatients and outpatients as follows: resting tremor, rigidity, akinesia, laterality of onset, postural instability, dementia, autonomic dysfunctions, and response to levodopa.

3. Results

3.1. Clinical characteristics and cause of death

Table 1 shows the clinical characteristics and causes of death in the 16 PD patients. Prior to autopsy, nine PD patients died of presumable causes: five patients died of aspiration pneumonia (cases 3, 8, 11, 13, and 15), two of myocardial infarction (cases 12 and 16), one of asphyxia (case 9), and one of dilated cardiomyopathy (case 5). Autopsy confirmed that the putative causes of death were compatible with the pathological ones. The remaining seven patients died suddenly of unknown causes (cases 1, 2, 4, 6, 7, 10, and 14). Autopsy revealed that the causes of death were asphyxia in two patients (cases 2 and 10) and perforation of a duodenal ulcer in one patient (case 6). Autopsy did not reveal the causes of sudden death in the remaining four patients (cases 1, 4, 7, and 14). Consequently, autopsy revealed that eight patients died of swallowing problems such as aspiration pneumonia and asphyxia, four of sudden death, three of cardiac problems, and one of a gastrointestinal problem.

Although the retrospective review did not find several descriptions, it revealed the following clinical findings in the period from disease onset to death: resting tremor was described in 11 out of 14 patients (except for cases 8, 11, and 14), rigidity in 14 out of 14 patients, akinesia in 14 out of 14 patients, laterality of onset in 13 out of 13 patients, postural instability in seven out of seven patients, dementia in 11 out of 15 patients (except for cases 5, 6, 10, and 16, who were Hoehn and Yahr stage 3 or 3.5), autonomic dysfunctions in 10 out of 11 patients (except for case 5, who was Hoehn and Yahr stage 3), and good response to levodopa in 10 out of 16 patients (except for cases 2, 3, 10, 11, 14, and 15).

The autonomic dysfunctions were constipation in seven out of eight patients (cases 6, 8, 12, 13, 14, 15, and 16), urinary disturbance in five out of six patients (cases 2, 8, 11, 14, and 16), and orthostatic hypotension in four out of five patients (cases 2, 4, 8, and 15). Bilateral vocal cord palsy with obstructive sleep apnea was observed in one patient (case 12). The duration from disease onset to dementia was 7.0 ± 4.3 years (range: 2–14 years). Dementia was observed only in patients with Hoehn and Yahr stage 4 or 5.

3.2. Cases of sudden death

In the following four cases, we could not find any satisfactory causes of death in the clinical and pathological findings. The mean ± SD age of sudden death was 77.3 ± 6.8 years (range: 68–84 years) and that of onset was 69.5 ± 7.6 years (range: 59–76 years). The duration of disease was 8.8 ± 1.3 years (range: 7–10 years). Hoehn and Yahr stage was 4 or 5. Autonomic dysfunctions were described in two patients (cases 4: orthostatic hypotension, case 14: urinary disturbance), although there were no descriptions on autonomic dysfunctions in the other two patients. The corrected-QT (QTc) interval was prolonged in two out of three patients (cases 4 and 14). We described the actual clinical situations of sudden death.

Case 1. The patient was a 76-year-old-male who presented with tremor. His past histories were unremarkable. The diagnosis of Parkinson's disease was made. At the age of 84, he became bedridden. Although he was admitted to our hospital to control his anti-Parkinsonian medicines, he went into cardiopulmonary arrest the next morning. His Hoehn and Yahr stage was 5. Total disease duration was nine years. Anti-Parkinsonian medicines at the time of death were levodopa-carbidopa 400 mg, bromocriptine 2.5 mg, amantadine 150 mg, and trihexyphenidyl hydrochloride 6 mg. The other medicines and QTc interval were unknown.

Case 4. The patient was a 69-year-old-male who presented with tremor and gait disturbance. His past history was urinary calculus. The diagnosis of Parkinson's disease was made. At the age of 77, he showed loss of appetite and difficulty in walking. He was admitted to our hospital to control his anti-Parkinsonian medicines. Head-up tilt test showed severe orthostatic hypotension (systolic blood pressure decreased from 138 mm Hg to 62 mm Hg). Plasma noradrenaline (NA) and vasopressin (ADH) did not change during the test (pre-standing NA 0.36 ng/ml, post-standing NA 0.37 ng/ml; pre-standing ADH 1.89 pmol/l, post-standing ADH 1.93 pmol/l). During the hospitalization, he died suddenly in the supine position in bed with his eyes open in front of doctors. His Hoehn and Yahr stage was 4. Total disease duration was nine years. Anti-Parkinsonian medicines were levodopa–carbidopa 600 mg and droxidopa 900 mg. The other medicines were midodrine hydrochloride 6 mg for orthostatic hypotension, flunitrazepam 1 mg for insomnia, and sennoside (tablet) 24 mg for constipation. QTc interval was 480 ms (>450 ms).

Case 7. The patient was a 59-year-old-female who presented with gait disturbance and akinesia. Her past history was unremarkable. At the age of 64, the diagnosis of Parkinson's disease was made. At the age of 67, she became bedridden due to femoral neck fracture. At the age of 68, she complained of a loss of appetite in the morning. In the evening, her caregiver found her in cardiopulmonary arrest at her house. She was transferred to our hospital, although she had already died. Her Hoehn and Yahr stage was 5. Total disease duration was 10 years. Anti-Parkinsonian medicines were levodopa–carbidopa 200 mg and trihexyphenidyl hydrochloride 4 mg. The other medicines were haloperidol 0.75 mg for agitation, clotiazepam 5 mg for insomnia, and sennoside (tablet) 24 mg for constipation. QTc interval was 433 ms (<450 ms).

Case 14. The patient was a 74-year-old-male who presented with gait freezing. His past history was benign prostatic hypertrophy surgically cured. At the age of 75, the diagnosis of Parkinson's disease was made. At the age of 80, he became bedridden and he was admitted to our hospital for treatment of aspiration pneumonia. A few days following post-recovery discharge, he suddenly lost consciousness and went into cardiopulmonary arrest after eating a negligible amount of rice. He was transferred to our hospital, although he had already died. His Hoehn and Yahr stage was 5. Total disease duration was seven years. Anti-Parkinsonian medicines were pergolide mesilate 150 μg and amantadine 100 mg. The other medicines were tiapride hydrochloride 25 mg for agitation, etizolam 0.5 mg for insomnia, and sennoside (granule) 1 mg for constipation. QTc interval was 454 ms (>450 ms).

4. Discussion

This paper described the following findings: (i) the most common cause of death was swallowing problems, aspiration pneumonia or asphyxia, (ii) the second most common cause of death was sudden death, (iii) some patients did not exhibit resting tremor before death, (iv) all patients exhibited rigidity, akinesia, and postural instability before death, (v) all patients with Hoehn and Yahr stage 4 or 5 exhibited dementia, (vi) average duration from disease onset to dementia was approximately seven years, (vii) almost all patients exhibited some autonomic dysfunctions except in a relatively young and mild case (case 5: the age of death was 48 years and Hoehn and Yahr stage was 3), and (viii) response to levodopa was not always good. Our study revealed that four out of 16 PD patients died of sudden death without any satisfactory causes of death determined even by autopsy. In the present study, there must be a bias for this high frequent sudden death, because all families requested a postmortem examination. Additionally, we analyzed only the PD patients who had a postmortem examination and did not show the clinical incidence of sudden death in PD patients who did not have a postmortem examination. Actually, the previous large clinical studies mentioned that only 10 out of 131 PD patients died of sudden death (7.6%) [12,13]. Thus, the actual incidence of sudden death would be less than that in our study. However, there are very few previous papers regarding sudden death in PD patients. In particular, there are few pathological studies on sudden death. Here, we discuss the causes of sudden death, which is the most important finding in our study. As assumed easily, the true causes of sudden death must be heterogeneous.

In our four cases, the clinical situations did not suggest any clues on the definite causes of sudden death. However, all these cases certainly died without any putative causes of death. The age at death and the disease severity in all cases with sudden death were relatively high, suggesting that these factors might be related to the mechanisms of sudden death. We assume that orthostatic hypotension might be related to sudden death as follows.

Actually, in one patient who died of sudden death (case 4), head-up tilt test showed that the plasma NA and ADH did not change during an abrupt drop in blood pressure, i.e. severe orthostatic hypotension, suggesting the neurogenic orthostatic hypotension due to the impairment of both post-ganglionic efferent fibers and afferent fibers in sympathetic nervous systems. Although the head-up tilt test was conducted only for the one patient, it suggests that orthostatic hypotension may be one of the causes of sudden death. Sato et al. (2006) reported that 10 out of 131 PD patients died of sudden death (7.6%) and that three out of the 10 patients died in the bathtub [12,13]. On the basis of the high frequency of death in the bathtub, they assume that one of the causes of sudden death might be related to orthostatic hypotension, because the vasodilation effect experienced in the bathtub could lead to fatal syncope in patients with severe orthostatic hypotension [13]. It is reported that severe syncope can cause cardiac sudden death [17]. Moreover, anti-Parkinsonian medicines such as levodopa and dopamine receptor agonists can exacerbate orthostatic hypotension [18,19].

On the other hand, QTc interval was prolonged in two patients who died of sudden death (cases 4 and 14). The prolongation of the QTc interval is well known as a risk factor of cardiac sudden death [20]. It is also reported that the QTc interval in PD patients was prolonged compared to that in normal controls [11,21,22]. Ishizaki et al. (1996) also reported that the QTc interval in two PD patients who died of sudden death was prolonged. Furthermore, drug-induced QTc prolongation is also well known as a risk factor of cardiac sudden death. Amantadine, taken by two patients in sudden death (cases 1 and 14), and psychotropic medicines, taken by three patients in sudden death (cases 4, 7, and 14), also could prolong the QTc interval [23,24]. Therefore, we propose the possibility that the prolongation of the QTc interval also might cause cardiac sudden death.

On the basis of our study, we assume that some of the true causes of sudden death may be related to orthostatic hypotension and QTc prolongation. To evaluate orthostatic hypotension, the various examinations on autonomic nervous system such as head-up tilt test, coefficient of variation of RR intervals (CVRR), heart rate variability (HRV), and ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy may be useful. To measure QTc interval, conventional electrocardiogram (ECG) is needed. Justifiably, there must be a lot of true causes of sudden death other than our assumptions. Furthermore, sudden death may be overlooked when interpreted as death from natural causes according to the insufficient postmortem examinations. To identify the true causes

of sudden death and to draw a final conclusion of the death from natural causes scientifically, a prospective study using clinical and pathological examinations including head-up tilt test, CVRR, HRV, MIBG scintigraphy, and QTc interval must be required.

In conclusion, our retrospective autopsy study revealed that eight out of 16 PD patients died of swallowing problems, whereas four out of 16 PD patients died of sudden death. Although there is a bias derived from a postmortem examination, we propose that a non-negligible number of PD patients die of sudden death.

Conflict of interest

There is no conflict of interest.

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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 $\text{OR}_{\varepsilon4/\varepsilon3}$ (95% confidence interval [CI]) = 4.81 (4.26–5.42) and $\text{OR}_{\varepsilon2/\varepsilon3}$ (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)			$P_{genotype}{}^{c}$	$OR_{CG\text{-}2}$ (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]$OR_{CG\text{-}2}$ (95% CI) for CG-2 (Ga-CC-TT, GG-Ct-TT, and GG-CC-Tc).

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SUPPLEMENTARY MATERIAL

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Influence of *APOE* Genotype and the Presence of Alzheimer's Pathology on Synaptic Membrane Lipids of Human Brains

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The *APOE* genotype is the major risk factor for Alzheimer's disease (AD); however, it remains unclarified how the ε4 allele accelerates whereas the ε2 allele suppresses AD development, compared with the more common ε3 allele. On the basis of the previous finding that the assembly of the amyloid-β protein (Aβ) into fibrils in the brain, an early and invariable pathological feature of AD, depends on the lipid environment, we determined the levels of synaptic membrane lipids in aged individuals of different *APOE* genotypes. In the comparison between amyloid-free ε2/ε3 and ε3/ε3 brains, the presence of the ε2 allele significantly decreased the level of cholesterol. Alternatively, in the comparison among ε3/ε3 brains, the presence of AD pathology substantially decreased the levels of cholesterol. This study suggests that the ε2 allele suppresses the initiation of AD development by lowering the cholesterol levels in synaptic membranes. © 2014 Wiley Periodicals, Inc.

Key words: Alzheimer's disease; cholesterol; ganglioside; synaptic plasma membrane; microdomain

The ε4 allele of the apolipoprotein E gene (*APOE*) increases the risk of Alzheimer's disease (AD), whereas the ε2 allele decreases it, compared with the most common ε3 allele (for review see Holtzman et al., 2012; Liu et al., 2013). Despite intensive efforts, the mechanisms underlying *APOE*-allele-linked modulation of AD development still remain to be clarified. However, it is at least confirmed that the *APOE* alleles can affect the risk of AD through a wide range of biological functions of apolipoprotein E, not through a simple function, such as the regulation of lipid transport, glucose metabolism, or neuroinflammation (for review see Holtzman et al., 2012; Liu et al., 2013). In addition, it is assumed that the *APOE* alleles modulate AD development upstream and downstream of amyloid deposition, a fundamental core of AD pathology, or through their effects on amyloid-dependent and amyloid-independent processes (Liu et al., 2013).

With regard to amyloid deposition in the brain, postmortem neuropathological examination and clinical amyloid imaging by positron emission tomography revealed that it is enhanced in the presence of the ε4 allele (Schmechel et al., 1993; Oyama et al., 1995; Polvikoski et al., 1995; Reiman et al., 2009) but suppressed in the presence of the ε2 allele (Benjamin et al., 1994; Nagy et al., 1995; Polvikoski et al., 1995; Lippa et al., 1997; Tiraboschi et al., 2004). Although the *APOE*-genotype-dependent effects on amyloid deposition also remain unknown, such effects likely are due to modulation of fibrillogenesis and/or clearance of the amyloid-β protein (Aβ), a proteinaceous component of amyloid (for review see Holtzman et al., 2012).

Accumulating evidence suggests that Aβ assembles into fibrils in the brain upon interaction with cellular membranes, especially through specific binding to gangliosides (for review see Yanagisawa, 2007; Ariga et al., 2008; Matsuzaki et al., 2010; Di Paolo and Kim, 2011). In our early studies with human *APOE* knock-in mouse brains, the ε4 knock-in brains, compared with the ε3 knock-in brains, showed significant increases in cholesterol levels in the exofacial leaflet of synaptic membranes (Hayashi et al., 2002) and ganglioside levels in an age-dependent manner in synaptic membrane microdomains (Yamamoto et al., 2004). Taken together with the evidence that amyloid deposition in the brain starts at

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presynaptic terminals (Bugiani et al., 1990; Probst et al., 1991), it is reasonable to assume that the effect of *APOE* genotype on amyloid deposition is attributable to the modulation of synaptic membrane lipids.

This study examined the frontal cortices of autopsied brains of aged individuals carrying the ε2/ε3, ε3/ε3, ε3/ε4, and ε4/ε4 alleles. Available brains from individuals carrying the ε4 allele all showed advanced AD pathology, making it difficult for us to perform fine analyses of synaptic membrane lipids. In contrast, available brains from individuals carrying the ε2 allele all lacked amyloid deposition. The extent of AD pathology varied in the brains with the ε3/ε3 allele; therefore, we selected the brains lacking or showing amyloid deposition. We focused on cholesterol and gangliosides because the major role of apolipoprotein E is the transport of cholesterol between cells in brains, and gangliosides likely are involved in the acceleration of Aβ assembly (Matsuzaki et al., 2010). Here we report the following. First, in the comparison between amyloid-free ε2/ε3 and ε3/ε3 brains, the presence of the ε2 allele decreased the levels of cholesterol in synaptic membranes and synaptic membrane microdomains. Second, in the comparison among ε3/ε3 brains, the presence of AD pathology decreased the levels of cholesterol in synaptic membranes and synaptic membrane microdomains and the levels of some species of gangliosides in synaptic membrane microdomains. This study suggests that ε2 allele suppresses AD development by keeping cholesterol in synaptic membranes and/or synaptic membrane microdomains under a certain level, which is prerequisite for the initiation of Aβ assembly into fibrils.

MATERIALS AND METHODS

Materials

The following antibodies were used: synaptophysin (Synaptic Systems, Gottingen, Germany), SNAP25 (Enzo Life Science, Farmingdale, NY), Bip/GRP78 and flotillin-1 (BD Transduction Laboratories, San Jose, CA), LAMP-1 (Millipore, Billerica, MA), transferrin receptor (TfR; Life Technologies, Carlsbad, CA), prion protein (PrP; Sigma, St. Louis, MO), anti-β amyloid 11–28 (12B2; IBL, Maebashi, Japan), and antiphosphorylated tau (AT8; Innogenetics, Temse, Belgium). Cholera toxin B subunit–peroxidase conjugate (CTB-HRP) and an Amplex-red cholesterol assay kit were purchased from Sigma-Aldrich and Life Technologies, respectively. Synthetic GM1(d18:1-^{13}C16:0) and GM3(d18:1-14:0) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Avanti Polar Lipids (Alabaster, AL), respectively. All the organic solvents used in liquid chromatography-mass spectrometry (LC-MS) were of LC-MS grade (Fluka, Sigma-Aldrich).

Tissue Source

Human brain specimens were obtained from the Brain Bank for Aging Research at the Tokyo Metropolitan Institute of Gerontology. This study was approved by the Ethics Committees of the National Center for Geriatrics and Gerontology and Tokyo Metropolitan Institute of Gerontology. Each specimen was taken from Brodmann area 8 or 9. Neuropathological analysis was performed by modified methenamine and Gallyas-Braak silver staining and immunohistochemical analysis with anti-Aβ 11–28 (12B2, monoclonal) and antiphosphorylated tau (AT8, monoclonal) antibodies as previously reported (Adachi et al., 2010). AD neuropathologies were classified in accordance with the criteria of Braak and Braak (1991).

Preparation of Synaptosomes

Synaptosomes were prepared as previously reported (Igbavboa et al., 1996; Yamamoto et al., 2008). Briefly, after removal of the white matter from a tissue piece, the gray matter was homogenized in ice-cold buffer A (10 mM HEPES, 0.32 M sucrose, 0.25 mM EDTA, pH 7.4) by motor-driven homogenization at 1,000 rpm with 10 strokes. After the removal of the nuclei by centrifugation at 580*g* for 8 min at 4°C, a crude mitochondrial pellet (CMP) was collected by centrifugation of the postnuclear supernatant (PNS) at 14,600*g* for 20 min at 4°C. The CMP was suspended in buffer B (10 mM HEPES, 0.32 M sucrose, pH 7.4) by hand homogenization, layered over 7.5% and 14% Ficoll in buffer B, and then centrifuged at 87,000*g* (SW50.1 rotor, Beckman) for 30 min at 4°C. The interface between 7.5% and 14% Ficoll solutions rich in synaptosomes was collected in 10 ml buffer B and then centrifuged at 18,550*g* for 15 min at 4°C.

Preparation of Synaptic Plasma Membranes

Synaptic plasma membranes (SPMs) were prepared as previously reported (Cotman and Matthews, 1971; Fontaine et al., 1980; Igbavboa et al., 1996). Briefly, synaptosomes were osmotically shocked by suspension in ice cold 5 mM Tris buffer (pH 8.5) and stirring on ice for 90 min with vortex mixing every 30 min. After centrifugation of the suspension at 43,700*g* for 20 min at 4°C, the resultant pellet was suspended in buffer B, layered over 25% and 32.5% sucrose in 10 mM HEPES (pH 7.4), and then centrifuged at 41,000*g* (SW50.1 rotor; Beckman) for 30 min at 4°C. The interface between 25% and 32.5% sucrose solutions rich in SPMs was collected in 9 volumes of 10 mM HEPES buffer (pH 7.4) and then centrifuged at 50,380*g* for 20 min at 4°C.

Isolation of Low-Density Membrane Microdomains From SPMs

Low-density membrane microdomains (LDMs), or lipid rafts, were isolated by a detergent-free method based on a previous report (Persaud-Sawin et al., 2009). Briefly, SPMs were suspended in buffer C (25 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing Complete protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany) and sonicated five times for 8 sec each on ice at a high power output with an ultrasonic disruptor (UD201; Tomy Seiko, Tokyo, Japan) at 1–2-min intervals. After adjusting to 40% sucrose by mixing with buffer C and 80% sucrose in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, pH 7.4), the suspension was placed at the bottom of a tube, and a discontinuous sucrose gradient was prepared by pouring 35%, 25%, 15%, and 5% sucrose in TBS sequentially on top of the suspension.

The gradient was ultracentrifuged at 261,000*g* (SW41 rotor; Beckman) for 18 hr at 4°C. Fractions (1 ml each) were collected from the top. Fractions 4, 5, and 6 were collected in 7 volumes of water and centrifuged at 450,000*g* for 20 min at 4°C (type 70.1 rotor; Beckman). The resultant pellets were used as LDM samples. In characterizations of the fractions by Western blotting, each sample was subjected to trichloroacetic acid (TCA) precipitation. The samples were mixed with an equal amount of 20% TCA solution and incubated for 1 hr at −20°C. After centrifugation at 20,400*g* for 15 min at 4°C, the pellets were washed with ice-cold 100% ethanol, and recentrifuged at 20,400*g* for 15 min at 4°C. The resultant pellets were air dried and analyzed by SDS-PAGE and Western blotting.

Measurement of Cholesterol Level

Cholesterol level was measured using an Amplex-red cholesterol assay kit, as previously reported (Oikawa et al., 2012).

Lipid Extraction

Lipids were extracted as previously reported (Oikawa et al., 2012). Briefly, whole lipids were extracted from SPMs and LDMs with chloroform–methanol (2:1; v/v) and chloroform–methanol–water (1:2:0.8; v/v/v). Gangliosides were separated by two-phase partitioning, as previously reported (Folch et al., 1957). After evaporation under N_2 gas, whole-lipid extract was redissolved in chloroform–methanol–water (8:4:3; v/v/v) and then centrifuged at 1,500 rpm for 10 min, and the upper phase was collected. Pure-solvent upper phase prepared from chloroform–methanol–water (8:4:3; v/v/v) was added to the resultant lower phase, followed by centrifugation at 1,500 rpm for 10 min. The upper phase was then collected. This step was repeated twice. The collected upper phases were combined, evaporated under N_2 gas, and redissolved in methanol. Synthetic GM1(d18:1-^{13}C16:0) and GM3(d18:1–14:0) were added to SPM and LDM samples, respectively, as internal standards for MS.

LC-MS

Gangliosides were analyzed by LC-MS, as previously reported, with slight modification (Nagafuku et al., 2012). Briefly, gangliosides were separated by LC using a Develosil ε30 column (1 mm i.d. × 50 mm; Nomura Chemical) and elution solvents, solvent A (12.5% [SPMs] or 20% [LDMs] water, 10 mM ammonium formate, and 0.1% formic acid in methanol) and solvent B (2.5% [SPMs and LDMs] water, 10 mM ammonium formate, 0.1% formic acid, 50% isopropyl alcohol in methanol), using a gradient elution of 20% B in A for 5 min, from 20% to 100% B in A for 20 min, and 100% B for 5 min. The flow rate in LC was 50 μl/min. MS was performed using a Shimadzu LC-IT-MS in the negative ion and automode with the mass range from m/z 200 to 2,000 and the detector voltage at 1.9 kV. The ratio of ganglioside signals to internal standard signals was measured using mass chromatograms monitoring $[M-H]^-$ ions. Ganglioside structures were confirmed by MS2 with collision-induced dissociation (CID) energy at 50% arbitrary, and ceramide structures were characterized by MS3 or MS4 with manual mode detection monitoring corresponding m/z values.

Statistical Analyses

All statistical analyses were performed in GraphPad Prism version 5. Data are presented as mean ± SEM (in cholesterol analysis) or mean (in ganglioside analysis). For comparisons between groups with different *APOE* genotypes and AD pathology, Student's *t*-test was used, and $P < 0.05$ were considered significant.

RESULTS

Demographics and Neuropathological Characteristics of Autopsied Brains

There were no significant differences between groups in any of the demographics, including age and post-mortem delay (Table I). Histopathological analysis showed no Aβ deposition in the amyloid-free ε2/3 and ε3/3 brains (Fig. 1A,B). In the ε3/3 brains with AD pathology, numerous senile plaques and neurofibrillary tangles were observed (Fig. 1C,D).

SPM and LDM Preparation From Brain Tissues

We first attempted to extract synaptosomes and SPMs from brain cortical tissues by density gradient fractionation (Igbavboa et al., 1996; Yamamoto et al., 2008). As previously reported, synaptic proteins, such as synaptophysin and SNAP25, were abundant in the synaptosome fraction, whereas an endoplasmic reticulum-localized protein, Bip/GRP78, and a lysosomal protein, LAMP-1, were limited in the fraction (Fig. 2A). Subsequently, SPMs were prepared from the collected synaptosomes by osmotic shock and sucrose density gradient fractionation, as previously reported (Cotman and Matthews, 1971; Fontaine et al., 1980). Next, to determine whether lipid distribution was also affected in synaptic membrane microdomains by the *APOE* genotype and the presence of AD pathology, LDMs were collected from SPMs by the detergent-free method, which potently prevents the artificial domain formation that commonly occurs in the conventional detergent method (Lichtenberg et al., 2005; Lingwood and Simons, 2007). For the detergent-free isolation of LDMs, we employed an original method (Persaud-Sawin et al., 2009), with minor modification as follows. The samples

TABLE I. Demographics and Neuropathologic Characteristics of the Autopsied Brains*

Group	ε2/ε3 (AF)	ε3/ε3 (AF)	ε3/ε3 (AD)
N (male/female)	8 (6/2)	8 (7/1)	8 (2/6)
Age (years; mean ± SD)	79 ± 2	78 ± 2	81 ± 2
PMD (hr; mean ± SD)	14.7 ± 8.0	16.0 ± 8.5	15.2 ± 19.9
Senile plaque stage	0	0	C
Braak stage (male/female)	I (5/0)	I (4/1)	V (2/6)
	II (1/1)	II (3/0)	
	III (0/1)		

*N, number of brain; AF, amyloid free; AD, neuropathologically diagnosed as Alzheimer's disease; PMD, post-mortem delay.