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 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.

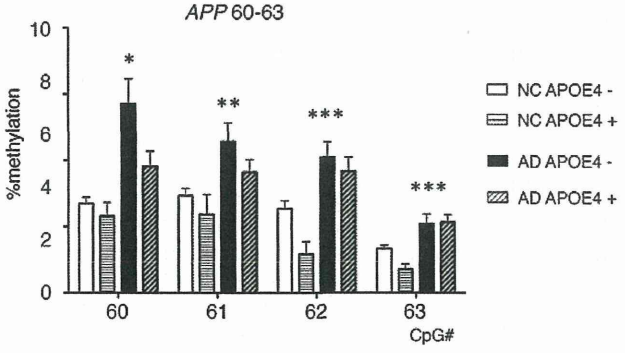

Figure 6. Subgroup analysis of the *APP* methylation status in temporal lobe samples by the presence or absence of APOE ε4 (APOE4). Overall significance was tested by two-way ANOVA and Bonferroni's multiple comparison tests, which revealed a statistically significant positive relationship $^{*}P < 0.0001$ 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.

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

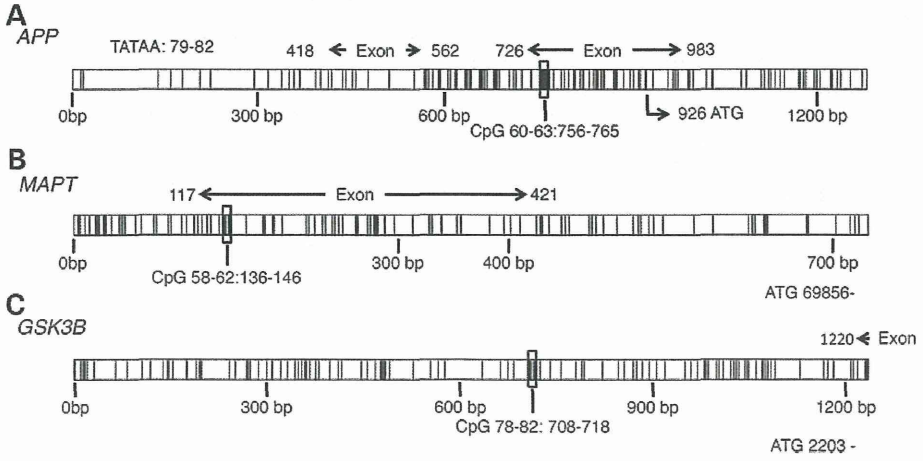

Figure 7. Structures of CpG islands analyzed in this study. Each vertical bar represents a CpG. Regions translated to mRNAs are shown as 'exon', and the first ATG positions are shown. Detected CpG regions are located below the sequences. (**A**) *APP*, (**B**) *MAPT*, (**C**) *GSK*3B.

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 ≥3 and amyloid stage ≥B. DLB samples were at Lewy body score ≥4, Braak stage ≤3 and amyloid stage ≤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′-TGGGCCGGATCAGCTGACTC-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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