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

Atsushi Iwata1,2,3,∗, Kenichi Nagata4, Hiroyuki Hatsuta5, Hiroshi Takuma6, Miki Bundo7, Kazuya Iwamoto3,7, Akira Tamaoka6, Shigeo Murayama5, Takaomi Saido4 and Shoji Tsuji2

1Department of Molecular Neuroscience on Neurodegeneration, Graduate School of Medicine and 2Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan
3Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan 4Laboratory for Proteolytic Neuroscience, RIKEN BSI, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan 5Department of Neuropathology, Tokyo Metropolitan Geriatric Hospital, 35-2 Sakaecho, Itabashi, Tokyo 173-0015, Japan 6Department of Neurology, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan 7Department of Molecular Psychiatry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan

Received July 25, 2013; Revised September 4, 2013; Accepted September 13, 2013

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 Aβ1–42, 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.

∗To whom correspondence should be addressed at: Department of Molecular Neuroscience on Neurodegeneration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan. Tel: 81 358008672; Email: iwata-tky@umin.ac.jp

© The Author 2013. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
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 rationale 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 e4 (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).

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 nuclei 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...
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 MAPT58–62 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
addition, since DNA is more stable than 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...
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 positions 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 GSK3B 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.

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 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...
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 (Invitrogen, Carlsbad, CA, USA). Next, 500 ng genomic DNA was subjected to the Epitect Bisulfite dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA). Next, 0.5 µl of the post-bisulfite reaction eluate was amplified via polymerase chain reaction (PCR) with a Pyromark PCR Kit (Qiagen), subject 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% CO2 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′-TGCCGACGGGGTGGGGGCG-3′ and 5′-TGGGCCGATCAGCTGAC-3′. The target sequence for MAPT was 5′-TTCTCCTGGGCCCAGTG-3′. The TALE effector sequence was confirmed by direct sequencing. DNMT3a cDNA (FXC03883) was purchased from Kazusa DNA Research Institute (Kisarazu, Chiba, Japan). The V777G mutation was introduced by PCR. Transfection was performed by Lipofectamine2000 (Lifetech, Carlsbad, CA, USA) following manufacturer’s protocol.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We are grateful for the technical support provided by Yuko Mitani, Yuki Inukai, and Yuko Naramoto. We are grateful to the Support Unit for Bio-Material Analysis, RIKEN BSI Research Resources Center, for the cell sorting.

Conflict of Interest statement. None declared.

FUNDING

This study was supported by JST PRESTO (Kawaguchi, Saitama), the Cell Science Research Foundation (Osaka, Japan), the Ichiro Kanehara Foundation for the Promotion of Medical Sciences and Medical Care (Tokyo, Japan), the
REFERENCES


