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.

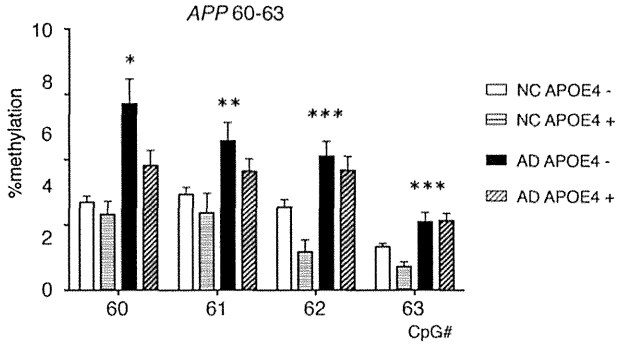

Figure 6. Subgroup analysis of the *APP* methylation status in temporal lobe samples by the presence or absence of APOE ε4 (APOE4). Overall significance was tested by two-way ANOVA and Bonferroni's multiple comparison tests, which revealed a statistically significant positive relationship $^{*}P < 0.0001$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

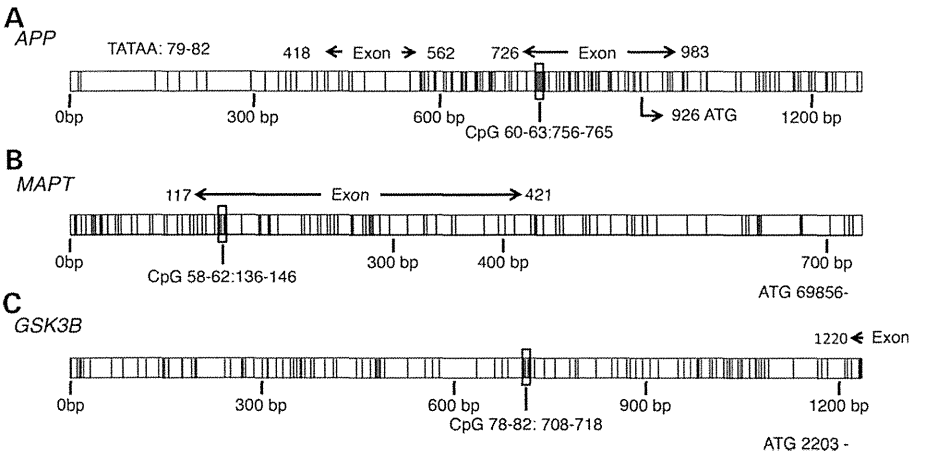

Figure 7. Structures of CpG islands analyzed in this study. Each vertical bar represents a CpG. Regions translated to mRNAs are shown as 'exon', and the first ATG positions are shown. Detected CpG regions are located below the sequences. (**A**) *APP*, (**B**) *MAPT*, (**C**) *GSK*3B.

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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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.

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.

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

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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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ORIGINAL PAPER

Argyrophilic grain disease as a neurodegenerative substrate in late-onset schizophrenia and delusional disorders

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Abstract To study the relationship between neurodegenerative diseases including argyrophilic grain disease (AGD) and late-onset schizophrenia and delusional disorders (LOSD; onset ≥40 years of age), we pathologically examined 23 patients with LOSD, 71 age-matched normal controls, and 22 psychiatric disease controls (11 depression, six personality disorder, two bipolar disorders, and three neurotic disorders cases). In all LOSD cases (compared to age-matched normal controls), the frequencies of Lewy body disease (LBD), AGD, and corticobasal degeneration (CBD) were 26.1 % (11.3 %), 21.7 % (8.5 %), and 4.3 % (0.0 %), respectively. There was no case of pure Alzheimer's disease (AD). The total frequency of LBD, AGD, and CBD was significantly higher in LOSD cases than in normal controls. Argyrophilic grains were significantly more severe in LOSD than in controls, but were almost completely restricted to the limbic system and adjacent temporal cortex. In LOSD patients whose onset occurred at ≥65 years of age (versus age-matched normal controls), the frequencies of LBD and AGD were 36.4 % (19.4 %) and 36.4 % (8.3 %), respectively, and AGD was significantly more frequent in LOSD patients than in normal controls. In LOSD patients whose onset occurred at <65 years of age, the frequencies of LBD, AGD, and CBD were 16.7, 8.3, and 8.3 %, comparable to those of age-matched normal controls (10.2, 5.1, and 0.0 %). In all psychiatric cases, delusion was significantly more frequent in AGD cases than in cases bearing minimal AD pathology alone. Given these findings, LOSD patients may have heterogeneous pathological backgrounds, and AGD may be associated with the occurrence of LOSD especially after 65 years of age.

Keywords Argyrophilic grain · α-Synuclein · Corticobasal degeneration · Four-repeat tau · Late onset · Tauopathy

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Introduction

Schizophrenia is most prevalent in early and middle life, before 40 years of age, but it is also known that this disorder is not infrequent in later life [1]. While there is no limitation regarding the age at onset in the current diagnostic criteria for schizophrenia, the International Classification of Diseases, Revision 10 (ICD-10) and Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) [2], it has been also considered that the pathogenic backgrounds of early- and late-onset schizophrenia may not be identical. Indeed, since a historic report on schizophrenia developing after 40 years of age, called late-onset schizophrenia, was published by Bleuler [3] in 1943, many studies have demonstrated that patients with late-onset schizophrenia have clinical features different from those in

early-onset cases: less severe affective flattening, less severe thought disorder, and a more favorable prognosis [4–9]. In 2000, the International Late-Onset Schizophrenia Group proposed that patients who develop symptoms of schizophrenia after 40 and 60 years of age should be differentiated from early-onset cases and the diseases called late-onset schizophrenia and very-late-onset schizophrenia-like psychosis, respectively [10]. In the present paper, we call psychotic disorders that occurred in cognitively preserved people older than 40 years of age "late-onset schizophrenia and delusional disorders" (LOSD).

Potential pathological backgrounds in patients with late-life depression have been explored mainly by focusing on cerebrovascular lesions [11–15], Alzheimer's disease (AD) [16], and Lewy body disease (LBD) [17–19], although several pathological studies suggested that vascular lesions and AD pathology are usually unrelated to the occurrence of late-life depression [20, 21]. Several studies demonstrated that the frequencies of AD [22, 23] and LBD pathologies [23] were not increased in elderly patients with chronic or residual schizophrenia. On the other hand, the available pathological data regarding psychoses that have developed in elderly people are limited. A few studies demonstrated that LOSD patients often had mild to moderate neurofibrillary tangles (NFTs) in the limbic region [24] and that pyramidal neurons in the hippocampus were spared in number [25, 26]. It is also known that some LBD cases show paranoia as the first symptom [27]. It was also reported that some cases of corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP), common four-repeat tauopathies, show psychosis along with characteristic motor disturbance [28–31]. Argyrophilic grain disease (AGD) is another of the four-repeat tauopathies that increases in frequency with age [32]. It was reported that cases bearing extensively and intensively distributed argyrophilic grains frequently show dementia [33] and that some AGD cases with dementia additionally show prominent psychiatric symptoms, such as aggression, irritability, depression, and psychosis [31, 34–38]. However, to our knowledge, no study that comprehensively examined these neurodegenerative changes common in the elderly in patients developing LOSD has been reported.

The primary aims of this study were to systematically examine the neurodegenerative bases in LOSD cases and to clarify whether AGD is associated with the occurrence of LOSD. To address these, we examined 23 LOSD cases, 71 age-matched normal controls, and 22 cases of various psychiatric disorders as a disease control using modern sensitive and standardized pathological methods. In this paper, we demonstrated that AGD may be a common pathology in LOSD cases that is comparable to LBD in frequency and that AGD may be associated with the occurrence of LOSD especially after the age of 65 years.

Materials and methods

Subjects

We selected 39 LOSD cases and 71 age-matched normal control cases without neurological or psychiatric disorders, as well as 22 cases having psychiatric disorders other than LOSD as disease controls (11 depression, six personality disorder (personality change), two bipolar disorder, and three neurotic disorder cases). All psychiatric cases were selected from an autopsy case series registered with the Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. The common selection criteria for the psychiatric cases including LOSD cases were as follows: (1) the initial psychiatric symptoms occurred after 40 years of age, (2) the absence of a history of neurological or psychiatric disorders before 40 years of age, (3) the absence of dementia in the early to middle stage of the course, and (4) the absence of episodes suggesting evident memory impairment, including delusion of theft. In this study, LOSD was defined as psychosis that developed after 40 years of age, fit the criteria of schizophrenia or delusional disorders of ICD-10, and lacked dementia at least in the early to middle stage of the course. Some of the LOSD cases were originally diagnosed as presenile-onset schizophrenia or senile-onset psychosis. Nine LOSD cases were excluded from the study because they had alcohol dependence, respiratory diseases, liver diseases, renal diseases, neurosyphilis, Huntington disease, or pathological evidence of large cerebral infarction or dentatorubral–pallidoluysian atrophy, which may be associated with the development of psychotic symptoms. Seven LOSD cases without detailed clinical data were also excluded. Finally, we pathologically re-examined 23 LOSD cases, 71 age-matched normal controls, and 22 disease control cases (11 depression, six personality disorder, two bipolar disorder, and three neurotic disorder cases) using modern standardized methods including a panel of immunohistochemistry and sensitive silver stains. Two psychiatrists (SN and OY) reviewed the available clinical information, interviewed clinicians if necessary, and made a consensus diagnosis based on ICD10. All of the LOSD cases but four died in psychiatric hospitals. Age-matched normal control cases ($n = 71$) were selected from an autopsy case series registered with the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology. Selection criteria for these normal control cases were as follows: (1) the absence of primary neurological and psychiatric disorders including dementia, stroke, and gait disturbance, (2) the absence of pathological evidence of large cerebral infarctions, and (3) the availability of medical and autopsy records and

Table 1 Demographic data of all cases

	Late-onset schizophrenia and delusional disorders	Normal controls	Other late-life psychiatric disorders			
			Depression	Bipolar disorders	Personality disorders	Neurotic disorders
N	23	71	11	2	6	3
Female, *n* (%)[a]	15 (65.2)	20 (28.2)	6 (54.5)	2 (100.0)	2 (33.3)	2 (66.7)
Age at onset, mean ± SD (years)	63.3 ± 12.9	–	62.3 ± 8.8	68.0	68.3 ± 10.9	65.7 ± 9.2
(Range, years)	(41–86)	–	(51–74)	(68)	(58–82)	(55–71)
Age at death, mean ± SD (years)	75.1 ± 7.5	72.3 ± 6.6	68.3 ± 7.2	78.5 ± 4.9	70.7 ± 11.0	73.0 ± 3.0
Disease duration, mean ± SD (years)	12.0 ± 7.7	–	7.1 ± 6.4	10.5 ± 4.9	2.3 ± 1.0	7.3 ± 6.8
Dementia in the last stage, *n* (%)[b]	7/19 (36.8)	0/71 (0.0 %)	4/9 (44.4)	0/2 (0.0)	1/6 (16.7)	1/3 (33.3)
Cause of death (*n*)						
Neoplasm	1	41	–	–	1	–
Acute myocardial infarction	2	5	–	–	–	–
Aortic aneurysm dissection	–	1	–	–	–	–
Acute respiratory distress syndrome	–	1	–	–	–	–
Heart failure	2	1	–	–	–	–
Pulmonary infarction	–	1	–	–	–	–
Pneumonia	5	3	5	–	3	1
Respiratory failure	4	4	–	–	1	–
Lung abscess	–	1	–	–	–	–
Gastrointestinal bleeding	1	1	1	–	–	–
Ileus	1	–	–	–	–	–
Hepatic failure	–	1	–	–	–	–
Liver cirrhosis	–	1	–	–	–	–
Goodpasture syndrome	–	1	–	–	–	–
Renal failure	–	3	–	–	–	–
Diabetes mellitus	–	2	–	–	–	–
Sepsis	1	1	–	1	–	–
Shock	1	1	–	–	–	–
Sudden death	1	2	1	1	1	1
Suicide	1	–	2	–	–	–
Not available	3	–	2	–	–	1

SD standard deviation

[a] The proportion of cases in each category of clinical diagnosis

[b] The proportion of cases that had dementia in the last stage of the course to all subjects which clinical data in the terminal stage was available

paraffin-embedded tissues. All subjects were autopsied after informed consent was obtained from family members. All experiments in this study were approved by the ethical committees of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, and the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology. The demographic data of all subjects are shown in Table 1.

Conventional neuropathological examination

Brains tissue samples were fixed postmortem with 10 % formaldehyde and embedded in paraffin. The median fixation time was 141 days (range 35–3,918 days, 25–75th percentile range 72–270 days) in LOSD cases for which data were available ($n = 11$, 47.8 %), 90 days (range 16–2,647 days, 25–75th percentile range 34–464 days) in psychiatric disease control cases ($n = 13$, 59.1 %), and 19 days (range 7–65 days, 25–75th percentile range 14–38 days) in age-matched normal control cases ($n = 46$, 63.4 %), respectively. The fixation time in age-matched normal controls was significantly shorter than those in LOSD cases and psychiatric disease control cases, respectively ($P < 0.0001$, respectively. Mann–Whitney *U* test [$\alpha/2$]). Ten-μm-thick sections from the frontal, temporal, parietal, occipital, insular, and cingulate cortices,

hippocampus, amygdala, basal ganglia, midbrain, pons, medulla oblongata, and cerebellum were prepared. Sections of the left hemisphere in psychiatric cases including LOSD cases and standard size sections including each anatomical region examined in age-matched normal control cases were stained with hematoxylin–eosin (H&E) and Klüver–Barrera (KB) stains. Selected regions were stained with modified Bielschowsky silver, methenamine silver, Gallyas–Braak silver methods, and Holzer stain.

Immunohistochemistry

Paraffin sections were cut at 6 μm thickness from regions for the standard assessments described below and immunostained by the immunoperoxidase method using 3′3-diaminobenzidine tetrahydrochloride as reported previously [39]. Antibodies used were against phosphorylated tau (AT8, mouse, monoclonal, 1:1,000, Innogenetics, Ghent, Belgium), tau (T46: mouse, monoclonal, 1:1,000, Invitrogen, Carlsbad, CA, USA), three-repeat (3R) tau (RD3: mouse, monoclonal, 1:3,000, Upstate, Syracuse, NY, USA), and four-repeat (4R) tau (RD4: mouse, monoclonal, 1:200, Upstate), Aβ11-28 (12B2, mouse, monocloncal, 1:2,000, Immuno-Biological Laboratories, Fujioka, Japan), Aβ42 (Aβ42, rabbit, polyclonal, 1:100, Immuno-Biological Laboratories), phosphorylated α-synuclein (psyn#64, mouse, monoclonal, 1:5,000, Wako, Osaka, Japan), α-synuclein (anti-α-synuclein, mouse monoclonal, 1:10,000, Invitrogen, Burlington, ON, Canada), phosphorylated TDP-43 (pS409/410-2, rabbit polyclonal, 1:5,000, Cosmo Bio, Tokyo, Japan), TDP-43 (anti-TDP-43, rabbit polyclonal, 1:1,000, ProteinTech, Chicago, IL, USA), and phosphorylated neurofilament (SMI31, mouse, monoclonal IgG, 1:10,000, Sternberger Monoclonals, Baltimore, MD, USA). When using anti-Aβ antibody, sections were pretreated with 70 % formic acid for 10 min for antigen retrieval. When using psyn#64, SMI31, anti-TDP-43, and pS409/410-2, sections were pretreated in a pressure cooker for 3 min in 10 mM sodium citrate buffer pH 6.0 to enhance immunoreaction. When using phosphorylation-independent anti-α-synuclein antibody, RD3 and RD4, sections were pretreated with 70 % formic acid for 10 min and heated for 3 min in 10 mM sodium citrate buffer in a pressure cooker. Sections were lightly counterstained with hematoxylin.

Assessment of histopathological changes

The distribution and severity of histopathological changes were assessed according to standardized methods. (1) The distribution of NFTs was assessed according to the Braak NFT stage (stage 0–VI) using AT8 immunohistochemistry [40]. (2) The distribution of senile plaques was assessed according to the Braak senile plaque stage using Aβ immunohistochemistry [41]. In statistical analyses, the original stages (i.e., none, A, B, and C) were indicated as stages 0, 1, 2, and 3, respectively. The pathological diagnosis of AD was made according to the NIA-Reagan criteria [42–44] using the modified Bielschowsky silver method, and tau and Aβ immunohistochemistry. (3) Lewy body-related pathology was classified into four histological subtypes (i.e., brain stem type, limbic type, diffuse neocortical type, amygdala-predominant type) according to the Third Consensus Guidelines for DLB [45] and a more recent report [46] with α-synuclein immunohistochemistry. (4) The distribution of argyrophilic grains was classified into four stages (stage 0–III) using a system proposed by Saito et al. [33]. For this evaluation, sections from the ambient gyrus, amygdala, entorhinal cortex, hippocampus, temporal, cingulate, and insular cortex, and orbital gyrus were stained with the Gallyas–Braak silver method and tau immunohistochemistry. In cases having argyrophilic grains, four-repeat tau-predominant accumulation was confirmed by RD4 and RD3 immunostaining. (5) The diagnoses of CBD and PSP were made according to established criteria [47, 48]: astroglial lesions (i.e., tufted astrocytes and astrocytic plaques), NFTs, pretangles, neuropil threads, and ballooned neurons were examined in the posterior superior and middle frontal gyri, primary motor cortex, parietal and temporal cortices, hippocampus, amygdala, caudate nucleus, putamen, globus pallidus, subthalamic nucleus, oculomotor nucleus, substantia nigra, pontine nucleus, inferior olivary nucleus, and dentate nucleus in the cerebellum using both the Gallyas–Braak silver method and tau immunohistochemistry. In this study, cases having not only sufficient NFTs but also astroglial lesions (tufted astrocytes or astrocytic plaques) were diagnosed with CBD or PSP. (6) TDP-43-positive lesions were assessed in the amygdala, entorhinal cortex, hippocampus, frontal and temporal cortices, and hypoglossal nuclei using an anti-TDP-43 antibody. The distribution of TDP-43-positive inclusions in the limbic region was classified into three pathological subtypes using the following system [39], which is similar to that reported by Amador-Ortiz et al. [49]: the amygdala type, in which inclusions were present only in the amygdala; the limbic type, in which inclusions extend to the amygdala, hippocampal dentate gyrus, entorhinal cortex, and fusiform gyrus, but not into the occipitotemporal gyrus; and the temporal type, in which inclusions are also present in the occipitotemporal gyrus. (7) Neuronal loss associated with gliosis and vascular lesions were assessed on H&E- and KB-stained sections according to the grading system employed in our previous studies [50, 51]. Cerebrovascular lesions in the cerebral cortex and basal ganglia were assessed on sections including the whole left hemisphere in LOSD cases ($n = 23$) and psychiatric disease control cases ($n = 22$)

according to a four-point grading system, respectively: grade 0, no lacuna; grade 1, one small lacuna; grade 2, two or more small lacuna without large infarction; grade 3, one or more large infarction. Then, the severity of vascular lesions was compared between LOSD cases and psychiatric disease controls. The severity (the number and size) of vascular lesions in LOSD cases was not compared with that in age-matched normal controls because in the latter group, only sections on standard size slides that included each anatomical region (cortices and nuclei) with only adjacent white matter were available.

Statistical analysis

The Mann–Whitney *U* test and Fisher's exact test were used to compare two groups. In multiple comparisons, Bonferroni correction was done. The odds ratio was used as the measure of the strength of association between binary variables. A P value <0.05 was accepted as significant. Statistical analyses were performed using Excel and statistical package R (http://www.r-project.org/). Clinical diagnosis subgroups were compared with age (the age at death)-matched control cases that were serially extracted from all 71 normal control cases, respectively: LOSD cases with the onset of $\geq$65 years of age (the age at death: median 79 years of age, range 72–91 years) were compared with 59 age-matched normal controls (median 76 years of age, range 73–90 years), and LOSD cases with the onset at <65 years of age (median 70 years of age, range 58–77 years) were compared with 36 age-matched normal controls (median 72 years of age, range 58–77 years). The variation in the number of normal control cases is due to this procedure.

Results

Frequencies of neurodegenerative changes in all LOSD and control cases

The pathological diagnoses of all LOSD and age-matched normal control cases are shown in Table 2 and Fig. 4. Of 23 LOSD cases, six cases (26.1 %) had LBD, five (21.7 %) had AGD, and one (4.3 %) had CBD. Two cases (8.7 %) had moderate AD pathology alone (Braak stage III–IV/0–C), and nine (39.1 %) had mild AD pathology alone (Braak stage I–II/0–C). Argyrophilic grains in all LOSD cases were almost completely restricted to the amygdala, hippocampus, and adjacent temporal cortex, corresponding to Saito's stages I–II (i.e., mild to moderate AGD). A few TDP-43-positive inclusions in the limbic region were found in two LOSD cases (one diffuse neocortical type LBD and one limbic type LBD). Representative AGD, LBD, and CBD cases that clinically exhibited LOSD are shown in Figs. 1, 2, 3. No LOSD case had pathological evidence of demyelinating diseases, neoplasms, or infections in the central nervous system. In all age-matched normal controls, eight cases (11.3 %) had LBD, and six (8.5 %) had AGD, respectively. In addition, one case (1.4 %) had moderate AD pathology alone (Braak stage III–IV), 52 (77.8 %) had minimal AD pathology alone (Braak stage 0–II), and four (5.6 %) lacked any degenerative change. No normal control case had CBD pathology. A few TDP-43-positive inclusions in the limbic region were found in one age-matched control case having Saito's stage II AGD.

None of our subjects was pathologically diagnosed as having pure AD (Braak NFT stage V–VI [44]), PSP, Pick's disease (with tau-positive Pick bodies), white matter tauopathy with globular glial inclusions [52], or frontotemporal lobar degeneration with TDP-43-positive inclusions. In addition, no case had senile dementia of the neurofibrillary tangle type (SD-NFT), a form of tangle-only dementia characterized by abundant extracellular NFTs, severe neuronal loss in the hippocampus, and no or minimal Aβ deposits [53].

A comparison of LOSD and control cases demonstrated a significant relationship between LOSD and the distribution of pathological diagnoses [$P = 0.0015$, Fisher's exact test ($\alpha/7$)]. The frequencies of AGD, LBD, and CBD in all LOSD cases tended to be higher than those in normal controls, although statistically not significantly [$P = 0.09$, 0.09, and 0.24, Fisher's exact test ($\alpha/7$)]. On the other hand, the total frequency of cases having either AGD, LBD, or CBD was significantly higher in LOSD cases than in controls [$P = 0.0037$, Fisher's exact test ($\alpha/7$)]. The frequency of cases having no or mild AD pathology alone (Braak stage 0–II) was significantly lower in LOSD cases than in control cases [$P = 0.0006$, Fisher's exact test ($\alpha/7$)], while the frequency of cases having moderate AD pathology alone (Braak stage III–IV) was not significantly different between two groups [$P = 0.15$, Fisher's exact test ($\alpha/7$)]. Odds ratio analyses demonstrated that patients who developed LOSD after the age of 40 years had a significantly increased risk of having either AGD, LBD, or CBD pathology [odds ratio 4.44, 95 % confidence interval (CI), 1.62–12.1] compared with normal controls.

The AGD stages in all LOSD cases were (versus normal controls): 75th percentile 0.5 (0); median 0 (0); and 25th percentile 0 (0). The AGD stage was significantly higher in LOSD cases than in control cases ($P = 0.0225$, Mann–Whitney *U* test). The Braak NFT stages in all LOSD cases were (versus normal controls): 75th percentile 3 (1); median 2 (1); and 25th percentile 2 (1). The Braak NFT stage was also significantly higher in all LOSD cases than that in controls ($P < 0.0001$, Mann–Whitney *U* test). The Braak stages of Aβ-positive senile

Table 2 Pathological diagnoses in all cases

Pathological diagnosis	Late-onset schizophrenia and delusional disorders ($n = 23$)	Normal controls ($n = 71$)	Other late-onset psychiatric disorders			
			Depression ($n = 11$)	Bipolar disorders ($n = 2$)	Personality disorders ($n = 6$)	Neurotic disorders ($n = 3$)
Argyrophilic grain disease						
AGD2 + NFT2-4 + SPB	2	–	–	–	–	–
AGD2 + NFT2 + SP0	1	–	1	–	–	–
AGD2 + NFT2 + SPA + TDPlim	–	1	–	–	–	–
AGD1 + NFT3 + SPB	–	1	–	–	–	–
AGD1 + NFT1-2 + SP0-A	2	4	–	–	–	–
Total (n)	5	6	1	–	–	–
Lewy body disease						
LBDdn + NFT5 + SPC + TDPlim	1	–	–	–	–	–
LBDdn + NFT5 + SP0	1	–	–	–	–	–
LBDdn + NFT1-3 + SPC	–	1	1	–	–	–
LBDlim + NFT4 + SPC + TDPlim	1	–	–	–	–	–
LBDlim + NFT1-2 + SPC	1	1	1	–	–	–
LBDbs + NFT1-2 + SPA	2	6	1	–	–	–
Total (n)	6	8	3	–	–	–
Corticobasal degeneration						
CBD + AGD3 + NFT3 + SPC	–	–	–	–	1	–
CBD + AGD1 + NFT1 + LBDbs	–	–	1	–	–	–
CBD + AGD1 + NFT2	1	–	–	–	–	–
CBD + AGD1 + NFT1	–	–	–	–	1	–
Total (n)	1	–	1	–	2	–
Mild to moderate AD pathology						
NFT3-4 + SP0-C	2	1	1	1	–	1
NFT1-2 + SPB-C	1	11	1	–	1	–
NFT1-2 + SP0-A	8	38	4	1	3	2
NFT0 + SPA-C	–	3	–	–	–	–
Total (n)	11	53	6	2	4	3
No degeneration						
Total (n)	–	4	–	–	–	–

NFT1, 2, 3, 4 and 5, Braak NFT stage I, II, III, IV, and V; SPA, B, and C, Braak senile plaque stage A, B, and C; LBDbs, brain stem type Lewy body disease; LBDlim, limbic type Lewy body disease; LBDdn, diffuse neocortical type Lewy body disease; TDPlim, limbic type TDP-43 pathology, AGD1, argyrophilic grain disease stage I; AGD2, AGD stage II, AGD3, AGD stage III; CBD, corticobasal degeneration; PSP, progressive supranuclear palsy

plaques in all LOSD cases were (versus normal controls): 75th percentile 2 (1); median 1 (1); and 25th percentile 0 (0). The Braak Aβ-positive plaque stage was not statistically different between two groups ($P = 0.83$, Mann–Whitney U test).

Frequencies of neurodegenerative changes in LOSD cases with onset age of ≥65 years

Of 11 LOSD cases with onset at ≥65 years of age (median age at death, 79 years), four cases (36.4 %) had AGD, four (36.4 %) had LBD, two (18.2 %) had moderate AD pathology (Braak stage III–IV) alone, and one (9.1 %) had minimal AD pathology (Braak stage I–II) alone (Fig. 4). In contrast, in 36 age-matched normal controls (median age at death, 76 years), three cases (8.3 %) had AGD, seven (19.4 %) had LBD, one (2.8 %) had moderate AD pathology (Braak stage III–IV) alone, and 25 (69.4 %) had minimal AD pathology (Braak stage I–II) alone.

The frequency of AGD was significantly higher in LOSD cases with onset at ≥65 years of age than in normal controls ($P = 0.0424$, Fisher's exact test). LOSD patients with onset at ≥65 years of age had a significantly increased risk of having AGD (odds ratio 6.29; 95 % CI 1.14–34.6) compared to age-matched normal controls.

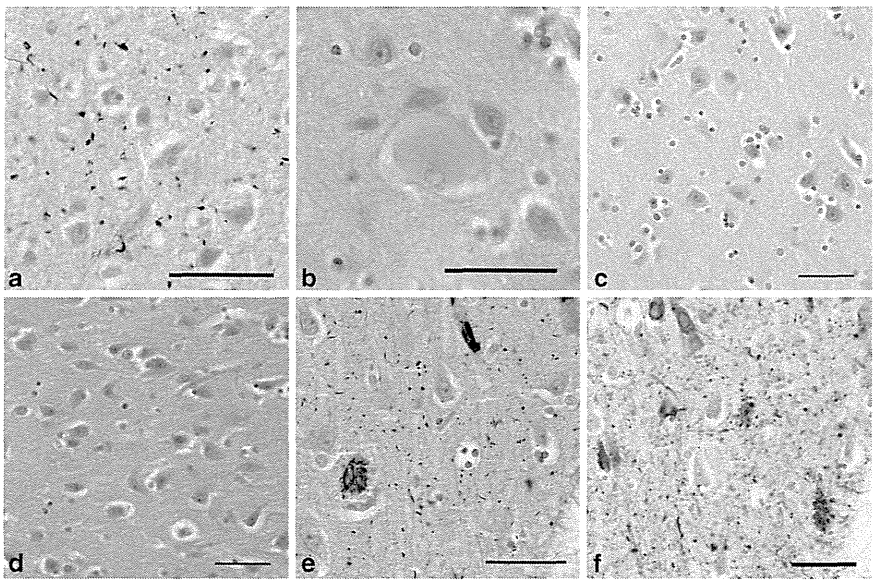

Fig. 1 Pathological findings in a representative argyrophilic grain disease case showing LOSD. Argyrophilic grain disease (AGD) seen in a 79-year-old woman who was diagnosed with senile-onset schizophrenia. She initially showed persecutory delusion and tactile hallucination at 68 years of age. Motor disturbance or dementia was absent throughout the course, and she died suddenly. The brain weighed 1,305 g. **a** Moderate argyrophilic grains in the ambient gyrus. **b** Ballooned neurons in the amygdala. **c** In contrast to the presence of argyrophilic grains, neuronal loss or gliosis is not noted in the amygdala. Pyramidal neurons in the hippocampal CA1 were well spared in number (**d**), but argyrophilic grains were densely distributed in the hippocampal CA1, corresponding to Saito's AGD stage II (**e**). **f** Tau-positive argyrophilic grains with a few neurofibrillary tangles in the hippocampus (Braak stage II). **a**, **e** Gallyas–Braak silver stain, **b**–**d** hematoxylin–eosin stain, **f** AT8 immunohistochemistry. All *scale bars* = 50 μm

Frequencies of neurodegenerative changes in LOSD cases with onset at <65 years of age

Of 12 LOSD cases with onset at <65 years of age (median age at death, 70 years), one had AGD (8.3 %), two (16.7 %) had LBD, and one (8.3 %) had CBD (Fig. 4). Of 59 age-matched normal controls (median age at death, 72 years), three (5.1 %) had AGD, and six (10.2 %) had LBD. The frequency of AGD was not significantly different between LOSD cases whose onset age was <65 years and control cases ($P = 0.53$, Fisher's exact test).

Comparison of severities of neurodegenerative changes between LOSD cases with and without dementia in last stage

The clinical data regarding the presence or absence of dementia in the last stage were available for 19 of 23 LOSD cases, and seven had dementia in the last stage. The Braak NFT stage (LOSD with vs. without dementia, median: 2 vs. 2), Braak Aβ stage (2 vs. 0.5), AGD stage (0 vs. 0), and the frequency of LBD (42.9 vs. 14.3 %) did not significantly differ between LOSD cases with and without dementia in the last stage ($P = 0.54$, 0.27, 0.33, and 0.12, Mann–Whitney U test and Fisher's exact test).

Frequencies of degenerative changes in cases of various psychiatric disorders with onset at ≥40 years of age

We additionally examined pathological changes in 22 patients who developed various psychiatric disorders other than LOSD after 40 years of age (Table 2).

AGD and LBD were found in some depression cases, but not in any case in the other clinical diagnosis groups. The frequencies of AGD (9.1 vs. 8.5 %) and LBD (27.3 vs. 11.3 %) were not statistically different between all depression cases and normal control cases, respectively [$P = 0.56$ and 0.12, Fisher's exact test (α/2), Table 2]. Of five depression patients with onset at ≥65 years of age, while no case had AGD, three had LBD. The frequency of LBD was significantly higher in depression patients whose onset age was ≥65 years of age than in normal controls ($n = 56$) (60.0 vs. 10.7 %, $P = 0.0198$, Fisher's exact test). On the other hand, of six depression patients whose onset age was <65 years of age, one had AGD and no case had LBD. The frequency of AGD in this subgroup of depression was not significantly different between LOSD and normal control cases ($n = 21$) [16.7 vs. 14.3 %, $P = 0.66$, Fisher's exact test (α/2)].

Pathological changes of CBD were found in one depression case and two cases of presenile-onset

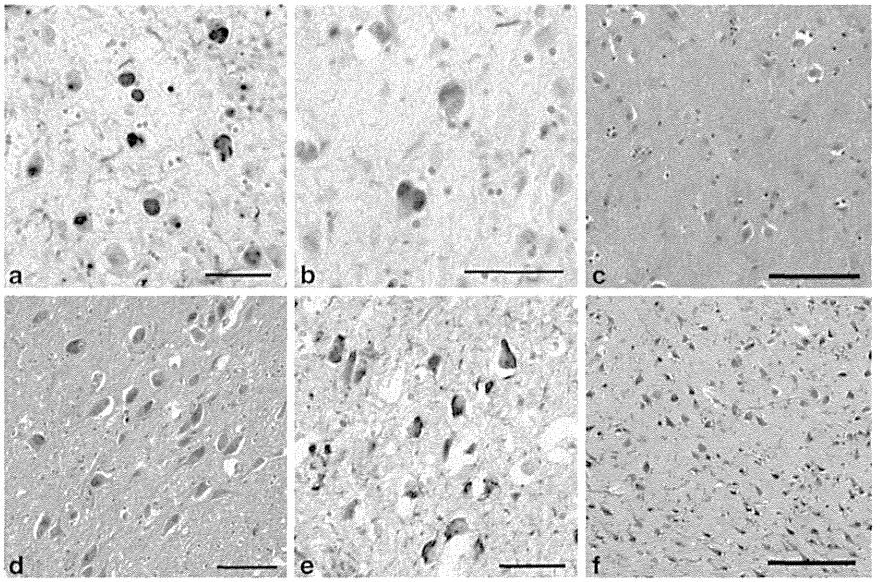

Fig. 2 Pathological findings in a representative Lewy body disease case showing LOSD. Limbic type Lewy body disease seen in a 82-year-old man diagnosed with senile-onset schizophrenia-like psychotic disorder. He initially developed delusion of observation and auditory hallucination at 72 years of age. His intelligence was mildly impaired after 77 years of age, but he did not exhibit dementia throughout the course. Although dysphasia was seen in the terminal stage, muscle rigidity or tremor was absent during the course. He died of pneumonia. The brain weighed 1,150 g. Many α-synuclein-positive Lewy bodies in the entorhinal cortex (**a**) and amygdala (**b**). **c** Moderate neuronal loss with astrocytosis in the amygdala. **d** Neurons in the substantia nigra were well spared in number. **e** Tau-positive neurofibrillary tangles (NFTs) in the hippocampal CA1. This case had NFTs in the insular cortex, corresponding to Braak NFT stage IV. **f** In contrast to NFTs, pyramidal neurons in the hippocampal CA1 were relatively well preserved in number. **a**, **b** Psyn#64 immunohistochemistry, **c**, **d** hematoxylin–eosin stain, **e** AT8 immunohistochemistry, **f** Klüver–Barrera stain. *Scale bars* = (**a**, **b**, **e**) 50 μm, (**c**, **d**, **f**) 100 μm

personality disorder. No psychiatric case had pathological evidence of demyelinating diseases, neoplasms, or infections in the central nervous system.

Comparison of vascular lesions between LOSD cases and psychiatric disease control cases

The severities of vascular lesions in the cerebral cortex in LOSD cases (versus psychiatric disease controls) were: 75th percentile 0.5 (0); median 0 (0); and 25th percentile 0 (0). The severity of vascular lesions in the cerebral cortex was not significantly different between the two groups ($P = 0.42$, Mann–Whitney U test). Likewise, the severities of vascular lesions in the basal ganglia in LOSD cases (versus psychiatric disease controls) were: 75th percentile 1 (1); median 0 (0); and 25th percentile 0 (0). The severity of vascular lesions in the basal ganglia was not significantly different between the two groups ($P = 0.78$, Mann–Whitney U test). The age at death in LOSD cases (the mean age at death 75.1 ± 7.5 years) was not significantly different from that in psychiatric disease control cases (70.5 ± 8.1 years) ($P = 0.082$, Mann–Whitney U test).

Clinical features in pathological diagnosis groups

The demographic data in four pathological diagnosis groups, i.e., AGD (AGD cases lacking LBD or CBD pathology), LBD (LBD cases lacking AGD or CBD pathology), CBD (cases having pathology of CBD), and non-degenerative disease groups (cases having minimal AD pathology of Braak stage I–II/0–A alone) are shown in Table 3. The ages of onset and death in CBD cases were about 10 years lower than those in AGD and LBD cases, respectively. Figure 5 shows the frequencies of clinical symptoms in each pathological diagnosis group. Delusion was significantly more frequent in the AGD group, and disinhibition was significantly more frequent in the CBD group than those in a non-degenerative disease group, respectively [$P = 0.0127$ and 0.0026, Fisher's exact test ($\alpha/3$)].

Discussion

To our knowledge, this is the first study that comprehensively examined the neurodegenerative bases in patients

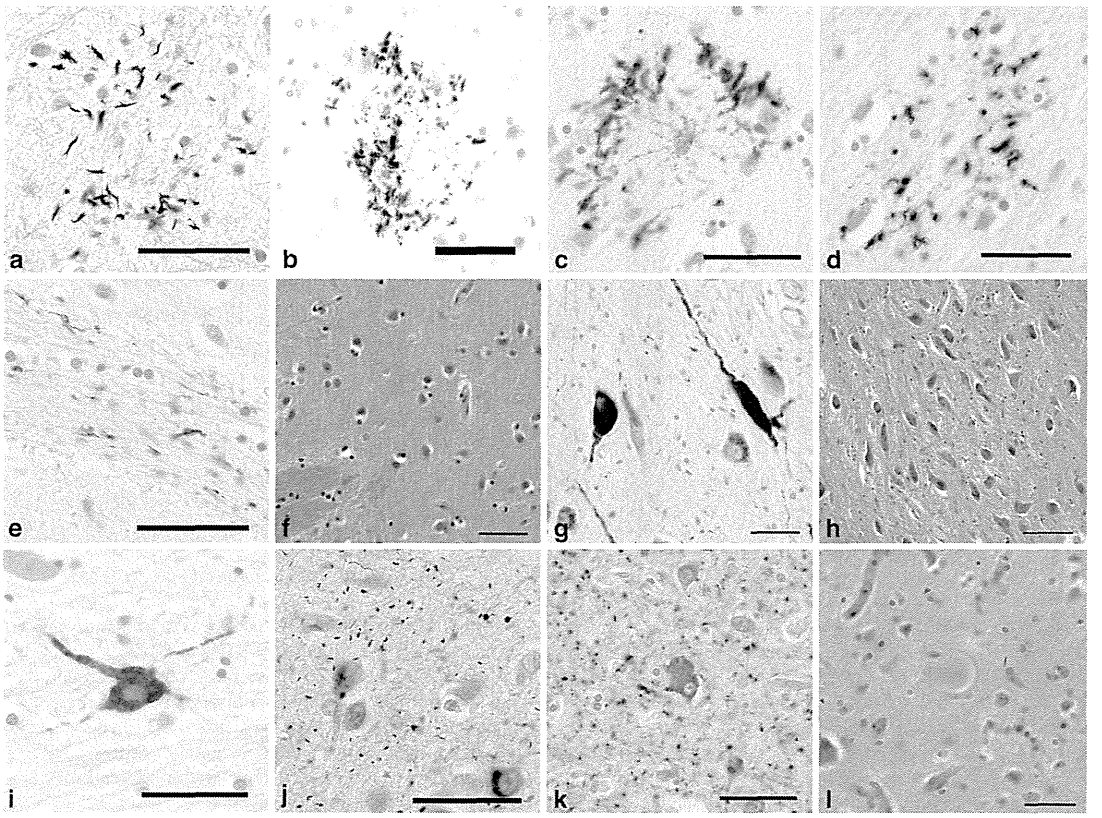

Fig. 3 Pathological findings in a representative corticobasal degeneration case showing LOSD. Corticobasal degeneration (CBD) seen in a 58-year-old man who was diagnosed with late-onset schizophrenia. His initial symptom was apathy at 41 years of age. Auditory hallucination and irritability occurred at 52 years of age. Motor disturbance, aphasia, or dementia was absent throughout the course. He died of chronic obstructive pulmonary disease. The brain weighed 1,480 g. Astrocytic plaques in the superior frontal gyrus (**a**), motor cortex (**b**), caudate nucleus (**c**), and putamen (**d**). **e** A small number of tau-positive threads were seen in the putamen. A small number of tau-positive and Gallyas-positive neurofibrillary tangles and threads were found in the frontal cortex, caudate nucleus, putamen, subthalamic nucleus, substantia nigra (**g**), oculomotor nucleus, pontine nucleus (**i**), and inferior olivary nucleus. Unlike classic CBD cases, neuronal loss with glial proliferation was not seen in the putamen (**f**) or substantia nigra (**h**). **j–l** This case also had Gallyas- and tau-positive argyrophilic grains (**j**, **k**), mild neurofibrillary tangles (Braak stage II), and ballooned neurons (**k**, **l**) in the ambient gyrus and amygdala. However, neuronal loss and gliosis were minimal in these regions (**l**). **a**, **j** Gallyas–Braak silver stain, **f**, **h**, **l** hematoxylin–eosin stain, (**b–e**, **g**, **i**, **k**) AT8 immunohistochemistry. *Scale bars* = (**a**, **c**, **d**, **e–g**, **i–l**) 50 μm, **b** 25 μm, **h** 100 μm

with LOSD and demonstrated a significant relationship between LOSD and AGD. The main findings in the present study were as follows: (1) AGD and LBD had comparably common pathological bases in our LOSD cases (21.7 and 26.1 %), frequencies about 2.5 times higher than those in normal controls (8.5 and 11.3 %). Argyrophilic grains in LOSD cases are almost completely restricted to the limbic system and adjacent temporal cortex. CBD was rarely found in LOSD but never in normal control cases. Consequently, LOSD patients who experienced onset after 40 years of age had about a fourfold increased risk of having either AGD, LBD, or CBD (odds ratio 4.44, 95 % CI 1.62–12.1) compared with normal controls. (2) AGD was significantly more frequent in LOSD patients whose onset occurred at ≥65 years of age than in normal controls, and the LOSD patients had about a sixfold increased risk of having AGD (odds ratio 6.29; 95 % CI 1.14–34.6) compared with normal controls. (3) In a psychiatric case series, the frequency of delusion in AGD cases was significantly more frequent than that in cases having minimal AD pathology alone. These findings suggest that LOSD cases may have heterogeneous pathological backgrounds, including AGD, LBD, and CBD and that mild to moderate argyrophilic grains may play an important role in the occurrence of LOSD, especially in elderly people.

It has been reported that some AGD cases with dementia show various psychiatric features, including delusion, hallucination, aggression, irritability, and obsession [31, 34–38]. However, as far as we know, there has been no study that demonstrated a significant relationship between AGD and LOSD in non-demented elderly people. In general, it is difficult to determine whether histopathological

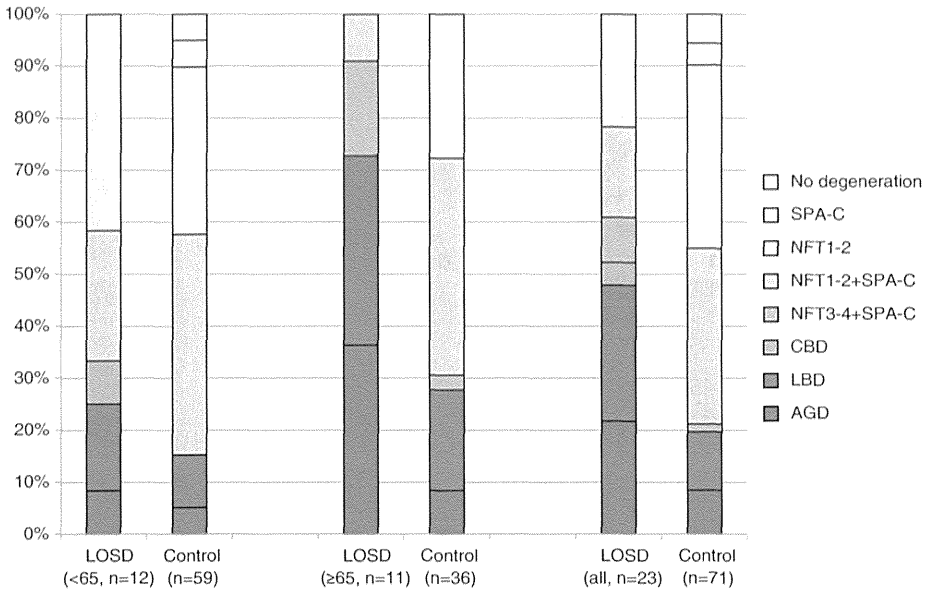

Fig. 4 Distribution of pathological diagnoses in LOSD cases and age-matched normal controls. *Right* A comparison of all LOSD and age-matched normal control cases. The frequencies of AGD and LBD in LOSD cases were about 2.5 times those in controls, and CBD was found only in LOSD cases. The total proportion of AGD, LBD, and CBD was significantly higher in LOSD cases than in controls [$P = 0.0037$, Fisher's exact test ($\alpha/7$)]. *Center* A comparison of LOSD patients ≥65 years of age at onset and age-matched normal controls. The frequency of AGD was significantly higher in LOSD cases than in controls ($P = 0.0424$, Fisher's exact test). *Left* A comparison of LOSD patients whose onset occurred at <65 years of age and age-matched normal controls. The frequency of AGD did not statistically differ between two groups (Fisher's exact test). *LOSD* late-onset schizophrenia and delusional disorders, *NFT* neurofibrillary tangles, *CBD* corticobasal degeneration, *LBD* Lewy body disease, *AGD* argyrophilic grain disease

Table 3 Demographic data by pathological diagnosis in cases with psychiatric disorders

Pathological diagnosis	*n*	Female *n* (%)	Age at onset (years) Mean ± SD	Age at death (years) Mean ± SD	Disease duration (years) Mean ± SD	Brain weight (g) Mean ± SD	Dementia in last stage *n* (%)[g]
Argyrophilic grain disease group[b]	6	4 (67.7)	67.0 ± 12.0	77.8 ± 7.3[f]	10.8 ± 5.9	1,179 ± 138.2	1/4 (25.0)
Lewy body disease group[a]	9	3 (33.3)	70.1 ± 8.3[e]	78.2 ± 5.7[e]	8.9 ± 6.1	1,273 ± 143.2	5/7 (71.4)
Corticobasal degeneration group[c]	4	1 (25.0)	60.5 ± 16.8	66.8 ± 12.4	6.3 ± 7.3	1,315 ± 124.8	2/4 (50.0)
Total	19	8 (42.1)	66.9 ± 11.6	75.7 ± 8.8	8.9 ± 6.1	1,240 ± 133.6	8/15 (53.3)
Non-degenerative group[d]	18	14 (77.8)	59.2 ± 9.4	68.3 ± 5.7	9.7 ± 9.2	1,204 ± 182.2	3/15 (20.0)

LBD Lewy body disease, *AGD* argyrophilic grain disease, *CBD* corticobasal degeneration, *PSP* progressive supranuclear palsy, *SD* standard deviation

[a] Lewy body disease (LBD) cases with variable degrees of Alzheimer's disease (AD) pathology but without argyrophilic grain disease (AGD), corticobasal degeneration (CBD), or progressive supranuclear palsy (PSP) pathology

[b] AGD cases without LBD, CBD, or PSP pathology

[c] All cases diagnosed pathologically as having CBD or PSP

[d] Cases having only minimal AD pathology (Braak NFT stage 0–II and/or Braak senile plaque stage 0–A)

[e] The onset age and age at death in the LBD group were significantly higher than those in the non-degenerative disease group, respectively [median age at onset: 71.5 vs. 61.0 years; median age at death: 77.0 vs. 69.0 years. $P = 0.0090$ and 0.00007. Mann–Whitney U test and Bonferroni correction ($P < 0.016$]

[f] The age at death in the AGD group was significantly higher than that in the non-degenerative disease group [median age at death: 76.0 vs. 69.0 years. $P = 0.0026$, Mann–Whitney U test and Bonferroni correction ($P < 0.016$)]

[g] The proportion of cases that had dementia in the last stage of the course of all subjects whose clinical data in the terminal stage was available

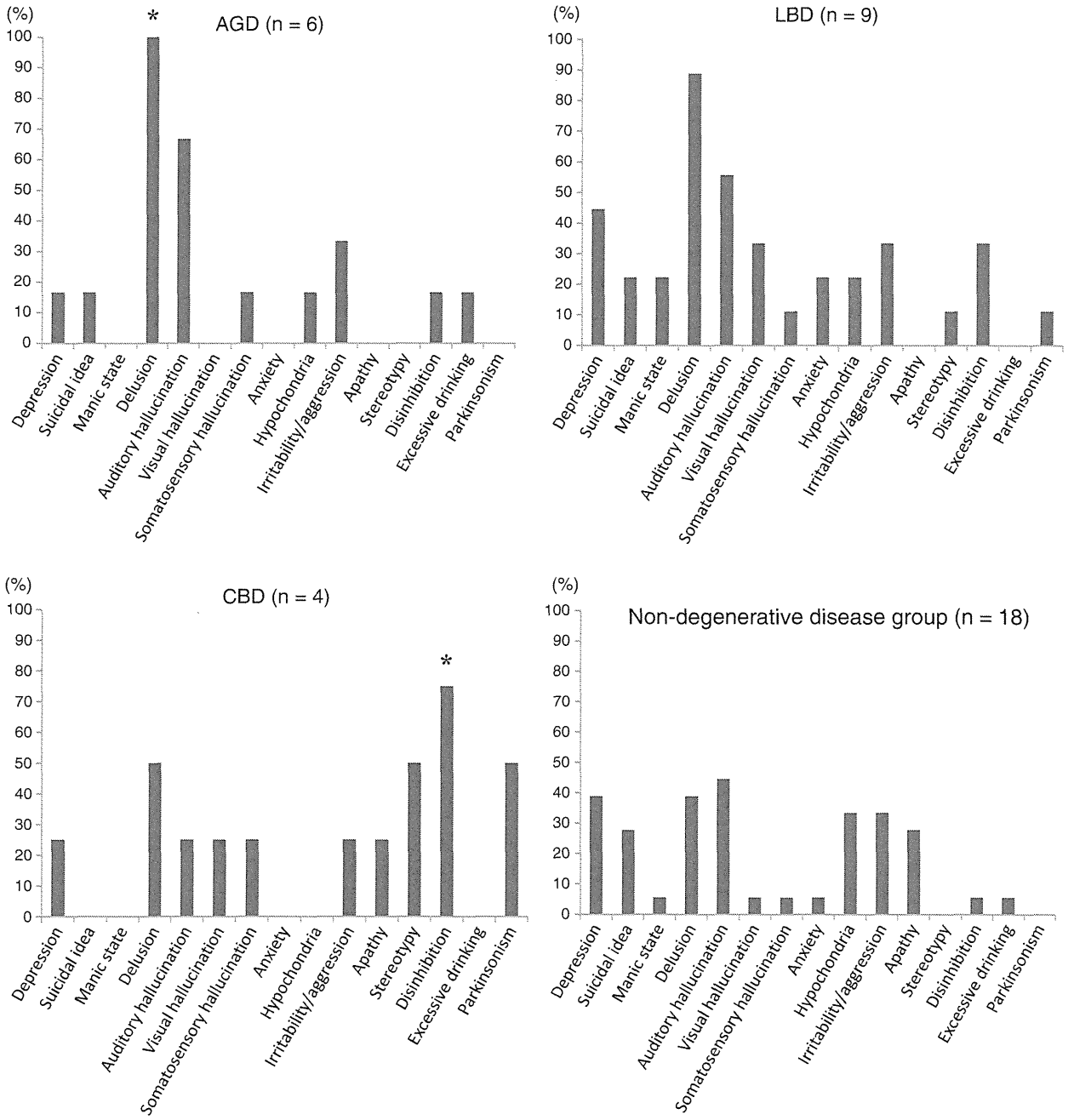

Fig. 5 Frequencies of clinical features by pathological diagnosis group. The frequencies of prominent clinical symptoms in the early stages of LBD ($n = 9$), AGD ($n = 6$), CBD ($n = 4$), and non-degenerative disease groups ($n = 18$). The frequency of delusion in the AGD group was significantly higher than those in a non-degenerative disease group [Fisher's exact test, $P = 0.0162$, Bonferroni correction ($\alpha/3$)]. The frequency of disinhibition in the CBD group was also significantly higher than that in a non-degenerative disease group [$P = 0.0026$, Fisher's exact test and Bonferroni correction ($\alpha/3$)]. See the definition of each pathological diagnosis group in the text. *LBD* Lewy body disease, *AGD* argyrophilic grain disease, *CBD* corticobasal degeneration

changes are causally related to young-onset psychiatric disorders because the initial symptoms usually occur several decades before death, and some major degenerative changes, e.g., argyrophilic grains [32], Lewy bodies [54], NFTs [55], and Aβ deposits [55], increase in frequency with age. However, our study demonstrated that the high frequency of AGD in LOSD cases may not be explained only by the age at death.

Argyrophilic grains in our LOSD cases tended to be less severe in topographical distribution (i.e., Saito's stage I–II)

compared with those in AGD cases showing dementia reported previously: some previous studies demonstrated that AGD cases with dementia frequently had argyrophilic grains more extensively distributed in the neocortex (i.e., Saito's stage III) [33]. In this context, it seems to be natural that our AGD cases lacking dementia, at least in the early to middle stage of the course, had less severe tau pathology. The impact of such mild to moderate argyrophilic grains on psychological functions, such as mood, anxiety, and thought, has hardly been explored. Our results suggest that LOSD without dementia at the onset may be one of the clinical presentations in elderly people having mild to moderate AGD. It is known that psychosis tends to occur secondarily when the limbic region and temporal cortex are involved in various diseases, such as cerebrovascular disease, traumatic brain injury, and epilepsy [56, 57]. These findings led us to consider that the occurrence of psychotic symptoms in AGD cases may be associated with the initial involvement of the limbic system by tau pathology in AGD [33].

It was reported that some LBD cases show systematized delusion [58]. The frequencies of delusion in LBD cases was reported to be 17–30 % in Parkinson's disease cases with or without dementia [59, 60] and 25–28.6 % in DLB cases [61, 62]. In our study also, although the difference did not reach statistical significance, the frequency of LBD in LOSD cases was about 2.5 times that in normal controls. Further, LBD was significantly more frequent in our depression cases with the onset at ≥65 years of age than in normal controls. On the other hand, AGD was not found in our depression cases. Because the number of cases examined in our study was small, whether the clinical spectrum in LBD cases is different from that in AGD cases cannot be concluded from these results. However, considering that the topographical distribution of degenerative changes, which is usually closely associated with clinical presentation, is different in AGD and LBD, it is plausible that these two degenerative diseases have different neuropsychiatric spectrums. For example, the limbic system and some brain stem nuclei (e.g., the raphe nuclei and locus coeruleus) frequently degenerate in LBD [45, 63], while argyrophilic grains consistently occur in the limbic regions but not in the brain stem nuclei. Dysfunction of the limbic system and brain stem nuclei was reported to be associated with depression [64]. Whether AGD is associated with the occurrence of depression or other psychiatric conditions should be explored by further studies using a larger sample.

In our study, although rare, some LOSD cases had CBD pathology. Several previous studies have also demonstrated that autopsy-confirmed CBD cases rarely showed psychotic symptoms [28–30, 65]. Interestingly, psychiatric symptoms occurred in all of our CBD cases younger than 65 years of age, in contrast to the relatively higher onset age in AGD and LBD cases (Table 3). Given these findings, in psychiatric practice, CBD should be considered one of the possible underlying pathologies in a patient who develops psychiatric symptoms before 65 years of age rather than after. The pathophysiological mechanism in the development of LOSD in CBD cases remains unclear. However, the coexistence of argyrophilic grains observed in all of our CBD cases might contribute at least partially to the occurrence of psychiatric symptoms.

Several previous studies, as well as this study, have consistently demonstrated that LOSD may not be associated with severe AD pathology (i.e., Braak NFT stage V–VI) [24, 25]. However, previous findings regarding the relationship between moderate NFTs in the limbic system and LOSD are not always consistent: while the severity of NFTs in the hippocampal CA1, entorhinal cortex, and temporal cortex was reported to be not significantly different between young-onset schizophrenia and LOSD cases [25], another study demonstrated that moderate NFTs distributed mainly in the limbic system and adjacent temporal cortex (Braak stage III–IV) may be associated with the development of LOSD [24]. In our study, the Braak NFT stage in LOSD cases was significantly higher than that in age-matched normal controls. However, our results cannot be simply compared with previous findings because various histological changes, including AD, LBD, AGD, and CBD pathologies, were not simultaneously evaluated in previous studies. For example, the high Braak NFT stage observed in our LOSD cases may be affected by the high proportions of LBD, AGD, and CBD cases having various severities of NFTs. Interestingly, in our LOSD cases, although the proportion of cases having only moderate NFTs (Braak stage III–IV) is not very large (8.7 %), it tends to be higher than that in normal controls (1.4 %). Considering our results together with previously reported findings, there may be LOSD patients whose onset is explained only by moderate numbers of NFTs distributed mainly in the limbic system; however, the proportion of such cases in all LOSD cases may not necessarily be large.

Limitations of our study are several. First, the sample sizes, especially those in clinical and pathological subgroups, are small. Therefore, our results may not always refute the possibility that LOSD is actually associated not only with AGD but also with LBD, CBD, and PSP. For example, in our study, although statistically not significant, the frequency of LBD in LOSD cases was over double that in age-matched normal controls (26.1 vs. 11.3 %), and CBD was found only in LOSD cases but not in age-matched normal controls. It is also known that LBD is found in some patients with paranoia [27, 59–61] and that clinically diagnosed PSP cases rarely show delusions [66]. Second, because almost all of our LOSD cases were psychiatric hospital inpatients, who probably had more severe clinical symptoms than outpatients, the case selection bias may

affect the frequency of each underlying pathology. Third, vascular lesions of the LOSD and age-matched normal control groups in this study could not be compared because the method of tissue sampling was different between these two groups. The impact of vascular changes on the occurrence of LOSD needs to be examined in the future. Fourth, in general, the immunoreactivity in tissue sections can be reduced by long fixation with formaldehyde, especially when using phosphorylation-dependent antibodies. However, in this study, AGD was explored by not only tau immunohistochemistry using phosphorylation-dependent and phosphorylation-independent anti-tau antibodies but also by Gallyas-Braak silver stain. Further, the fixation time in LOSD cases was longer than that in age-matched normal controls. Therefore, the significantly high frequency of AGD in LOSD cases observed in this study could not be explained by the effect of fixation time. Finally, whether neuronal loss and gliosis occur in AGD cases with LOSD are less severe than that in AGD cases with dementia should be also examined by further studies.

Although the present study demonstrated that LOSD patients have heterogeneous neurodegenerative backgrounds, including AGD, it may be still difficult to predict the underlying pathology in LOSD patients in life. Based on our results, biomarkers for tauopathies [67, 68] and α-synucleinopathies [67, 69], which continue to be developed for the precise clinical diagnosis of neurodegenerative dementias, might be useful to predict the pathogenic background in LOSD patients. Further clinicopathological studies are awaited to provide precise prognostic information to families based on biological findings and to develop novel therapeutic strategies for patients with LOSD.

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Conflict of interest None.

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