Supplementary material

Supplementary file 1

Supplementary file 2

Supplementary file 1

Materials and methods

Conventional neuropathological examination

Brain tissue samples were fixed postmortem with 10% formaldehyde and embedded in paraffin. 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. In some cases, spinal cord tissue was not obtained at autopsy. These sections were stained with hematoxylin-eosin, Klüver-Barrera, Gallyas silver, and modified Bielschowsky silver stains.

Immunohistochemistry

Six-µm-thick paraffin sections were immunostained by the immunoperoxidase method using 3, 3'-diaminobenzidine tetrahydrochloride. Antibodies used were against tau phosphorylated at Ser 202 (AT8, mouse, monoclonal, 1:1000, Innogenetics, Ghent, Belgium), three repeat (3R) tau (RD3, mouse, monoclonal, 1:2000, Millipore, Temecula, CA, USA), 4R tau (anti-4R tau, rabbit, polyclonal, 1:2000, Cosmo Bio Co., Tokyo, Japan), phosphorylated TDP-43 (pS409/410-2, rabbit, polyclonal, 1:5000, Cosmo Bio), TDP-43 (10782-2-AP, rabbit, polyclonal, 1:2000, Proteintech, Rosemont, IL, USA), FUS (HPA008784, rabbit, polyclonal, 1:200, Sigma-Aldrich, St. Louis, MO, USA), phosphorylated neurofilament (SMI31, mouse, monoclonal, 1:1000, Sternberger, Lutherville, MD, USA), N-terminus of p62 protein (p62-N, pig, 1:100, Progen Biotechnik GmbH, Heidelberg, Germany), C-terminus of p62 protein (p62-C, guinea pig, polyclonal, 1:500, Progen Biotechnik GmbH), Aβ (11-28) (12B2, mouse, monoclonal, 1:100, IBL, Fujioka, Fujioka, Japan), and phosphorylated α-synuclein (pSyn#64, mouse, monoclonal, 1:5000, Wako Co. Ltd., Osaka, Japan).

Deparaffinized sections were incubated with 1% H2O2 in methanol for 20 min to eliminate endogenous peroxidase activity in the tissue. Sections were washed in phosphate-buffered saline (PBS, pH 7.4). After blocking with 10% normal serum, sections were incubated overnight at 4°C with one of the primary antibodies in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1% Tween and 15 mM NaN3. When using AT8, p62-N, p62-C, pSyn#64, pS409/410-2, 10782-2-AP, HPA008784, and SMI31, sections were autoclaved for 10 min in 10 mM sodium citrate buffer at 121°C for antigen retrieval. When using 12B2, RD3, and 4R-tau, sections were autoclaved for 10 min in 10 mM sodium citrate buffer at 121°C and treated with 70% formic acid for 10 min. After three 10-min washes in PBS, sections were incubated in biotinylated anti-rabbit or anti-mouse or anti-pig secondary antibody for 1 h, and then in avidin-biotinylated horseradish peroxidase complex (ABC Elite kit, Vector, Burlingame, CA) for 1 h. The peroxidase labeling was visualized with diaminobenzidine-nickel as the chromogen.

Pathological diagnoses and semiquantitative assessment of granular fuzzy astrocytes (GFAs)

The Braak neurofibrillary stage [1,2], Braak pretangle stage [3], Thal Aβ phase [4], the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuritic plaque score [5], Alzheimer's Disease (AD) [6], primary age-related tauopathy (PART) [7], Lewy body disease [8], Braak Parkinson's disease stage [9], Saito argyrophilic grain disease (AGD) stage [10], progressive supranuclear palsy (PSP) [11], corticobasal degeneration (CBD) [12], TAR DNA-binding protein 43 (TDP-43) pathology histological subtype and distribution [13–17], limbic-predominant age-related TDP-43 encephalopathy (LATE) neuropathologic change stage [18], and tau-positive astrocytic lesions (tufted astrocytes, astrocytic plaques, and granular fuzzy astrocytes (GFAs)) [19] were evaluated in all

subjects using established criteria, respectively. The diagnoses of PSP [11] and CBD[12] were routinely determined except when regions of interest were not available.

Double staining by Gallyas method and AT8 immunohistochemistry

To examine whether argyrophilic grains occur in association with GFAs, double staining using the Gallyas method and AT8 immunohistochemistry was done in representative AGD cases. Sections were first stained by the Gallyas method, followed by immunostaining with AT8. The peroxidase labeling was visualized with Vector Blue Alkaline Phosphatase Substrate (Vector Laboratories, Inc.) as the chromogen. Sections were lightly counterstained with hematoxylin.

Statistical analysis

The Mann-Whitney U test was used to compare the variables between two groups. Kruskal-Wallis and Steel-Dwass tests were used when comparing the ordinal variables and continuous variables between four groups (i.e., amygdala GFA stage 0, stage 1, stage 2, and stage 3 groups). When comparing the categorical variables between the four groups, chi-square ($\chi 2$) tests and residual analyses were used. Spearman rankorder correlation analysis was applied to determine correlations between two variables. Binomial logistic regression analysis was used to assess the effects of predictor variables (age at death, Braak stage, Thal phase, frontal lobe GFA stage, caudate nucleus GFA stage, putamen GFA stage, or amygdala GFA stage) on the occurrence of AGD. The effects were described as odds ratios and 95% confidence interval (CI). A P value <0.05 was accepted as significant. Statistical analysis was performed using Bell Curve for Excel 2.15 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

References

1. Braak H, Braak E: Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 1991;82:239-259. https://doi.org/10.1007/BF00308809.

2. Braak H, Thal DR, Ghebremedhin E, et al.: Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. J Neuropathol Exp Neurol 2011;70:960-969. https://doi.org/10.1097/NEN.0b013e318232a379.

3. Braak H, Del Tredici K: The pathological process underlying Alzheimer's disease in individuals under thirty. Acta Neuropathol 2011;121:171-181. https://doi.org/10.1007/s00401-010-0789-4.

4. Thal DR, Rüb U, Orantes M, et al.: Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology 2002;58:1791-1800. https://doi.org/10.1212/wnl.58.12.1791.

5. Mirra SS, Heyman A, McKeel D, et al.: The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991;41:479-486. https://doi.org/10.1212/wnl.41.4.479.

6. Montine TJ, Phelps CH, Beach TG, et al.: National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. Acta Neuropathol 2012;123:1-11. https://doi.org/10.1007/s00401-011-0910-3.

7. Crary JF, Trojanowski JQ, Schneider JA, et al.: Primary age-related tauopathy (PART): a common pathology associated with human aging. Acta Neuropathol 2014;128:755-766. https://doi.org/10.1007/s00401-014-1349-0.

8. McKeith IG, Dickson DW, Lowe J, et al.: Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium. Neurology 2005;65:1863-1872. https://doi.org/10.1212/01.wnl.0000187889.17253.b1.

9. Braak H, Del Tredici K, Rüb U, et al.: Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 2003;24:197-211. https://doi.org/10.1016/s0197-4580(02)00065-9.

10. Saito Y, Ruberu NN, Sawabe M, et al.: Staging of argyrophilic grains: an age-associated tauopathy. J Neuropathol Exp Neurol 2004;63:911-918. https://doi.org/10.1093/jnen/63.9.911.

11. Hauw JJ, Daniel SE, Dickson D, et al.: Preliminary NINDS neuropathologic criteria for Steele-Richardson-Olszewski syndrome (progressive supranuclear palsy). Neurology 1994;44:2015-2019. https://doi.org/10.1212/wnl.44.11.2015.

12. Dickson DW, Bergeron C, Chin SS, et al.: Office of Rare Diseases neuropathologic criteria for corticobasal degeneration. J Neuropathol Exp Neurol 2002;61:935-946. https://doi.org/10.1093/jnen/61.11.935.

13. Cairns NJ, Bigio EH, Mackenzie IR, et al.: Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration. Acta Neuropathol 2007;114:5-22. https://doi.org/10.1007/s00401-007-0237-2.

14. Mackenzie IR, Neumann M, Baborie A, et al.: A harmonized classification system for FTLD-TDP pathology. Acta Neuropathol 2011;122:111-113. https://doi.org/10.1007/s00401-011-0845-8.

15. Brettschneider J, Del Tredici K, Toledo JB, et al.: Stages of pTDP-43 pathology in amyotrophic lateral sclerosis. Ann Neurol 2013;74:20-38. https://doi.org/10.1002/ana.23937.

16. Brettschneider J, Del Tredici K, Irwin DJ, et al.: Sequential distribution of pTDP-43 pathology in behavioral variant frontotemporal dementia (bvFTD). Acta Neuropathol 2014;127:423-439. https://doi.org/10.1007/s00401-013-1238-y.

17. Lee EB, Porta S, Michael Baer G, et al: Expansion of the classification of FTLD-TDP: distinct pathology associated with rapidly progressive frontotemporal degeneration. Acta Neuropathol 2017;134:65-78. https://doi.org/10.1007/s00401-017-1679-9.

18. Nelson PT, Dickson DW, Trojanowski JQ, et al.: Limbic-predominant age-related TDP-43 encephalopathy (LATE): consensus working group report. Brain 2019;142:1503-1527. https://doi.org/10.1093/brain/awz099.

19. Kovacs GG, Ferrer I, Grinberg LT, et al.: Aging-related tau astrogliopathy (ARTAG): harmonized evaluation strategy. Acta Neuropathol 2016;131:87-102. https://doi.org/10.1007/s00401-015-1509-x.

Supplementary file 2

Table 2. Pathological features in our case series after stratification by amygdala GFAs and AGD

		Status of amygdala GFAs and AGD			
		Amygdala GFA-/AGD-	Amygdala GFA+/AGD-	Amygdala GFA+/AGD+	*P* value[a]
N [female (%)]		127 (39.3)	72 (29.0)	40 (44.7)	0.1844[b]
Age at death (y, mean ± SD)		65.2±14.8	72.7±9.8	78.6±8.6	<0.001[c]
Brain weight (g, mean ± SD)		1,187.5±227.3	1,223.0±174.7	1,168.5±165.1	0.3855[d]
Braak NFT stage					
Median (25-75th percentiles)		2 (1-5)	2 (2-3)	2 (2-4)	0.1186[d]
Thal phase					
Median (25-75th percentiles)		1 (0-4)	1 (0-3)	1 (0-3)	0.7960[d]
LBD					
Diffuse neocortical type [N (%)]		9 (7.1)	7 (9.7)	2 (5.0)	
Limbic type [N (%)]		9 (7.1)	3 (4.2)	4 (10.0)	
Brain stem-predominant type [N (%)]		3 (2.4)	9 (12.5)	2 (5.0)	
Amygdala-predominant type [N (%)]		6 (4.7)	0 (0.0)	0 (0.0)	
Olfactory Lewy bodies only [N (%)]		0 (0.0)	1 (1.4)	0 (0.0)	
All LBD cases [N (%)]		27 (21.3)	20 (27.8)	8 (20.0)	0.0510[e]
TDP-43 proteinopathy					
LATE-NC stage 3 [N (%)]		0 (0.0)	0 (0.0)	0 (0.0)	
LATE-NC stage 2 [N (%)]		14 (11.0)	5 (6.9)	3 (7.7)	
LATE-NC stage 1 [N (%)]		4 (3.1)	3 (4.2)	3 (7.7)	
All LATE-NC cases [N (%)]		18 (14.2)	8 (11.1)	6 (15.4)	0.7870[e]
ALS-TDP cases [N (%)]		23 (18.1)	11 (15.3)	6 (15.4)	0.8317[e]
FTLD-TDP cases [N (%)]		4 (3.1)	2 (2.8)	0 (0.0)	0.5318[e]
GFA (N, GFA stage)					
Frontal cortex	[N (%)]	6 (4.7)	18 (25.0)	22 (55.5)	
	[Median (25-75th percentiles)]	0 (0-0)	0 (0-0.5)	1 (0-1)	<0.001[f]
Caudate nucleus	[N (%)]	10 (7.9)	12 (16.7)	19 (47.5)	
	[Median (25-75th percentiles)]	0 (0-0)	0 (0-0)	0 (0-1)	<0.001[g]
Putamen	[N (%)]	18 (14.2)	11 (15.3)	22 (55.0)	
	[Median (25-75th percentiles)]	0 (0-0)	0 (0-0)	1 (0-1)	<0.001[h]
Amygdala	[N (%)]	0 (0.0)	72 (100.0)	40 (100.0)	
	[Median (25-75th percentiles)]	0 (0-0)	1 (1-1)	2 (1-3)	<0.0001[i]

GFA: granular fuzzy astrocyte, AGD: argyrophilic grain disease, N: number of cases, NFT: neurofibrillary tangles, LBD: Lewy body disease, LATE-NC: Limbic-predominant age-related TDP-43 encephalopathy neuropathological change, ALS-TDP: amyotrophic lateral sclerosis with TDP-43-positive inclusions, FTLD-TDP: frontotemporal lobar degeneration with TDP-43-positive inclusions. a) All data except for the amygdala GFA data were compared between the three groups. The amygdala GFA stage was compared between amygdala GFA+/AGD- and amygdala GFA+/AGD+ groups. b) The sex ratio was not significantly different between the three groups (chi-square (χ2) test). c) The age

at death in the amygdala GFA-/AGD- group was significantly lower than those in amygdala GFA+/AGD- and amygdala GFA+/AGD groups ($p<0.001$, respectively), and the age at death in the amygdala GFA+/AGD- group was significantly lower than that in the amygdala GFA+/AGD+ group ($p=0.0035$) (Kruskal-Wallis and Steel-Dwass tests). d) The brain weight, Braak stage, and Thal phase did not significantly differ between the three groups, respectively (Kruskal-Wallis test). e) The proportions of all LBD, all LATE, ALS-TDP, and FTLD-TDP cases did not significantly differ between the three groups ($\chi 2$ test). f) The frontal lobe GFA stage in the amygdala GFA+/AGD+ group was significantly higher than those in the amygdala GFA+/AGD- and amygdala GFA-/AGD- groups ($p=0.0030$ and <0.0001, respectively, Kruskal-Wallis and Steel-Dwass tests), and the frontal lobe GFA stage in an amygdala GFA+/AGD- group was significantly higher than that in the amygdala GFA-/AGD- group ($p<0.0001$, respectively, Kruskal-Wallis and Steel-Dwass tests). g) The caudate nucleus GFA stage in the amygdala GFA+/AGD+ group was significantly higher than those in the amygdala GFA+/AGD- and amygdala GFA-/AGD- groups ($p=0.0016$ and <0.001, respectively, Kruskal-Wallis and Steel-Dwass tests). h) The putamen GFA stage in the amygdala GFA+/AGD+ group was significantly higher than those in the other two groups ($p<0.0001$, respectively, Kruskal-Wallis and Steel-Dwass tests). i) The amygdala GFA stage in the amygdala GFA+/AGD+ group was significantly higher than that in the amygdala GFA+/AGD- group ($p<0.0001$, Mann-Whitney U test).