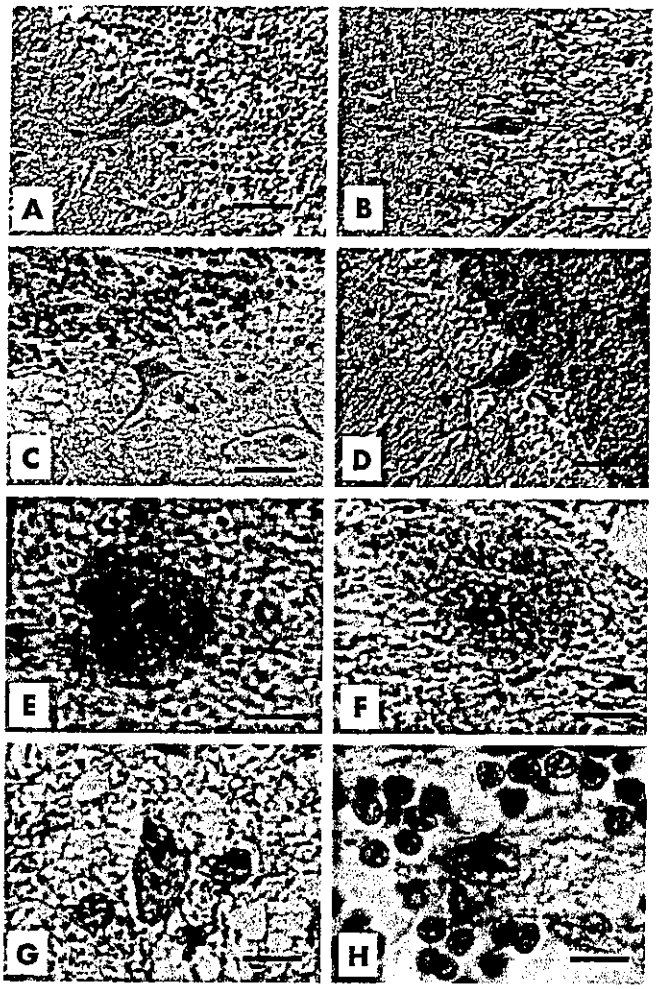

Figure 1 Ubiquitin-like immunoreactivity (A–G) in cerebellar cortex of SCA1 (A), SCA2 (B), SCA3 (C), and DRPLA (D). Neither aggregates nor inclusions were present in nuclei of Purkinje cells in any of cases examined (bars=100 μm; A–D: the same magnification). NIs were occasionally found in dentate neurons in SCA3 (E), DRPLA (F) and rarely found in Golgi cells, as seen in SCA1 (G). These NIs were also immuostained with the antibody against expanded polyglutamine (1C2), as seen in Golgi cells in SCA1 (H) (bars=10 μm; E–H: the same magnification).

Table 1 Number of Pukinje cells in hereditary ataxias

	Cases (n)	Range	Average
SCA1	3	310–600	420
SCA2	3	140–160	150
SCA3	5	1290–3720	2320
DRPLA	4	1600–4560	3130

NIs in any of the remaining Pj cells irrespective of the disease examined.

DISCUSSION

Cerebellar ataxia is one of the important clinical features of SCA1, SCA2, SCA3, and DRPLA, all exhibit cerebellar degeneration of different degree. Cerebellar cortex is severely affected in SCA2 and SCA1, while relative preservation of Pj cells is a morphological feature of SCA3 and DRPLA.[8] Different reports, including ours, confirmed the absence of NIs in Pj cells of SCA2[9 10] and DRPLA[11] brains. In this study, we tried to expand this observation by comparing a variety of cases with different degree of cerebellar degeneration associated in common with pathological expansion of CAG repeat. Although several animal models were successfully generated to reproduce NI formation and neurodegeneration in the cerebellar cortex,[12] our in vivo observation based on a series of human necropsied cases, which includes a variety of hereditary ataxias, failed to identify NIs in any of Pj cells. Because some NIs were identified in dentate neurones or Golgi cells on the same ubiquitin immunolabelled preparations, the absence of NIs in Pj cells was not attributable to a technical failure. Furthermore, we confirmed the absence of NIs in Pj cells by immunohistochemical examination with 1C2, which labels the pathological moiety of abnormal gene product found in NIs.

At least two conflicting interpretations are possible to explain this consistent absence of NIs in Pj cells. Pj cells containing NIs may be more susceptible to neuronal degeneration and degenerate rapidly, leaving only those not containing NIs. If Pj cells develop NIs before degeneration, however, some NIs should have appeared at least once during the degenerative process. The consistent absence of NIs in Pj cells with various severity of degeneration linked to different genetic abnormalities, therefore, is against this hypothesis. On the other hand, accumulating evidence claimed that neurodegeneration and NI formation were not necessarily correlated[13] or even inversely correlated[7] with each other, consistent with a possible protective role of NIs against neuronal degeneration. Indeed, inactivation of ubiquitin-proteasome cascade was reported to accelerate cell death and attenuate formation of NIs induced in cultured cell lines transfected with expanded CAG tagged to ataxin-1.[6] Inversely, inactivation of caspases, which counteracted cell death, was reported to promote NI formation.[7] This inverse relation between NI formation and cell death has been verified also in necropsied brains with Huntington's disease[4] or with NIHID,[5] which exhibit similar ubiquitin-immunopositive inclusions. It supposes, therefore, an intrinsic cellular mechanism that links cell death and NI formation in opposite directions, although both of them are triggered by the expansion of CAG/polyglutamine repeat. As shown in this study, Pj cells without NIs could, therefore, be more vulnerable if NI formation is linked to a mechanism counteracting a cascade that accelerates neuronal death.

It remains to be proved, however, why Pj cells hardly develop NIs in human brains. Because targeted expression of a mutated gene (for example, ataxin-1) to Pj cells induces NIs in murine brains,[14] mechanisms involved after the expression of the mutated gene may be different in human Pj cells. Otherwise, smaller repeat size in the cerebellum, a common phenomenon in several CAG/polyglutamine repeat expansion disorders known as "somatic mosaicism", may locally influence the phenotype by modifying cell death and NI formation. Recent reports demonstrated, however, that transcribed repeat size in Pj cells of DRPLA brains was longer than that in cerebellar granule cells,[15 16] suggesting that the absence of NI in Pj cells is not related to shorter repeat size. Anyway, this study demonstrated that Pj cells, more or less affected in these hereditary ataxias, were uniformly characterised by the paradoxical absence of NIs in necropsied human brains with different CAG/polyglutamine expansion disorders with ataxia. If NI formation is linked to a protective mechanism against cell death, its complete absence renders Pj cells more susceptible to cell death. Further studies are required to clarify cellular and molecular mechanism to explain these particular characteristics of human Pj cells seen commonly in these hereditary ataxias. It may provide clues to understanding a common mechanistic link, which involves cell death and NI formation both triggered by CAG repeat expansion.

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Addendum

A recent study on knocked-in mice with SCA1 gene flanking expanded CAG repeat demonstrated an extreme scarcity of intranuclear inclusions in degenerated cerebellar Purkinje cells. This corroborated our findings on human necropsied brains (*Neuron* 2002;**34**:905–19).

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REFERENCES

1 **Zoghbi HY**, Orr HT. Glutamine repeats and neurodegeneration. *Annu Rev Neurosci* 2000;**23**:217–47.
2 **Holmes SE**, O'Hearn E, McInnis MG, *et al*. Expansion of a novel CAG trinucleotide repeat in the 5′ region of PPP2R2B is associated with SCA12. *Nat Genet* 1999;**23**:391–2.
3 **Nakamura K**, Jeong S-Y, Uchihara T, *et al*. SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. *Hum Mol Genet* 2001;**10**:1441–8.
4 **Kuemmerle S**, Gutekunst C-A, Klein AM, *et al*. Huntingtin aggregates may not predict neuronal death in Huntington's disease. *Ann Neurol* 1999;**46**:842–9.
5 **Takahashi J**, Tanaka J, Arai K, *et al*. Recruitment of nonexpanded polyglutamine proteins to intranuclear aggregates in neuronal intranuclear hyaline inclusion disease. *J Neuropathol Exp Neurol* 2001;**60**:369–76.
6 **Cummings CJ**, Reinstein E, Sun Y, *et al*. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Cell* 1999;**24**:879–92.
7 **Saudou F**, Finkbeiner S, Devys D, *et al*. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 1998;**95**:55–66.
8 **Iwabuchi K**, Tsuchiya K, Uchihara T, *et al*. Autosomal dominant spinocerebellar degenerations. Clinical, pathological and genetic correlations. *Rev Neurol (Paris)* 1999;**155**:255–70.
9 **Huynh DP**, DelBigio MR, Ho DH, *et al*. Expression of ataxins-2 in brains from normal individuals and patients with Alzheimer' s disease and spinocerebellar ataxia 2. *Ann Neurol* 1999;**45**:232–41.
10 **Koyano S**, Uchihara T, Fujigasaki H, *et al*. Neuronal intranuclear inclusions in spinocerebellar ataxia type 2: triple-labeling immunofluorescent study. *Neurosci Lett* 1999;**273**:117–20.
11 **Hayashi Y**, Kakita A, Yamada M, *et al*. Hereditary dentatorubral-pallidoluysian atrophy : detection of widespread ubiquitinated neuronal and glial intranuclear inclusions in the brain. *Acta Neuropathol* 1998;**96**:547–52.
12 **Skinner PJ**, Koshy BT, Cummings CJ, *et al*. Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures. *Nature* 1997;**389**:971–4.
13 **Huynh DP**, Figueroa K, Hoang N, *et al*. Nuclear localization or inclusion body formation of ataxin-2 are not necessary for SCA2 pathogenesis in mouse or human. *Nat Genet* 2000;**26**:44–50.
14 **Klement IA**, Skinner PJ, Kaytor MD, *et al*. Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell* 1998;**95**:41–53.
15 **Hashida H**, Goto J, Suzuki T, *et al*. Single cell analysis of CAG repeat in brains of dentatorubral-pallidoluysian atrophy. *J Neurol Sci* 2001;**190**:87–93.
16 **Watanabe H**, Tanaka F, Doyu M, *et al*. Differential somatic CAG repeat instability in variable brain cell lineage in dentatorubral pallidoluysian atrophy (DRPLA): a laser-captured microdissection (LCM)-based analysis. *Hum Gen* 2000;**107**:452–7.

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REGULAR PAPER

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Constant involvement of the Betz cells and pyramidal tract in amyotrophic lateral sclerosis with dementia: a clinicopathological study of eight autopsy cases

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Abstract We investigated clinicopathologically pyramidal signs, including hyperreflexia, Babinski sign, and spasticity, and the involvement of the primary motor cortex and pyramidal tract, in eight Japanese autopsy cases of amyotrophic lateral sclerosis (ALS) with dementia. Pyramidal signs were observed in seven (88%) of the eight autopsy cases. Hyperreflexia and Babinski sign were evident in seven (88%) and three (38%) patients, respectively, but spasticity was not observed in any of the eight patients. Loss of Betz cells in the primary motor cortex was evident in the seven cases in which this structure was examined. Astrocytosis in the fifth layer of the primary motor cortex was noticed in three cases. In all eight cases, involvement of the pyramidal tract was obvious in the medulla oblongata, but no involvement of the pyramidal tract was found in the midbrain. Involvement of the pyramidal tract in the spinal cord, particularly of large myelinated fibers, was observed in all six cases in which the spinal cord was examined. In ALS with dementia, pyramidal signs were shown to be present more frequently than previously believed, and the clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract was obvious. Constant involvement of Betz cells and the pyramidal tract in ALS with dementia has not been reported. Our clinicopathological findings may make a contribution to the understanding of the clinicopathological hallmarks of this disorder. Furthermore, we believe that this study will also contribute to the elucidation of the nosological status of ALS with dementia.

Keywords Amyotrophic lateral sclerosis · Betz cells · Dementia · Pyramidal signs · Pyramidal tract degeneration

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder, clinically characterized by upper and lower motor neuron signs, with degeneration of the upper and lower motor neurons of the brain and spinal cord [41, 42]. The familial form of the disease accounts for about 5–10% of patients with ALS and is usually transmitted by an autosomal dominant trait [38, 39, 40,

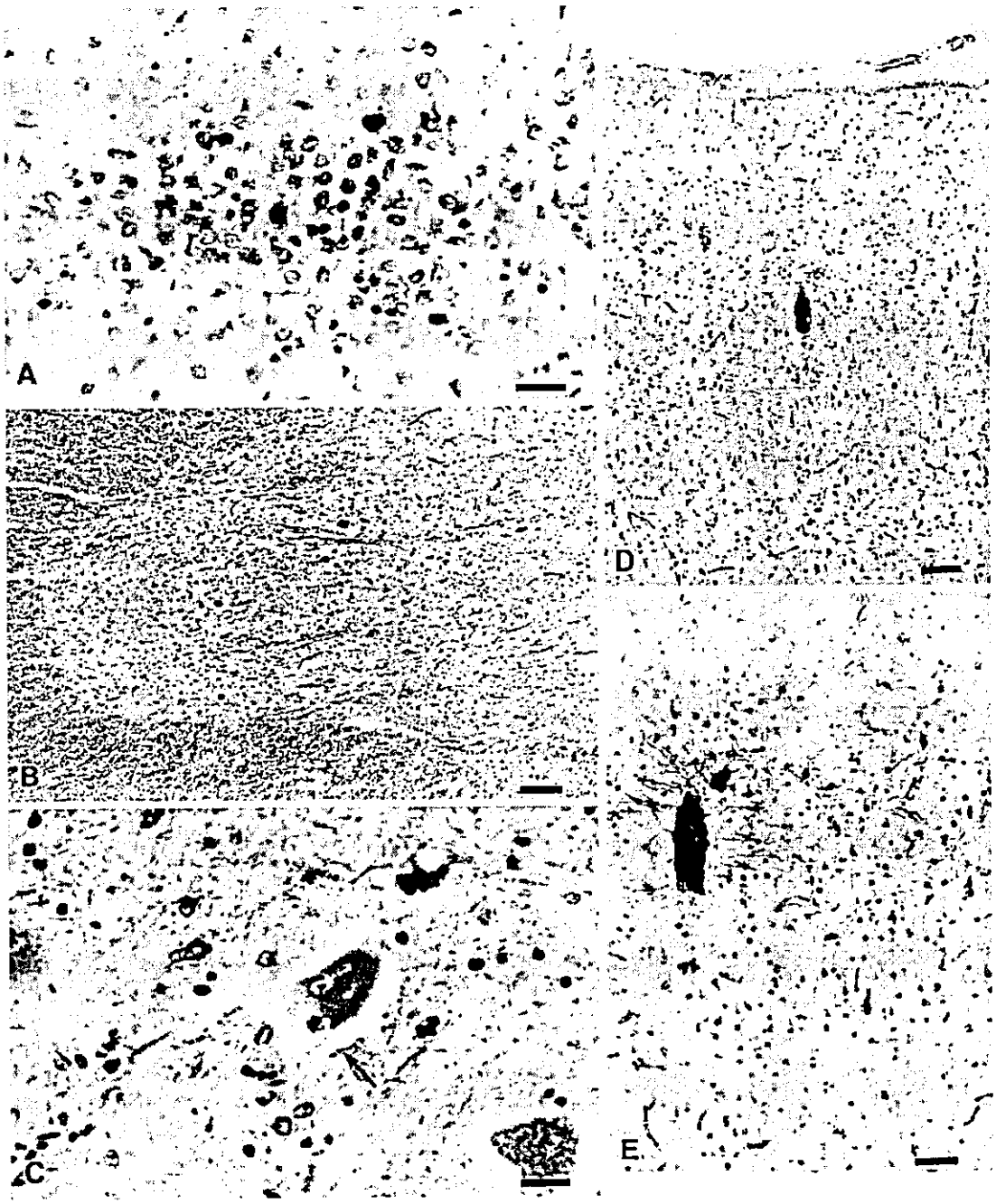

Fig. 1A–I Case 2. **A** Ubiquitin-immunoreactive intraneuronal inclusions in the hippocampal dentate granular cells. **B** Right anterior horn of the lower cervical cord showing prominent neuronal loss; Klüver-Barrera stain. **C** Bunina body (*arrow*) of the lumbar cord; H&E stain. **D, E** Left parahippocampal gyrus showing obvious neuronal loss with gliosis; **D** H&E stain, **E** Holzer stain. **F, G** Left amygdala showing overt neuronal loss with gliosis; **F** H&E stain, **G** Holzer stain. **H, I** Left substantia nigra showing obvious neuronal loss with gliosis; **H** Klüver-Barrera stain, **I** Holzer stain. *Bars* **A** 0.03 mm; **B, D** 0.1 mm; **C** 0.02 mm, **E–I** 0.05 mm

45, 46]. An association between dementia and ALS was first described by Meyer in 1929 [18], but this has since become increasingly recognized [9]. In Japan, a clinical case of ALS with dementia (ALS+D) was first noticed by Yuasa in 1964 [53], and thereafter, as autopsy cases of ALS+D accumulated [23, 37, 54], Mitsuyama and Mitsuyama et al. [20, 21, 22] proposed ALS+D as a new clinicopathological entity. Recently, motor neuron disease-inclusion dementia (MNDID) has been shown to be part of a subgroup of dementia of frontal lobe type [10, 11, 47]. MNDID, as proposed by Jackson et al. in 1996 [11], is a variant of frontotemporal dementia characterized not only clinically by progressive dementia without clinical evidence of ALS, but also pathologically by variable macroscopic atrophy and neuronal loss in the frontotemporal lobes with intraneuronal ubiquitin-immunoreactive inclusions in the hippocampal dentate granular cells and residual neurons in layer II of the frontotemporal cortex with-

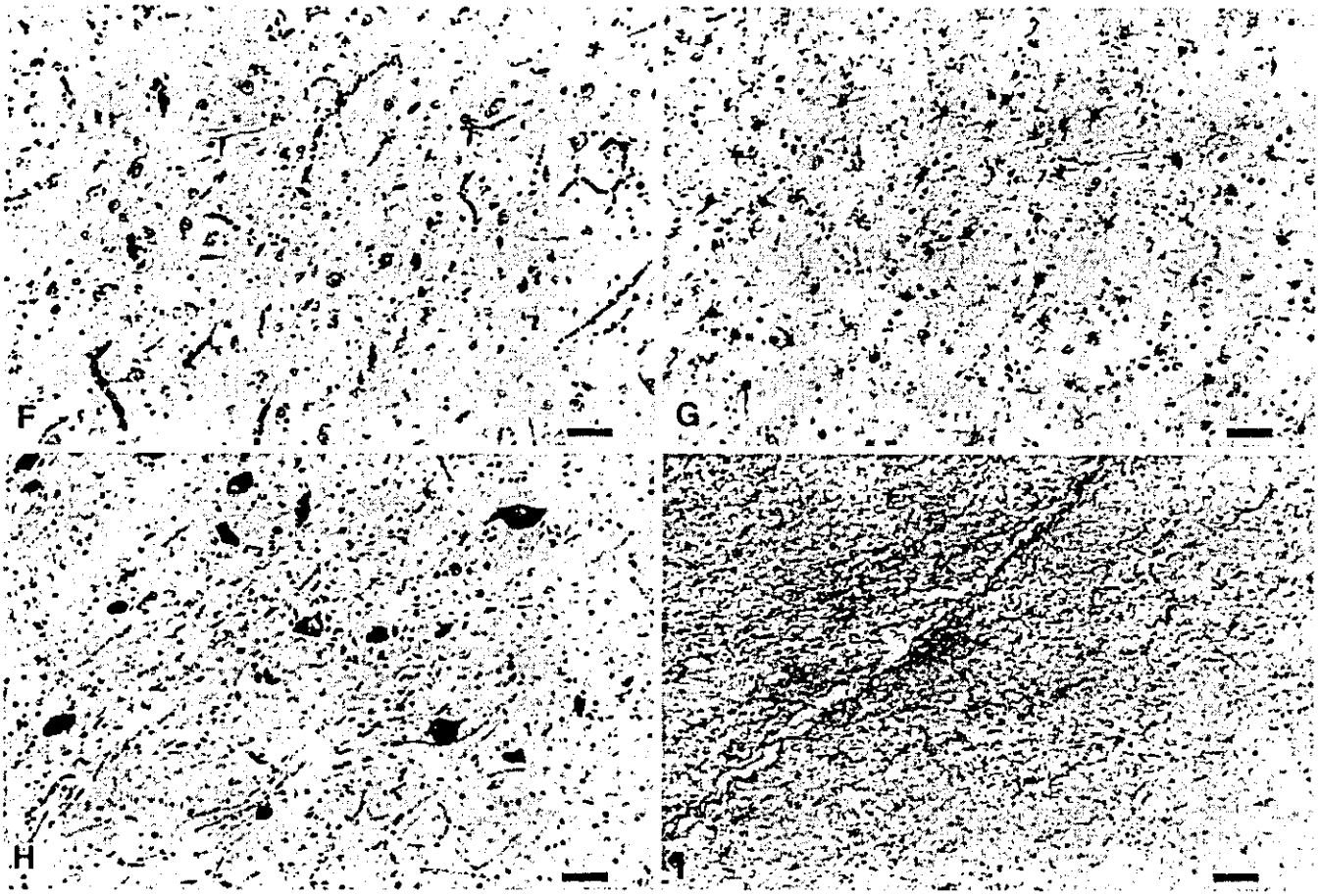

Fig. 1F–I

out pathological evidence of motor system degeneration. Furthermore, in MNDID, it is known that ubiquitin-immunoreactive dystrophic neurites are prominent in areas of cortical microvacuolation [10]. Whether or not ALS+D [2, 3, 4, 7] or motor neuron disease-type dementia (MNDD) [16, 28, 29, 48] is a rare variant of ALS, making up a few percent of ALS cases [50], and/or a subgroup of frontotemporal group, including MNDID, remains to be elucidated.

It is generally believed that pyramidal signs, including hyperreflexia, Babinski sign, and spasticity, are rarely observed in case of ALS+D [20, 21, 22]. Furthermore, it has been reported that in ALS+D, loss of Betz cells of the primary motor cortex is not usually noted, and that despite the loss of anterior horn cells, degeneration of the pyramidal tract within the spinal cord and brain stem is rarely seen in patients with ALS+D [28, 29], except for a few unusual cases of ALS+D having prominent degeneration of the pyramidal tract [47].

The purpose of this report is to describe the clinicopathological features of ALS+D in eight Japanese autopsy cases, including pyramidal signs and involvement of the pyramidal tract and primary motor cortex, and to examine the clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract. We also address in the discussion the nosological status of ALS+D, and whether this disorder belongs to a variant of ALS or a new disorder.

Materials and methods

The present study was carried out on eight Japanese autopsy cases of ALS+D from a Japanese Institution. The clinical records and tissue specimens in all eight cases are stored at the Department of Neuropathology, Tokyo Institute of Psychiatry.

After fixation in formalin, the brains of the eight cases were sectioned in the coronal plane. Cerebral hemisphere blocks, including the frontal, temporal, parietal, and occipital lobes, and the striatum, pallidum, subthalamic nucleus, thalamus, amygdala, and hippocampus, were taken. Additional tissue blocks were taken from the midbrain, including the substantia nigra, brain stem, and cerebellum. The spinal cord was examined in six of eight cases. The brains and spinal cords were embedded in paraffin and cut at a thickness of about 10 μm. The sections were stained with hematoxylin-eosin (H&E), and by the Klüver-Barrera, Holzer, Bodian, methenamine silver, and modified Gallyas-Braak methods. Immunocytochemistry was also performed using antibodies against monoclonal ubiquitin (from Dr. H. Mori; Osaka City University), and glial fibrillary acidic protein (GFAP).

The neuropathological diagnosis of ALS+D in the eight cases was made on the basis of the findings described below, which included not only presence of ubiquitin-immunoreactive intraneuronal inclusions [33, 34, 35, 51] in the hippocampal dentate granular cells, frontotemporal cortical layer II neurons, and motor neurons of the brain stem and spinal cord, but also neuronal loss with astrocytosis in the dorsomedial portion of the anterior first temporal gyrus, subiculum, parahippocampal gyrus, amygdala, and sub-

stantia nigra [25, 26, 27], in addition to the degeneration of the upper and lower motor neuron consistent with the pathology of ALS (Fig. 1).

The clinicopathological findings for some of these cases have been reported previously (case 1 [43], cases 5 and 6 [19], cases 6 and 7 [30, 31], and case 8 [49]). Previously, we reported cases 6 (case 2 [30, 31]) and 7 (case 1 [30, 31]) as „Pick's disease with amyotrophic lateral sclerosis" because of the prominent lobar atrophy of the temporal lobes found in both cases, in spite of the presence of ubiquitin-immunoreactive inclusions of the hippocampal dentate granular cells in case 7. Jellinger [12] mentioned that our cases 6 and 7 might be frontotemporal dementia with motor neuron disease, and ALS+D, respectively. Since then, we have encountered not only an autopsy case of ALS+D with circumscribed lobar atrophy that clinically showed rapidly progressive aphasia [43], but also an atypical autopsy case of ALS+D mimicking frontal Pick's disease with a clinical course of 15 years [47]. Therefore, we reinvestigated cases 6 and 7 pathologically, also using immunohistochemisty. The clinical and pathological features of all cases are summarized in Tables 1 and 2.

Pyramidal signs were judged to be present in patients who showed one or more signs of hyperreflexia in the extremities, Babinski sign, and spasticity in the extremities. Pyramidal tract degeneration was also judged as present in cases showing definite loss of myelinated fibers determined by myelin staining, including Klüver-Barrera stain, Holzer stain, and immunohistochemistry using an antibody against GFAP (Fig. 2). Loss of Betz cells was judged to be present in cases that showed small grouping of lipofuscin-laden macrophages in the holes, from which Betz cells had presumably disappeared, in the primary motor cortex showing the presence of normal and degenerated Betz cells in the absence of internal granular layer (Fig. 3). Astrocytosis of primary motor area layer V was considered present in cases showing definite astrocytosis determined by Holzer staining or immunohistochemistry using an antibody against GFAP. The determination of loss of Betz cells, astrocytosis of primary motor cortex layer V, and pyramidal tract degeneration described above was fundamentally consistent with that in multiple system atrophy (MSA), as described by Tsuchiya et al. [44]. The pertinent data are summarized in Table 3.

Case reports

Clinical course and neuropathological findings in case 2

The patient was in good health until the age of 77, when she became aware of weakness in the right hand, followed by dysarthria 4 months later and memory disturbance 6 months after the disease onset. A neurological examination when the patient was 78 years old, about 1 year 2 months after the onset of the disease, revealed dementia, dysphagia, fasciculation and atrophy of the tongue, weakness and atrophy of the upper and lower extremities, prominently in the distal parts of the upper extremities, and hyperreflexia in the lower extremities. Neurological examination, about 1 year 3 months after disease onset, showed bilateral Babinski signs without spasticity. She developed respiratory difficulty 1 year 10 months after the disease onset. She died of suffocation at age 79, about 2 years after the onset of the disease. No respiratory support was given throughout the clinical course. She was clinically diagnosed as having ALS+D.

The weight of the brain was 1,125 g. Macroscopic examination revealed atrophy of the frontotemporal lobes, with accentuation of the anterior portion, bilateral parahippocampal gyri, and bilateral amygdalae, with depigmentation of the substantia nigra and atrophy of the anterior horns and anterior roots of the cervical cord. A histological examination showed neuronal loss with astrocytosis in the frontotemporal lobes, parahippocampal gyrus, subiculum, amygdala, substantia nigra, right trigeminal motor nucleus, hypoglossal nucleus, and anterior horns of the spinal cord (Fig. 1b, d–i). Ubiquitin-immunoreactive intraneuronal inclusions were found in the hippocampal dentate granular cells, frontotemporal cortical layer II neurons, hypoglossal nucleus, and anterior horns of the spinal cord (Fig. 1a). Bunina bodies were also evident in the right trigeminal motor nucleus, hypoglossal nucleus, and anterior horns of the spinal cords (Fig. 1c). Degeneration of the pyramidal tract was evident in the spinal cord and medulla oblongata, but was not obvious in the midbrain (Fig. 2). No senile plaques were seen with methenamine silver staining. A moderate number of neurofibrillary changes, including neurofibrillary tangles and neuropil threads, were found in the hippocampal CA1 and parahippocampal gyrus, compatible with stage III of Braak's classification [6], using Gallyas-Braak staining.

Clinical course and neuropathological findings in case 3

The patient was well until the age of 39, when she lost interest in household affairs and became antisocial, followed by apathy 5 months later. Abnormal behavior and dysarthria appeared about 7 months after the disease onset, followed by emotional lability and character changes 2 months later. Aspontaneity became apparent 1 year 3 months after the onset of the disease. Dysphagia became obvious 1 year 7 months after the disease onset. A neurological examination when the patient was 41 years old, about 2 years after the disease onset, showed atrophy and fasciculation of the tongue, weakness of the upper and lower extremities, prominently in the upper extremities, and hyperreflexia in the lower extremities. Tube feeding was started 2 years 4 months after the onset of the disease. She developed a respiratory disorder about 4 months before her death. She died of the respiratory disorder at age 42, about 3 years 2 months after the disease onset. No respiratory support was given throughout the clinical course. She was clinically diagnosed as having ALS+D.

The weight of the brain was 1,200 g. Macroscopic examination showed slight atrophy of the frontotemporal lobes. A histological examination revealed neuronal loss with astrocytosis in the frontotemporal lobes, including the dorsomedial portion of the anterior first temporal gyrus, the subiculum, parahippocampal gyrus, amygdala, caudate nucleus, thalamus, substantia nigra, facial nucleus, and hypoglossal nucleus. Ubiquitin-immunoreactive intraneuronal inclusions were found in the hippocampal dentate granular cells, frontotemporal cortical layer II neurons, and facial nucleus. Bunina bodies were also evident in the facial nucleus and hypoglossal nucleus. Degeneration of the pyramidal tract was evident in the medulla oblongata, but was not obvious in the midbrain. No senile plaques were seen using methenamine silver staining. A few neurofibrillary changes were found in the parahippocampal gyrus, without neurofibrillary changes in the hippocampal CA1, compatible with stage I of Braak's classification [6], using Gallyas-Braak staining.

Clinical course and neuropathological findings in case 4

The patient was in good health until the age of 72, when she became aware of weakness in the distal part of the upper extremities. Memory disturbance appeared about 8 months after the disease onset. A neurological examination when the patient was 73 years old, about 11 months after the onset of the disease, revealed dementia, dysarthria, dysphagia, atrophy and fasciculation of the tongue, muscle atrophy of the upper extremities, fasciculation in the upper and lower extremities, hyperreflexia in the lower extremities, and absence of Babinski sign. She died of respiratory difficulty at age 73, about 1.5 years after the onset of the disease. No respiratory support was given throughout the clinical course. She was clinically diagnosed as having ALS+D.

Brain weight was 1,202 g. Macroscopic examination revealed slight atrophy of the frontal lobe and anterior parahippocampal gyrus, with depigmentation of the substantia nigra. A histological examination showed neuronal loss with astrocytosis in the frontotemporal lobes, including the dorsomedial portion of the anterior first temporal gyrus, the subiculum, parahippocampal gyrus, amygdala, caudate nucleus, substantia nigra, facial nucleus, hypoglossal nucleus, and anterior horns of the spinal cord. Ubiquitin-

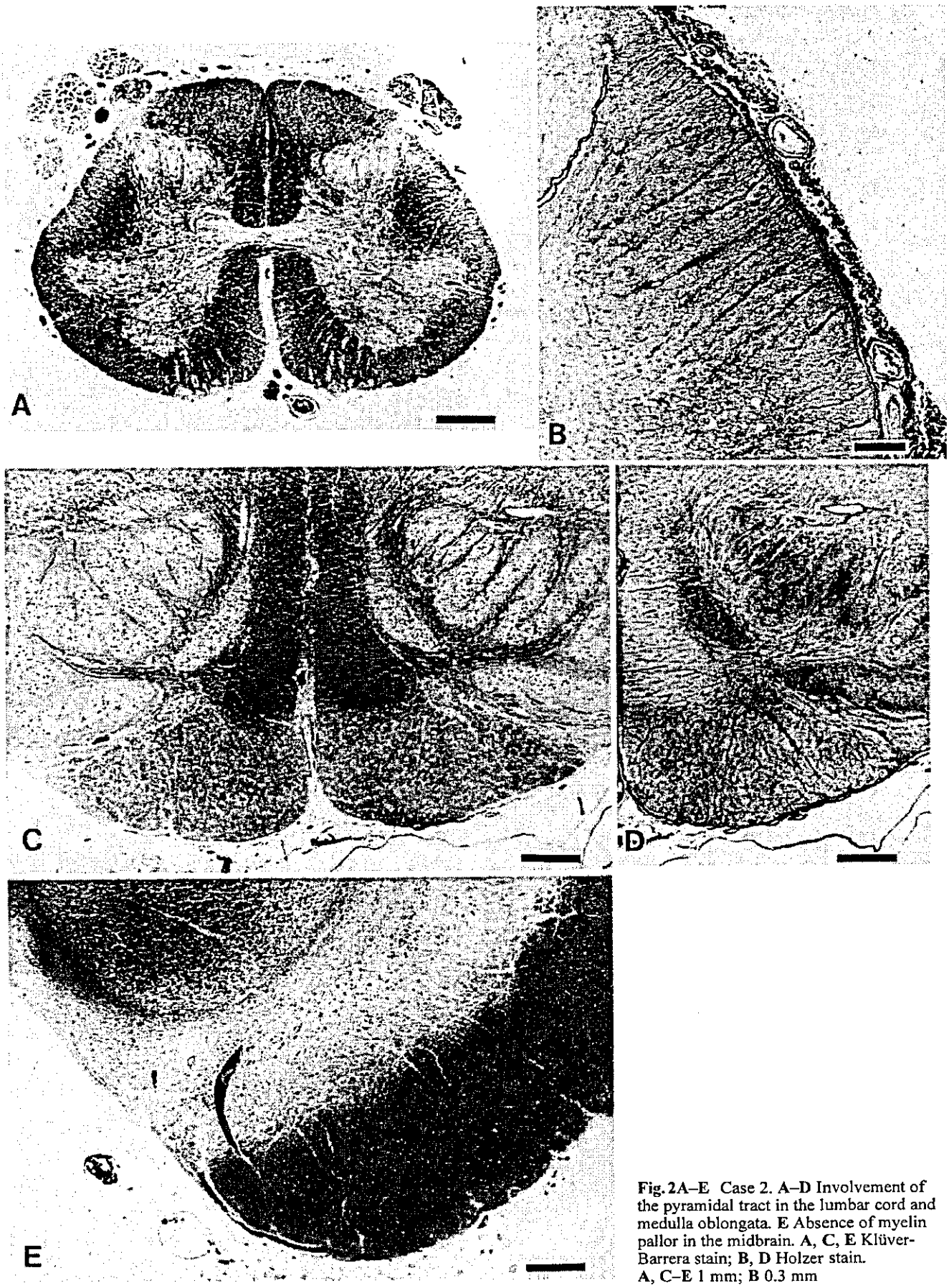

Fig. 2A–E Case 2. **A–D** Involvement of the pyramidal tract in the lumbar cord and medulla oblongata. **E** Absence of myelin pallor in the midbrain. **A, C, E** Klüver-Barrera stain; **B, D** Holzer stain. **A, C–E** 1 mm; **B** 0.3 mm

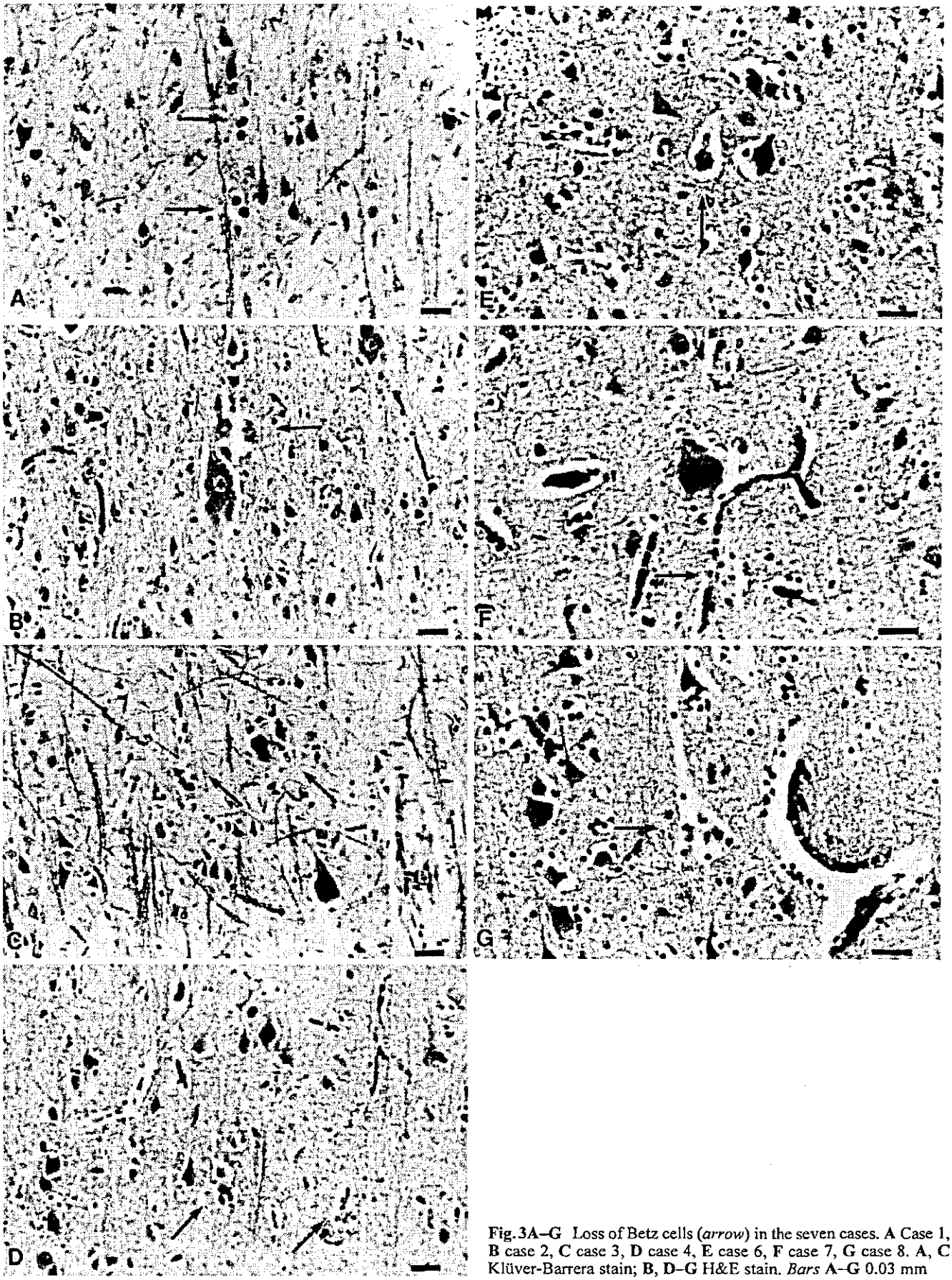

Fig. 3A–G Loss of Betz cells (*arrow*) in the seven cases. **A** Case 1, **B** case 2, **C** case 3, **D** case 4, **E** case 6, **F** case 7, **G** case 8. **A**, **C** Klüver-Barrera stain; **B**, **D**–**G** H&E stain. *Bars* **A**–**G** 0.03 mm

immunoreactive intraneuronal inclusions were evident in the hippocampal dentate granular cells, frontotemporal cortical layer II neurons, amygdala, facial nucleus, hypoglossal nucleus, and anterior horns of the spinal cord. Bunina bodies were also obvious in the facial nucleus, hypoglossal nucleus, and anterior horns of the spinal cord. Degeneration of the pyramidal tract was evident in the spinal cord and medulla oblongata, but was not obvious in the midbrain. No senile plaques were seen using methenamine silver staining. A few neurofibrillary changes were found in the hippocampal CA1 and amygdala, with a small quantity of neurofibrillary changes in the parahippocampal gyrus, compatible with stage II of Braak's classification [6], using Gallyas-Braak staining.

Results

Clinical features

The main clinical information on the eight patients (one male, seven female) is summarized in Table 1. The patients had no hereditary burden. The age at onset of symptoms was from the fourth to eighth decade of life, with an average of about 61 years 5 months. The duration of the disease ranged from 10 months in case 1 to 3 years 2 months in case 3; the mean duration was 1 year 11 months. Four patients presented with psychiatric signs, including abnormal behavior and indifference, or dementia as the initial sign. Three patients initially developed lower motor neuron signs, including dysarthria, dysphagia, and weakness of the upper extremities. Dementia and slight memory disturbance were observed in all eight cases. Lower motor neuron signs were found in seven cases, but not in case 8. Five patients were diagnosed as having ALS+D, one aphasia + ALS, one ALS, and one frontal lobe dementia.

Pathological features

The neuropathological data are summarized in Table 2. Brain weights at autopsy ranged from 1,070 to 1,365 g (average 1,221.5 g). Ubiquitin-immunoreactive intraneuronal inclusions were evident in the hippocampal dentate granular cells, frontotemporal cortical layer II neurons, and motor neurons in the brain stem and/or spinal cord in all eight cases. Neuronal loss with astrocytosis and presence of Bunina bodies was obvious in the motor neurons

Table 1 Summary of clinical features (+ present, – absent, *ALS* amyotrophic lateral sclerosis, *D* dementia, *y* years, *m* months)

Case	1	2	3	4	5	6	7	8
Sex	F	F	F	F	F	M	F	F
Heredity	–	–	–	–	–	–	–	–
Age at onset (years)	74	77	39	72	56	49	65	59
Duration of the disease	10 m	2 y	3 y 2 m	1 y 6 m	2 y 3 m	2 y 10 m	2 y	1 y
Initial sign	Aphasia, dysphagia	Weakness of the right hand	Indifference	Weakness of the distal part of the upper extremities	Dementia	Dementia	Dysarthria, dysphagia	Abnormal behavior
Dementia	+	+	+	+	+	+	Slight memory disturbance	+
Lower motor neuron sign	+	+	+	+	+	+	+	–
Clinical diagnosis	Aphasia + ALS	ALS+D	ALS+D	ALS+D	ALS+D	ALS+D	ALS	Frontal lobe dementia

Table 2 Summary of pathological features (+ present, – absent, *N.E.* not examined)

Case	1	2	3	4	5	6	7	8
Brain weight (g)	1,070	1,125	1,200	1,202	1,210	1,260	1,340	1,365
Ubiquitin-immunoreactive intraneuronal inclusions in the hippocampal dentate granular cells, frontotemporal cortical layer II neurons, and motor neuron in the brain stem and/or spinal cord	+	+	+	+	+	+	+	+
Neuronal loss with Bunina bodies in the motor neuron of the brain stem and/or spinal cord	+	+	+	+	+	+	+	+
Neuronal loss with astrocytosis in the dorsomedial portion of the anterior first temporal gyrus	+	N.E.	+	+	+	+	+	N.E.
Neuronal loss with astrocytosis in the subiculum	+	+	+	+	+	N.E.	+	+
Neuronal loss with astrocytosis in the parahippocampal gyrus	+	+	+	+	+	+	+	+
Neuronal loss with astrocytosis in the amygdala	+	+	+	+	+	+	+	+
Neuronal loss with astrocytosis in the substantia nigra	+	+	+	+	+	+	+	+

Table 3 Clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract (+ present, – absent, *N.R.* not recorded, *N.E.* not examined)

Case	1	2	3	4	5	6	7	8
Pyramidal sign	+	+	+	+	+	+	+	–
Hyperreflexia	+	+	+	+	+	+	+	–
Babinski sign	+	+	N.R.	–	–	–	+	–
Spasticity	N.R.	–	N.R.	N.R.	N.R.	N.R.	N.R.	–
Loss of Betz cells	+	+	+	+	N.E.	+	+	+
Astrocytosis of the primary motor cortex layer V	+	–	+	–	N.E.	+	–	–
Degeneration of the pyramidal tract								
Midbrain	–	–	–	–	–	–	–	–
Medulla oblongata	+	+	+	+	+	+	+	+
Spinal cord	+	+	N.E.	+	+	+	+	N.E.

of the brain stem and/or spinal cord in all cases. Neuronal loss with astrocytosis in the dorsomedial portion of the oral first temporal gyrus was observed in all six cases in which this structure was examined. Neuronal loss with astrocytosis in the subiculum was found in all seven cases examined. In all eight cases, neuronal loss with astrocytosis was evident in the parahippocampal gyrus, amygdala, and substantia nigra.

Clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract

Pyramidal signs and involvement of the pyramidal tract are summarized in Table 3. Pyramidal signs, including hyperreflexia and Babinski sign, were noted in seven cases. Spasticity was not found in any patients with ALS+D. Loss of Betz cells was observed in all seven cases in which the primary motor cortex was examined. In contrast, astrocytosis of the primary motor cortex layer V, detected by Holzer and GFAP staining, was obvious in three cases (cases 1, 3, and 6). Degeneration of the pyramidal tract, particularly of large myelinated fibers, was observed in all eight cases, and the distal portion was more affected than the proximal portion.

Discussion

Clinical features

According to Mitsuyama and colleagues [20, 21, 22], who proposed ALS+D as a new clinicopathological entity, pyramidal signs, including hyperreflexia, Babinski sign, and spasticity, were rarely observed in ALS+D. Furthermore, they noticed that the clinical hallmarks of motor neuron involvement in ALS+D were usually bulbar-type ALS, and that the lower motor neuron signs were more predominant than the upper motor neuron signs. Neary et al. [28] also reported that in MNND there was a relative absence of severe spasticity of the limbs. In contrast, there are few reports concerning obvious pyramidal signs in cases of ALS+D. According to Abe et al. [1], who conducted a single-photon emission computed tomographic investigation of 11 patients with motor neuron disease, including four cases of ALS+D, hyperreflexia was evident in all four cases of ALS+D, and Babinski sign was also found in three cases of ALS+D. Matsumoto et al. [17], who reported an autopsy case of ALS+D with ubiquitin-immunoreactive intraneuronal inclusions in the small neurons of temporal cortex, noted that, in their case, spasticity and brisk tendon reflexes were evident. According to Kato et al. [13], who investigated the topographic distribution of degenerative changes in the cerebral cortices and subcortical structures using large brain sections from eight autopsy cases of ALS, including five autopsy cases of ALS+D, hyperreflexia was evident in four (80%) of five cases of ALS+D and Babinski sign was positive in three (60%) of five cases of ALS+D. Nakano [26], who reviewed 36 autopsy cases of ALS+D reported in Japan up to 1996, noticed that hyperreflexia was observed in 17 (57%) of 30 autopsy cases in which clinical features were adequately reported, with Babinski sign being found in 10 cases (33%).

When we consider the frequency of pyramidal signs in ALS+D, our data showing that pyramidal signs were observed in seven (88%) of eight autopsy cases deserve mention.

Pathological features

It is thought that Betz cells of the primary motor area in ALS+D are relatively preserved. In 1990, Neary et al. [28] reported that in two autopsy cases of MNDD, Betz cells of the precentral gyrus were largely preserved in number, though many were grossly shrunken. In 2000, Mitsuyama [21] noted that in ALS+D, Betz cells of the primary motor cortex were usually intact. In contrast, loss of Betz cells in ALS+D has been rarely reported. In 1970, Shirabe et al. [37] reported an autopsy case of ALS+D having a clinical course of 1 year 3 months, and noticed that in their case Betz cells in the primary motor area were almost depleted. In 1976, Miyamoto et al. [23] also reported a Japanese autopsy case of ALS+D with parkinsonism and a clinical course of 2 years, and noted that

Betz cells in the primary cortex were diminished in number. Furthermore, in 1986, Kuroda et al. [15] reported an autopsy case of ALS+D with neck extension and a clinical course of 1 year 5 months, and noted that in their case the primary motor area showed marked diminution of Betz cells with astrocytic proliferation. In 1999, Noda et al. [32], who investigated Gallyas- and tau-positive glial structures in three autopsy cases of ALS+D in which the ages at onset were 70, 74, and 71 years, respectively, noticed that in their three cases Betz cells in the primary motor cortex were depleted.

From a perusal of the literature concerning the involvement of Betz cells in ALS+D, it has become obvious that there have been few reports showing loss of Betz cells in the primary motor cortex of these patients. Thus, our data, showing that there is constant loss of Betz cells in cases of ALS+D, are notable.

As mentioned in the introduction, it has been reported that the pyramidal tracts in patients with ALS+D are relatively preserved. In 1984, Mitsuyama [20], who reviewed 26 cases of ALS+D reported in Japan up to 1984, including 18 autopsies cases, noted that although half of the cases of ALS+D showed pyramidal tract degeneration, it was very mild compared with that seen in the classical sporadic ALS. In 1990, Neary et al. [28], who reported four patients with MNDD, including two autopsy cases, noticed that in their autopsy cases there was no obvious demyelination within the corticospinal tracts in the pons, medulla oblongata, and spinal cord. In 1998, Mann [16] noted that in MNDD despite the loss of anterior horn cells, the long white matter tracts within the spinal cord and brain stem remained well myelinated, and that no overt loss of axons was seen. In contrast, there are a few reports concerning the involvement of the pyramidal tract in ALS+D. In 1992, Okamoto et al. [35], who elucidated the constant presence of ubiquitin-positive intraneuronal inclusions in the hippocampal dentate granular cells and small neurons of the superficial layers of the frontotemporal lobes in ten cases of ALS+D, also noted that pyramidal tract degeneration was present in seven of ten cases. In 1996, Bergman et al. [5], who conducted a neuropathological and immunohistochemical study on 20 cases of frontotemporal dementia, including five cases of motor neuron disease with dementia, noted that the pyramidal tracts were affected in four of five cases of motor neuron disease with dementia. In 1998, Kawashima et al. [14], who reported ubiquitinated skein-like inclusions in the striatum from an autopsy case of ALS+D, also noticed that in their case the pyramidal tracts in the spinal cord had slightly degenerated.

Therefore, our data showing that there is constant involvement of the pyramidal tract in ALS+D patients is an interesting finding. Furthermore, the involvement of the pyramidal tracts in our eight cases was more prominent in the distal portion than in the proximal portion. Dominant involvement of the pyramidal tract in the distal portion in our cases is also fundamentally compatible with the results of a study by Yoshida et al. [52].

Clinicopathological correlation between pyramidal signs and involvement of the pyramidal tracts

There have been very few investigations on the clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract in cases of ALS+D. Morita et al. [24], who reviewed 34 Japanese cases of ALS+D reported, including 26 neuropathologically confirmed autopsy cases, noted that hyperreflexia was seen in only 27% of patients, with Babinski sign being evident in 18%. Furthermore, they noticed that degeneration of the pyramidal tract was found in only 27% of autopsy cases of ALS+D, and proposed that from the point of view of motor neuron disease, ALS+D should be considered as an atypical spinal progressive muscular atrophy rather than ALS. However, Morita et al. [24] did not comment on whether there was any clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract in 26 Japanese autopsy cases of ALS+D. However, Yoshida et al. [52] clinicopathologically investigated 13 cases of ALS+D, comprising 11 sporadic cases and 2 familial cases, and reported the pathological features of 6 of the sporadic cases and 1 familial case. They noted that in each of the 6 sporadic autopsy cases of ALS+D, hyperreflexia, including jaw jerk, was found, with Babinski sign being evident in 4 cases (67%). Furthermore, they noticed that in the spinal cords of these 6 cases, degeneration of the pyramidal tract, with preferential involvement of the distal portion of the pyramidal tract, was evident, and that, in contrast to the previous studies [20, 22, 24], there was an obvious clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract.

On the basis of our data showing that pyramidal signs were observed in seven of eight autopsy cases, and that involvement of the pyramidal tract was obvious in all cases, we believe that there is an obvious clinicopathological correlation between pyramidal signs and involvement of the pyramidal tract in ALS+D.

Nosological status of ALS+D

Mitsuyama and Takamiya [22], who reported an additional Japanese female case of ALS+D, who had a clinical course of 3 years, and proposed ALS+D as a new disease entity, noted that in ALS+D there were not only no definite pyramidal signs or pathological reflexes, but also a low incidence of pyramidal tract degeneration, inconsistent with the pathology of classic ALS. Therefore, they believed that ALS+D was a new disease entity rather than a variant of ALS. In contrast, Hudson [9], who reviewed 42 cases of sporadic ALS accompanied by dementia and/or parkinsonism reported up to 1981 throughout the world, noted that the sporadic type of ALS+D or ALS associated with dementia and parkinsonism might be a variant of classical ALS. Salazar et al. [36], who reviewed 231 cases of dementia with early lower motor neuron signs, including 65 cases referred to their laboratory, con-

cluded that the syndromes of dementia and lower motor neuron disease were closely related to the usual forms of sporadic and familial ALS. Furthermore, Horoupian et al. [8], who reported three autopsy cases of ALS+D in which dementia without neurofibrillary tangles or Pick bodies antedated amyotrophy by several years and in which pyramidal tract degeneration was found in all three cases, considered that their cases might represent a subset of motor neuron disease.

Based on our data for eight autopsy cases showing a high frequency (88%) of pyramidal signs and constant involvement of Betz cells and the pyramidal tract, and on the obvious clinicopathological correlation between pyramidal signs and pyramidal tract involvement, we believe that ALS+D is not a new clinicopathological entity, but a rare variant of ALS.

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References

1. Abe K, Fujimura H, Toyooka K, Hazama T, Hirono N, Yorifuji S, Yanagihara T (1993) Single-photon emission computed tomographic investigation of patients with motor neuron disease. Neurology 43:1569–1573
2. Arima K, Ogawa M, Sunohara N, Nishio T, Shimomura Y, Hirai S, Eto K (1998) Immunohistochemical and ultrastructural characterization of ubiquitinated eosinophilic fibrillary neuronal inclusions in sporadic amyotrophic lateral sclerosis. Acta Neuropathol 96:75–85
3. Bak TH, Hodges JR (1999) Cognition, language and behaviour in motor neurone disease: evidence of frontotemporal dysfunction. Dement Geriatr Cogn Disord 10 [Suppl 1]:29–32
4. Bak TH, O'Donovan DG, Xuereb JH, Boniface S, Hodges JR (2001) Selective impairment of verb processing associated with pathological changes in Brodmann areas 44 and 45 in the motor neurone disease-dementia-aphasia syndrome. Brain 124: 103–120
5. Bergmann M, Kuchelmeister K, Schmid KW, Kretzschmar HA, Schröder R (1996) Different variants of frontotemporal dementia: a neuropathological and immunohistochemical study. Acta Neuropathol 92:170–179
6. Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239–259
7. Dickson DW, Horoupian DS, Thal LJ, Davies P, Walkley S, Terry RD (1986) Klüver-Bucy syndrome and amyotrophic lateral sclerosis: a case report with biochemistry, morphometrics, and Golgi study. Neurology 36:1323–1329
8. Horoupian DS, Thal L, Katzman R, Terry RD, Davies P, Hirano A, DeTeresa R, Fuld PA, Petito C, Blass J, Ellis JM (1984) Dementia and motor neuron disease: morphometeric, biochemical, and Golgi studies. Ann Neurol 16:305–313
9. Hudson AJ (1981) Amyotrophic lateral sclerosis and its association with dementia, parkinsonism and other neurological disorders: a review. Brain 104:217–247
10. Ince PG, Lowe J, Shaw PJ (1998) Amyotrophic lateral sclerosis: current issues in classification, pathogenesis and molecular pathology. Neuropathol Appl Neurobiol 24:104–117
11. Jackson M, Lennox G, Lowe J (1996) Motor neuron disease-inclusion dementia. Neurodegeneration 5:339–350
12. Jellinger KA (1997) Pick's disease with amyotrophic lateral sclerosis. J Neurol Sci 152:227–228
13. Kato S, Oda M, Hayashi H, Kawata A, Shimizu T (1994) Participation of the limbic system and its associated areas in the dementia of amyotrophic lateral sclerosis. J Neurol Sci 126:62–69
14. Kawashima T, Kikuchi H, Takita M, Doh-ura K, Ogomori K, Oda M, Iwaki T (1998) Skein-like inclusions in the neostriatum from a case of amyotrophic lateral sclerosis with dementia. Acta Neuropathol 96:541–545
15. Kuroda S, Kuyama K, Hayashi Y, Morioka E (1986) An autopsy case of amyotrophic lateral sclerosis with dementia and neck extension (in Japanese with English abstract). Brain nerve (Tokyo) 38:655–659
16. Mann DMA (1998) Dementia of frontal type and dementias with subcortical gliosis. Brain Pathol 8:325–338
17. Matsumoto S, Kusaka H, Ito H, Shibata N, Asayama T, Imai T (1996) Sporadic amyotrophic lateral sclerosis with dementia and Cu/Zn superoxide dismutare-positive Lewy body-like inclusions. Clin Neuropathol 15:41–46
18. Meyer A (1929) Über eine der amyotrophischen Lateralsklerose nahestehende Erkrankung mit psychischen Störungen. Zugleich ein Beitrag zur Frage der spastischen Pseudosklerose (A. Jacob). Z Gesamte Neurol Psychiatr 121:107–138
19. Mimura M, Tominaga I, Kashima H, Honda M, Kosaka K, Kato Y (1998) Presenile non-Alzheimer dementia with motor neuron disease and laminar spongiform degeneration. Neuropathology 18:19–26
20. Mitsuyama Y (1984) Presenile dementia with motor neuron disease in Japan: clinico-pathological review of 26 cases. J Neurol Neurosurg Psychiatry 47:953–959
21. Mitsuyama Y (2000) Dementia with motor neuron disease. Neuropathology 20:79–81
22. Mitsuyama Y, Takamiya S (1979) Presenile dementia with motor neuron disease in Japan. A new entity? Arch Neurol 36:592–593
23. Miyamoto Y, Ukida G, Kato N, Tanaka J, Ishihara Y (1976) A case of sporadic amyotrophic lateral sclerosis with parkinsonism and dementia (in Japanese with English abstract). Neurol Med (Tokyo) 4:424–429
24. Morita K, Kaiya H, Ikeda T, Namba M (1987) Presenile dementia combined with amyotrophy: a review of 34 Japanese cases. Arch Gerontol Geriatr 6:263–277
25. Nakano I (1993) Temporal lobe lesions in amyotrophic lateral sclerosis with or without dementia: a neuropathological study. Neuropathology 13:215–227
26. Nakano I (1996) Amyotrophic lateral sclerosis (ALS) and dementia (in Japanese with English abstract). Shinkei Kenkyu no Shinpo 40:63–74
27. Nakano I, Iwatsubo T, Hashizume Y, Mizutani T, Mannen T (1992) Amyotrophic lateral sclerosis with dementia – lesions in the apical cortex and some deeper structures of the temporal lobes. Neuropathology 12:69–77
28. Neary D, Snowden JS, Mann DMA, Northen B, Goulding PJ, Macdermott N (1990) Frontal lobe dementia and motor neuron disease. J Neurol Neurosurg Psychiatry 53:23–32
29. Neary D, Mann DMA, Snowden JS (1998) Frontotemporal dementia with motor neuron disease. In: Kertesz A, Munoz DG (eds) Pick's disease and Pick complex. Wiley-Liss, New York, pp 145–158
30. Niizato K, Tsuchiya K, Tominaga I, Kato Y, Ikeda K (1997) Pick's disease with amyotrophic lateral sclerosis (ALS): report of two autopsy cases and literature review. J Neurol Sci 148: 107–112
31. Niizato K, Tsuchiya K, Ikeda K (1997) Reply to K.A. Jellinger's letter to the editor: Pick's disease with amyotrophic lateral sclerosis. J Neurol Sci 152:230
32. Noda K, Katayama S, Watanabe C, Yamamura Y, Nakamura S (1999) Gallyas- and tau-positive glial structures in motor neuron disease with dementia. Clin Neuropathol 18:218–225
33. Okamoto K (1998) Temporal lobe pathology in patients with amyotrophic lateral sclerosis. Neuropathology 18:222–227

34. Okamoto K, Hirai S, Yamazaki T, Sun X, Nakazato Y (1991) New ubiquitin-positive intraneuronal inclusions in the extramotor cortices in patients with amyotrophic lateral sclerosis. Neurosci Lett 129:233–236
35. Okamoto K, Murakami N, Kusaka H, Yoshida M, Hashizume Y, Nakazato Y, Matsubara E, Hirai S (1992) Ubiquitin-positive intraneuronal inclusions in the extramotor cortices of presenile dementia patients with motor neuron disease. J Neurol 239: 426–430
36. Salazar AM, Masters CL, Gajdusek DC, Gibbs CJ Jr (1983) Syndromes of amyotrophic lateral sclerosis and dementia: relation to transmissible Creutzfeldt-Jakob disease. Ann Neurol 14:17–26
37. Shirabe T, Inoue N, Izumi K, Kikuchi M (1970) An autopsy case of amyotrophic lateral sclerosis with dementia (in Japanese with English abstract). Kyushu Neuropsychiatry 16:208–215
38. Takahashi H, Oyanagi K, Ikuta F, Tanaka M, Yuasa T, Miyatake T (1993) Widespread multiple system degeneration in a patient with familial amyotrophic lateral sclerosis. J Neurol Sci 120:15–21
39. Takahashi H, Makifuchi T, Nakano R, Sato S, Inuzuka T, Sakimura K, Mishina M, Honma Y, Tsuji S, Ikuta F (1994) Familial amyotrophic lateral sclerosis with a mutation in the Cu/Zn superoxide dismutase gene. Acta Neuropathol 88:185–188
40. Tanaka J, Nakamura H, Tabuchi Y, Takahashi K (1984) Familial amyotrophic lateral sclerosis: features of multisystem degeneration. Acta Neuropathol 64:22–29
41. Tsuchiya K, Shintani S, Kikuchi M, Kondo H, Kamaya T, Ohbu S, Kato S, Hayashi H, Ikeda K, Nakano I (1999) Sporadic amyotrophic lateral sclerosis of long duration mimicking spinal progressive muscular atrophy: a clinicopathological study. J Neurol Sci 162:174–178
42. Tsuchiya K, Arai M, Matsuya S, Nishimura H, Ishiko T, Kondo H, Ikeda K, Matsushita M (1999) Sporadic amyotrophic lateral sclerosis resembling primary lateral sclerosis: report of an autopsy case and a review of the literature. Neuropathology 19:71–76
43. Tsuchiya K, Ozawa E, Fukushima J, Yasui H, Kondo H, Nakano I, Ikeda K (2000) Rapidly progressive aphasia and motor neuron disease: a clinical, radiological, and pathological study of an autopsy case with circumscribed lobar atrophy. Acta Neuropathol 99:81–87
44. Tsuchiya K, Ozawa E, Haga C, Watabiki S, Ikeda M, Sano M, Ooe K, Taki K, Ikeda K (2000) Constant involvement of the Betz cells and pyramidal tract in multiple system atrophy: a clinicopathological study of seven autopsy cases. Acta Neuropathol 99:628–636
45. Tsuchiya K, Shintani S, Nakabayashi H, Kikugawa K, Nakano R, Haga C, Nakano I, Ikeda K, Tsuji S (2000) Familial amyotrophic lateral sclerosis with onset in bulbar sign, benign clinical course, and Bunina bodies: a clinical, genetic, and pathological study of a Japanese family. Acta Neuropathol 100:603–607
46. Tsuchiya K, Matsunaga T, Aoki M, Haga C, Ooe K, Abe K, Ikeda K, Nakano I (2001) Familial amyotrophic lateral sclerosis with posterior column degeneration and basophilic inclusion bodies: a clinical, genetic and pathological study. Clin Neuropathol 20:53–59
47. Tsuchiya K, Ikeda K, Haga C, Kobayashi T, Morimatsu Y, Nakano I, Matsushita M (2001) Atypical amyotrophic lateral sclerosis with dementia mimicking frontal Pick's disease: a report of an autopsy case with a clinical course of 15 years. Acta Neuropathol 101:625–630
48. The Lund and Manchester Groups (1994) Clinical and neuropathological criteria for frontotemporal dementia. J Neurol Neurosurg Psychiatry 57:416–418
49. Uchihara T, Sato T, Suzuki H, Ikeda K, Akiyama H, Takatori T (2001) Bunina body in frontal lobe dementia without clinical manifestations of motor neuron disease. Acta Neuropathol 101:281–284
50. Wakabayashi K, Piao Y.-S, Hayashi S, Kakita A, Yamada M, Takahashi H (2001) Ubiquitinated neuronal inclusions in the neostriatum in patients with amyotrophic lateral sclerosis with and without dementia – A study of 60 patients 31 to 87 years of age. Clin Neuropathol 20:47–52
51. Wightman G, Anderson VER, Martin J, Swash M, Anderton BH, Neary D, Mann D, Luthert P, Leigh PN (1992) Hippocampal and neocortical ubiquitin-immunoreactive inclusions in amyotrophic lateral sclerosis with dementia. Neurosci Lett 139:269–274
52. Yoshida M, Murakami N, Hashizume Y, Takahashi A (1992) A clinicopathological study on 13 cases of motor neuron disease with dementia (in Japanese with English abstract). Clin Neurol 32:1193–1202
53. Yuasa R (1964) Amyotrophic lateral sclerosis with organic dementia: report of a case (in Japanese with English abstract). Clin Neurol 4:529–534
54. Yuasa R (1970) Amyotrophic lateral sclerosis with dementia (in Japanese with English abstract). Clin Neurol 10:569–577

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Heterogeneity of Nigral and Cortical Lewy Bodies Differentiated by Amplified Triple-Labeling for Alpha-Synuclein, Ubiquitin, and Thiazin Red

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Alpha-synuclein(α-S) and ubiquitin(Ub) are constituents of the Lewy bodies (LBs), composed of fibrillary structures. To clarify morphological heterogeneity of LBs, we looked for localization of these epitopes in relation to fibrillary structure possibly detectable by a fluorochrome, thiazin red (TR). On the sections of the substantia nigra (SN) and the cingulate gyrus (CG) obtained from Parkinson's disease brains, double amplification by CARD fluorescent immunohistochemistry with anti-α-S monoclonal (LB509) and anti-Ub polyclonal antibodies was performed, followed by staining with TR. These triple-labeled images were captured by a confocal laser microscope and subsequently stained with Campbell–Switzer method, a silver staining specific for LBs. Staining profiles of LBs were different between those in the SN and in the CG. Immunolabeling either with the anti-α-S or anti-Ub antibody was diffuse without halo structure in LBs of CG. In addition to this diffuse staining, a lot of LBs of SN exhibited a halo structure immunopositive for α-S and Ub, probably representing later stages of LB evolution. Irrespective of the presence of this halo structure, the TR signal was always concentrated in the center of LBs, as the silver-stained material was, suggesting that fibrillary components in the central portion of LBs undergo some conformational changes detectable by TR and the silver-staining. This technique reveals different epitopes in relation to LB evolution in vivo. Heterogeneity in staining profile of LBs, as clarified by this method, may represent evolutional changes of LBs, related to conformational states of their constituents. © 2002 Elsevier Science (USA)

Key Words: **Lewy body; triple-labeling immunohistochemical study; CARD; thiazin red; Alpha-synuclein; ubiquitin; substantia nigra; cingulate gyrus; confocal laser microscope; Campbell–Switzer method.**

INTRODUCTION

Lewy bodies (LBs) are recognized as eosinophilic inclusions found in restricted areas of the brain. Their morphology and prevalence are greatly varied according to their location or disease condition (1, 2). Although morphological heterogeneity of LBs is more apparent on immunohistochemical (3, 4) and electron-microscopic (1, 5–16) examination, it is now known that they are commonly composed of fibrils or aggregates of alpha-synuclein(α-S) (17, 18). It is, therefore, probable that factors other than α-S itself may be responsible to explain the morphological heterogeneity of LBs (19–26). Because α-S in LBs is woven into fibrils, it is likely that difference in composition of fibrils and in its underlying conformation may be responsible for these heterogeneity (3, 16–18). Inversely, these heterogeneities between LBs (inter-LB heterogeneity) or in internal structure of each LB (intra-LB heterogeneity) may represent different conformation or fibril composition. In the present study, we attempted to define these inter- and intra-LB heterogeneities on the basis of immunohistochemistry, silver staining and a fluorochrome, thiazin red, that has an affinity to fibrillary structures such as neurofibrillary tangles (27). Dual enhancement of double immunofluorescent signals by CARD, as we have established recently (28), enabled immunofluorescent signals from α-S and ubiquitin (Ub) to be quantified precisely. Simultaneous detection of TR signal from the same structure provided additional information, possibly related to fibrillary status of the LB under observation. Finally, superimposition of silver-stained image, specific for LBs (29), onto this triple-labeled fluorescence image enabled to exclude non-specific fluorescence not related to LBs. The

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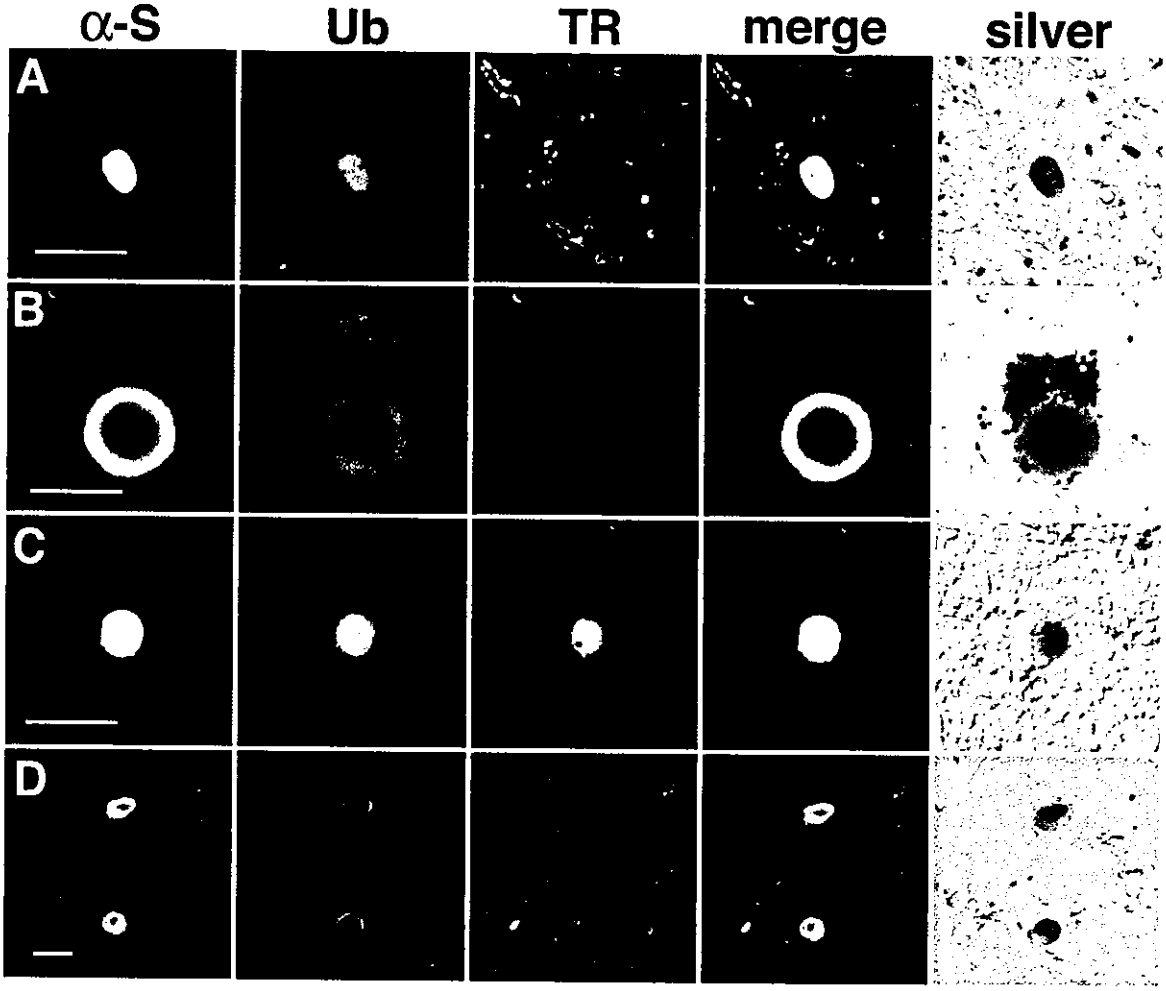

FIG. 1. Various types of Lewy bodies (LBs) differentiated by triple-fluorescence labeling. Green alpha-synculein (α-S) labeled by FITC; blue: ubiquitin (Ub) labeled by Cy-5; red: stained by thiazin red (TR); and merge: merged image of the three, and stained by Campbell-Switzer silver pyridine technique (silver). Five images, arranged horizontally, were obtained from the same LB. A, B are two representative types of LBs in the substantia nigra. C, D are two representative types of LBs in the cingulate gyrus. Bars, 20 μm.

present study clarified inter-LB and intra-LB heterogeneities and their possible relation to evolution of LBs and to conformational states will be discussed.

MATERIALS AND METHODS

Five patients with the clinicopathological diagnosis of PD (1 male/4 females; age at death, range 60–78 years), obtained from the Department of Pathology, Tokyo Metropolitan Neurological Hospital, Tokyo, Japan, were enrolled in this study. Duration of the disease ranged 2–20 years. Clinical diagnosis of PD was confirmed postmortem on the basis of neuronal loss and the presence of LBs in the substantia nigra (SN), locus coeruleus, and dorsal motor vagal nucleus. All these were compatible with limbic/transitional type of DLB according to the consensus guidelines (30). Formalin-fixed, paraffin-embedded blocks were sampled from the cingulate gyrus (CG) and the SN at the level of the red nucleus. And sections were deparaffinized after cutting at 4 μm.

In order to observe localization of the two epitopes more clearly without crosstalk, each fluorescent signal from the two epitopes was amplified as described previously as dual enhancement of double immunofluorescent signals by CARD (28). After treatment with 2% H_2O_2 to inactivate endogenous peroxidases, sections were incubated with the monoclonal antibody against α-S LB509 (18) (1:300, courtesy of Professor Iwatsubo, University of Tokyo, Tokyo, Japan) diluted with 0.01M phosphate buffered saline (pH 7.4) containing 0.03% Triton-X 100 (PBST) and 5% normal goat serum for 48 h at 4°C. Sections were washed for ten minutes three times with PBST between the steps throughout the procedure. After incubation with an anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:500, Kirkegaard & Perry, Gaithersburg, MD) for 2 h, the HRP signal was directly visualized with FITC-conju-

gated tyramide (1:200, FITC-tyramide, NEN Life Science Products, Boston, MA) in the dark. Next, HRP was again blocked by incubating the section with 2% H_2O_2 in 0.1M Tris-buffered saline for 30 min. The sections were then incubated with a rabbit polyclonal antibody against Ub (1:500, Dako, Denmark) at 4°C for 48 h. After incubation with an anti-rabbit IgG conjugated with HRP (1:500, Pierce, Rockford, IL) for 2 h, the HRP signal was amplified with biotinylated tyramide (1:1000) (31) for 10 min. The amplified signal was visualized with Cy5-conjugated streptavidin (1:200, Kirkegaard & Perry). After washing, stained sections were then immersed in 0.01 M phosphate-buffered saline containing TR (1:30,000; Wako, Japan) for 30 min. After washing, they were mounted with buffered glycerol containing *p*-phenylenediamine to minimize photobleaching. Sections were observed under a fluorescence microscope combined with laser confocal system (TCS-SP, Leica, Heidelberg, Germany). This system is equipped with spectrophotometer consisting of a prism and two sets of movable slit in front of the detection photomultiplier, which select arbitrarily whatever wavelength between 400–800 nm for the light path (LP) to be detected. For balanced excitation of fluorochromes, Ar-Kr laser is combined with acoustico-optical tunable filter (AOTF) system, which enables to adjust the individual intensity of each of the three laser beams (488, 568, and 647 nm) independently. FITC, which labeled α-S epitope, was excited by 488 nm beam and was detected through a LP ranging 500–540 nm. Cy-5, which labeled Ub epitope, was excited by 647 nm beam and was detected through a LP ranging 690–730 nm. TR (emission peak: 620 nm) was excited by 568 nm beam and was detected through LP ranging 600–640 nm. After triple-stained images were photographed and recorded on magneto-optical disks, the same section was subjected to Campbell-Switzer silver pyridine technique, which is an advanced silver-staining, specific for LBs (29). Those stained by this silver technique were identified as LBs. This procedure enabled the relationship between four different staining features; α-S-like immunoreactivity (IR), Ub-like IR, TR positivity, and argyrophilia by the silver staining, to be assessed on each LB. Morphological profile of each LB was classified based on both these staining features and its morphology (presence or absence of halo). At least twenty LBs on sections from the CG and from SN from each case were screened for these morphological profiles.

RESULTS

Dual enhancement of two different fluorescent signals enabled to detect highly intense signals from α-S (FITC) and from Ub (Cy-5) without cross talk between the TR signal (Fig. 1). Because the same section was subjected to the silver staining, the same LB, already recorded as multilabeling fluorescence images, was identified on the silver-stained section to assure that the silver-stained inclusions were nothing other than LBs. No structures lacking argyrophilia were neither immunopositive for α-S nor Ub. Other structures such as corpora amylacea, which often exhibit nonspecific immunolabeling and fluorescence were, therefore, excluded because they were not stained by this silver staining (29).

Although α-S-like IR and Ub-like IR looked similar in most of the LBs in general, Ub-like IR localized to the periphery of LB and always surrounded α-S-like IR. In other words, α-S-like IR never extended over the area occupied by the Ub-like IR. TR-positive materials or silver-stained materials were more or less concentrated at the center of the LB (Fig. 1). These staining profiles were, however, different in the SN and CG.

Substantia Nigra

Nearly two-thirds (64.7%, Table 1) of nigral LBs exhibited peripheral accentuation of α-S-like IR, recognized as so-called "brain stem-type" of LBs with halo (Figs. 1B and 1D). Most of them (47.5%) exhibited concomitant accentuation of Ub-like IR at the periphery. This peripheral accentuation of Ub-like IR was, however, only seen when α-S like IR was accumulated at the periphery. On the other hand, significant proportion (35.8%) of nigral LBs lacked this peripheral accentuation of these IRs, and most of them (33.3%) exhibited both α-S and Ub epitopes without peripheral accumulation (Fig. 1A). TR signal, present only in 24.2% of nigral LBs, was always restricted to the center of LBs even when α-S or Ub accumulated at its periphery (Figs. 1B and 1D). Although all of these LBs were stained by the silver impregnation technique, silver-stained materials were more or less concentrated at the center of each LB (Figs. 1A–1D) as TR signal was.

Cerebral Cortex (Cingulate Gyrus)

Over two-thirds (69.6%) of LBs in CG lacked peripheral accentuation of α-S-like or Ub-like IR, and were recognized as so-called "cortical type" of LBs without halo (Fig. 1C). Frequency of TR positive-LBs was higher (82.6%) compared with that in SN (24%). This contrast was still evident even when diffusely immunostained-LBs without halo were compared between CG (C) and SN (A). The TR signal in LBs was more prevalent in CG $62/(18 + 62) = 77.5\%$ than in SN $10/(3 + 30 + 10) = 30.3\%$. The TR signal was always concentrated at the center of LB as seen in SN. Staining profiles of LBs in the cingulate gyrus were, therefore, more homogeneous than those observed in SN.

DISCUSSION

Multi-labeling immunohistochemistry is usually confronted with a difficulty in visualizing different epitopes without cross talk. This difficulty is more serious when dealing with fluorescent labeling, which is sometimes hampered by a significant background staining, especially when multiple epitopes, possibly colocalizing to each other, are labeled by different fluorochromes. Dual enhancement of double immunofluorescent signal by CARD (28) overcame this difficulty by obtaining two intense fluorescent signals from two different epitopes, α-S and Ub, differently represented on LBs. Use of FITC and Cy-5, whose emission peaks were widely separated, enabled to combine another fluorochrome, TR, which has an affinity to fibrillary structures possibly representing peculiar conformational state such as neurofibrillary tangles (27). Identity of LBs, sometimes difficult to verify on immunofluorolabeled sections because of their diverse morphology and non-specific fluorescence, was ultimately confirmed on the same section, subsequently stained by the silver-staining method. This allowed us to pick up only genuine LBs and to analyse their morphology in relation to α-S, Ub and their conformational state in a quantitative way at an optimum condition. Although α-S is now considered to be a major constituent of LBs and Ub is an essential component during formation of LBs, we found some difference in staining profiles between LBs found in the SN and those in the CG. As expected, most of LBs in SN exhibited halo-like structure, which contained both α-S and Ub, representing typical brain-stem type of LBs. Most of LBs in CG lacked this structure and contained both α-S and Ub in the center. Regardless of this heterogeneity in their morphology, TR signal, when detectable, was always concentrated at the center of LBs. Lack of TR signal at the periphery of brain stem-type LBs, where both α-S and Ub are concentrated, suggests that the presence of α-S or Ub and their accumulation itself hardly explain this positive TR signal. Although we only picked up silver-stained structures as genuine LBs, silver-stained materials were also concentrated at the center of LBs, where TR signal was also accumulated. This copositivity with TR as well as with the silver-stain may, therefore, represent some conformational states, which is represented at the center of LBs.

Previous electron microscopic studies demonstrated that brain stem-type LBs consisted of radiating filaments at the periphery while their central domain was densely packed with granular materials and vesicular substances. In addition, the central domain contains many filaments that are similar to those in the periphery but are arranged haphazardly (1, 5). On the other hand, cortical LBs consist of similar, randomly oriented filaments intermingled with some membranous profiles as seen in the center of brain stem-type of LBs (6–9), while they lacked the peripheral counterpart consisting of radiating filaments (10–14). It is, therefore, speculated that formation of radiating filaments at the periphery of LBs, as seen in brain stem-type LBs, may represent later stage of formation of LBs (10, 15, 16). Indeed, the packing density of filamentous structures in cortical LBs is less dense than that in brain stem-type LBs, again suggesting that LBs of cortical type may represent a precursor stage preceding brain stem-type LBs (1, 4–16, 18, 32). These morphological heterogeneities of LBs may, otherwise, represent an influence dependent on the neuronal types in which they are formed. Anyway, both types of LBs, in spite of their morphological heterogeneity, share similar ultrastructural profile in their center as mentioned above. In addition, accumulation of mitochondria in the central part of LBs is another feature shared by both types of LBs (10, 13, 14). These similarities in ultrastructural profiles, seen in the center of both types of LBs, may correspond to some conformational states detected with both TR and silver staining on fixed histological sections.

We do not yet know whether differences in staining profiles seen in LBs of SN and CG represent different mechanism of LB formation or different stages of LB formation shared by LBs in SN and those in CG. The later possibility seems more plausible because there were significant overlap in the staining profiles between LBs in SN and those in CG. As previously hypothesized, simple deposition of α-S as amorphous deposits may occur as an early event (Fig. 2①), as shown in Fig. 2. Subsequently, Ub is then integrated into LBs (Fig. 2②A) followed by some conformational changes detected by TR (Fig. 2②C). Halo-like structures then appear, when α-S and Ub are redistributed to the periphery of LBs (Fig. 2②D). Some of them finally may evolve into mature LBs, when TR positivity is absent (Fig. 2③). Our quantitative approach clarified that LBs in SN exhibited full range of these different staining profiles, while LBs in CG exhibited only limited staining profiles restricted to Figs. 2A, 2C, and 2D, as encased in the rectangle (Fig. 2). This suggests that LBs in CG exhibit only limited stages of their morphological evolution. Formation of halo-like structure, exclusively observed in LBs in SN, is correlated with an effacement of α-S-like IR, Ub-like IR or TR positivity at the center of LBs. This effacement may represent disappearance of these epitopes or change in their conformational state. It may, otherwise, be related to progressive accumulation of dense materials in the center of LBs, that will modify epitopes or disarrange accumulated fibrils. At any rate, this change in staining profile represents evolution of LBs, which is unique to SN.

Although it is considered that α-S is one of the major constituents of LBs, some studies demonstrated that simple overexpression of α-S in cultured cells or high

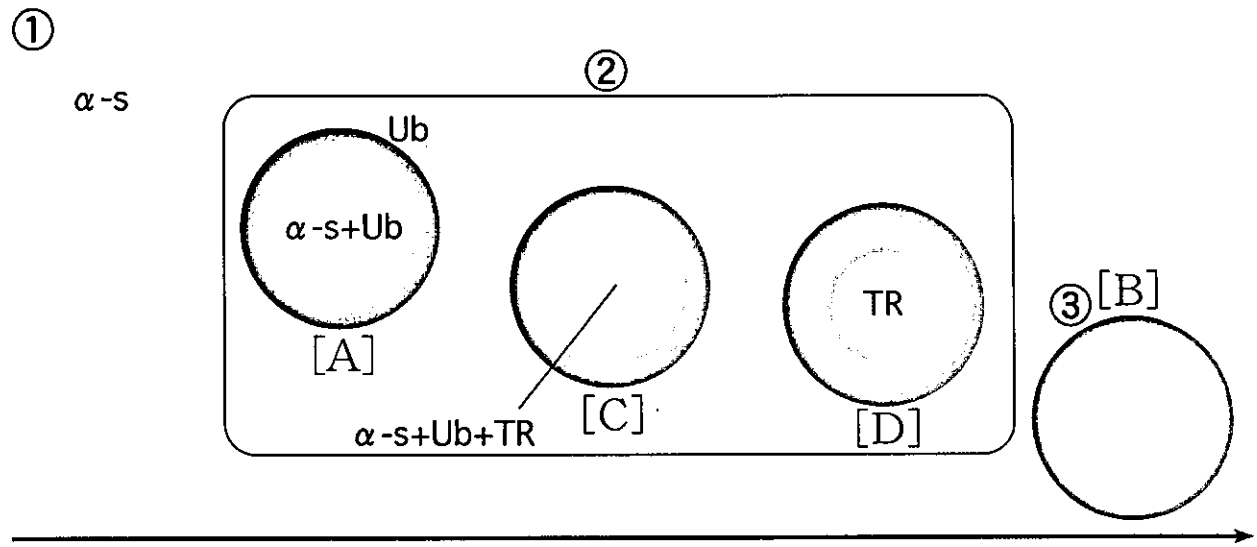

FIG. 2. Evolution of Lewy bodies (LBs) based on immunohistochemistry for alpha-synuclein (α-S), ubiquitin (Ub), and positive staining with thiazin red (TR). Most of LBs in the cingulate gyrus (CG) could be encased in the rectangle as in Table 1, while those in the substantia nigra (SN) exhibit more variable staining profiles. Putative direction of LB evolution is indicated with arrows. A, B, C, and D correspond to staining profiles shown in Figs. 1A, 1B, 1C, and 1D, respectively.

concentration of α-S in vitro failed to reproduce structures reminiscent of LBs seen in diseased human brains (19–23). It is, therefore, likely that intervention of factors other than translated α-S fragment may be necessary to mimic LBs outside human brains.

One of the factors is Ub. Its localization to LBs (3, 4) is now considered to be more and more relevant to the pathogenesis of PD, especially after the identification of parkin gene, because it codes for an E3 ubiquitin ligase and some of its mutations are linked to an autosomal recessive type of Parkinsonism (25). Double labeling immunofluorescence study at our optimum condition revealed that area occupied by Ub-like IR was larger than that occupied by α-S-like IR without exception. This is in contrast with previous immunofluorescence studies on LBs, which demonstrated that Ub-like IR of LBs was surrounded by α-S-like IR (3, 4). Difference of primary antibodies used or difference in staining procedure without amplification method in the previous studies may explain this discrepancy, which awaits, however, further confirmation. Similar images as we obtained; α-S-like IR surrounded by Ub-like IR, however, has been observed on cultured cells which overexpress α-S, thereby forming its aggregates surrounded by Ub-like IR (33). Interestingly, similar composition was noted in cells overexpressing wild-type α-S, when they were exposed to paraquat, a potent inducer of intracellular oxidative stress by producing superoxide (33). Although it remains to be proved where in neurons or in LBs α-S and Ub interact, one of the recent studies indicates that ubiquitin-proteasome system is down regulated where protein aggregates are accumulated as in the center of LBs (34). These experimental data corroborate our observation on LBs that Ub is localized to their outermost rim.

Other modifications of α-S molecule such as glycosylation (25), nitration (38–40), or phosphorylation (35–37) have been noted. Although nitration occurs in a wide variety of conditions, not restricted to LBs, it has been reported that this event preferentially takes place only when α-S has assembled into filaments (38) and stabilizes pre-assembled filaments (39). Because nitro-

TABLE 1

Heterogeneity in the Staining Profile of Lewy Bodies (LBs) in the Substantia Nigra and Cingulate Gyrus

Nigra/120	(%)	α-s	Ub	TR	Cortex/115	(%)
3	2.5	●	(−)	(−)		
30 (A)*	25.0	●	●	(−)	18	15.7
10	8.3	●	●	●	62 (C)**	53.9
		●	○	●	13	11.3
2	1.7	○	●	●	8	7.0
12	10.0	○	○	●	12 (D)	10.4
5	4.2	○	(−)	●		
13	10.8	○	●	(−)	2	1.7
45 (B)*	37.5	○	○	(−)		

Note. Stained structures positive for the silver staining were counted as LBs. In addition to positive (+) or negative (−) staining each for alpha-synuclein (α-S), ubiquitin (Ub), and thiazin red (TR), staining pattern was classified as either diffuse (●) or halo-like (○).

* Staining profiles predominant in the substantia nigra (SN).

** Staining profiles predominant in the cingulate gyrus (CG). Staining profiles with TR positivity were encased in the rectangle which include most of LBs in CG. Staining profiles of LB in SN were more variable. A, B, C, and D correspond to staining profiles shown in Figs. 1A, 1B, 1C, and 1D, respectively.

tyrosine accumulates in the center of LB (40), it is probable that assembled filaments decorated by nitration, which are accumulated in the center of LB, undergo some conformational changes. Accentuation of TR signal in the center of LB may correspond to this pathological conformation.

Apart from modification of α-S molecule itself, several molecules or structures have been reported to be present in LBs and some of them may interact with α-S. Among them, microtubule-associated protein-2 (MAP-2) (41), lipids (4) and microglia (42) as well as their related factors (43) are accumulated in the center of LBs, which may possibly be relevant to conformational changes of LB as similarly detected by TR at the center of LBs. Presence of synphilin-1 in LBs (44) and its capacity to interact with α-S and parkin (24–26, 45) are attracting increasing attention. Relative scarcity of synphilin-1-like IR at the periphery of LB (44) again suggests its possible link to a conformational state corresponding to more consolidated area of LB, as the center of LB.

Although the list of molecules or epitopes possibly linked to the formation of LBs is still growing, we do not yet know which modification or which interacting molecule is most relevant to the formation of LBs. Although it is reasonable and promising to reproduce this process in vitro or in cellular or animal model, parallel examination of human brain is another indispensable counterpart to verify what is really going on in the disease brains. Our double-labeling method with dual amplification of signals combined with TR clarified the morphological heterogeneities between LBs, which may correspond to their different evolutional stages. Because this method is also advantageous to look for structural heterogeneity in each LB, it will provide an optimum condition to look for other candidate molecules or epitopes possibly involved in the formation of LBs during their evolution from amorphous deposits to concentric classical LBs.

REFERENCES

1. Galloway, P. G., P. Mulvihill, and G. Perry. 1992. Filaments of Lewy bodies contain insoluble cytoskeletal elements. *Am. J. Pathol.* **140:** 809–822.
2. Wakabayashi, K., S. Hayashi, M. Yoshimoto, H. Kudo, and H. Takahashi. 2000. NACP/alpha-synuclein-positive filamentous inclusions in astrocytes and oligodendrocytes of Parkinson's disease brains. *Acta Neuropathol. (Berlin)* **99:** 14–20.
3. Gomez-Tortosa, E., K. Newell, M. C. Irizarry, J. L. Sanders, and B. T. Hyman. 2000. alpha-Synuclein immunoreactivity in dementia with Lewy bodies: Morphological staging and comparison with ubiquitin immunostaining. *Acta Neuropathol. (Berlin)* **99:** 352–357.
4. Gai, W. P., H. X. Yuan, X. Q. Li, J. T. Power, P. C. Blumbergs, and P. H. Jensen. 2000. *In situ* and *in vitro* study of colocalization and segregation of alpha-synuclein, ubiquitin, and lipids in Lewy bodies. *Exp. Neurol.* **166:** 324–333.
5. Hill, W. D., V. M. Lee, H. I. Hurtig, J. M. Murray, and J. Q. Trojanowski. 1991. Epitopes located in spatially separate domains of each neurofilament subunit are present in Parkinson's disease Lewy bodies. *J. Comp. Neurol.* **309:** 150–160.
6. Bethlem, J., and W. A. D. H. Jager. 1960. The incidence and characteristic of Lewy bodies in idiopathic paralysis agitans (Parkinson's disease). *J. Neurol. Neurosurg. Phychiatry* **23:** 74–80.
7. Forno, L. S., and R. L. Norville. 1976. Ultrastructure of Lewy bodies in the stellate ganglion. *Acta Neuropathol. (Berlin)* **34:** 183–197.
8. Hainfellner, J. A., P. Pilz, H. Lassmann, G. Ladurner, and H. Budka. 1995. Diffuse Lewy body disease as substrate of primary lateral sclerosis. *J. Neurol.* **242:** 59–63.
9. Masliah, E., D. Galasko, C. A. Wiley, and L. A. Hansen. 1990. Lobar atrophy with dense-core (brain stem type) Lewy bodies in a patient with dementia. *Acta Neuropathol. (Berlin)* **80:** 453–458.
10. Forno, L. S. 1987. The Lewy body in Parkinson's disease. *Adv Neurol* **45:** 35–43.
11. Fukuda, T., J. Tanaka, K. Watabe, R. T. Numoto, and M. Minamitani. 1993. Immunohistochemistry of neuronal inclusions in the cerebral cortex and brain-stem in Lewy body disease. *Acta Pathol. Jpn.* **43:** 545–551.
12. Pappolla, M. A., D. L. Shank, J. Alzofon, and A. W. Dudley. 1988. Colloid (hyaline) inclusion bodies in the central nervous system: Their presence in the substantia nigra is diagnostic of Parkinson's disease. *Hum. Pathol.* **19:** 27–31.
13. Roy, S., and L. Wolman. 1969. Ultrastructural observations in Parkinsonism. *J. Pathol.* **99:** 39–44.
14. Takahashi, H., K. Iwanaga, S. Egawa, and F. Ikuta. 1994. Ultrastructural relationship between Lewy bodies and pale bodies studied in locus ceruleus neurons of a non-Parkinsonian patient. *Neuropathology* **14:** 73–80.
15. Dale, G. E., A. Probst, P. Luthert, J. Martin, B. H. Anderton, and P. N. Leigh. 1992. Relationships between Lewy bodies and pale bodies in Parkinson's disease. *Acta Neuropathol. (Berlin)* **83:** 525–529.
16. Wakabayashi, K., S. Hayashi, A. Kakita, M. Yamada, Y. Toyoshima, M. Yoshimoto, and H. Takahashi. 1998. Accumulation of alpha-synuclein/NACP is a cytopathological feature common to Lewy body disease and multiple system atrophy. *Acta Neuropathol. (Berlin)* **96:** 445–452.
17. Baba, M., S. Nakajo, P. H. Tu, T. Tomita, K. Nakaya, V. M. Lee, J. Q. Trojanowski, and T. Iwatsubo. 1998. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am. J. Pathol.* **152:** 879–884.
18. Trojanowski, J. Q., and V. M. Lee. 1998. Aggregation of neurofilament and alpha-synuclein proteins in Lewy bodies: Implications for the pathogenesis of Parkinson disease and Lewy body dementia. *Arch. Neurol.* **55:** 151–152.
19. Conway, K. A., J. D. Harper, and P. T. Lansbury. 1998. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat. Med.* **4:** 1318–1320.
20. Crowther, R. A., R. Jakes, M. G. Spillantini, and M. Goedert. 1998. Synthetic filaments assembled from C-terminally truncated alpha-synuclein. *FEBS Lett.* **436:** 309–312.
21. Masliah, E., E. Rockenstein, I. Veinbergs, M. Mallory, M. Hashimoto, A. Takeda, Y. Sagara, A. Sisk, and L. Mucke. 2000. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: Implications for neurodegenerative disorders. *Science* **287:** 1265–1269.
22. Feany, M. B., and W. W. Bender. 2000. A Drosophila model of Parkinson's disease. *Nature* **404:** 394–398.

23. Goldberg, M. S., and P. T. Lansbury, Jr. 2000. Is there a cause-and-effect relationship between alpha-synuclein fibrillization and Parkinson's disease? *Nat. Cell Biol.* **2:** E115–119.
24. Engelender, S., Z. Kaminsky, X. Guo, A. H. Sharp, R. K. Amaravi, J. J. Kleiderlein, R. L. Margolis, J. C. Troncoso, A. A. Lanahan, P. F. Worley, V. L. Dawson, T. M. Dawson, and C. A. Ross. 1999. Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. *Nat. Genet.* **22:** 110–114.
25. Shimura, H., M. G. Schlossmacher, N. Hattori, M. P. Frosch, A. Trockenbacher, R. Schneider, Y. Mizuno, K. S. Kosik, and D. J. Selkoe. 2001. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: Implications for Parkinson's disease. *Science* **293:** 263–269.
26. Chung, K. K., Y. Zhang, K. L. Lim, Y. Tanaka, H. Huang, J. Gao, C. A. Ross, V. L. Dawson, and T. M. Dawson. 2001. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: Implications for Lewy-body formation in Parkinson disease. *Nat. Med.* **7:** 1144–1150.
27. Uchihara, T., A. Nakamura, M. Yamazaki, and O. Mori. 2000. Tau-positive neurons in corticobasal degeneration and Alzheimer's disease—Distinction by thiazin red and silver impregnations. *Acta Neuropathol. (Berlin)* **100:** 385–389.
28. Uchihara, T., A. Nakamura, U. Nagaoka, M. Yamazaki, and O. Mori. 2000. Dual enhancement of double immunofluorescent signals by CARD: Participation of ubiquitin during formation of neurofibrillary tangles. *Histochem. Cell Biol.* **114:** 447–451.
29. Sandmann-Keil, D., H. Braak, M. Okochi, C. Haass, and E. Braak. 1999. Alpha-synuclein immunoreactive Lewy bodies and Lewy neurites in Parkinson's disease are detectable by an advanced silver-staining technique. *Acta Neuropathol. (Berlin)* **98:** 461–464.
30. McKeith, I. G., D. Galasko, K. Kosaka, E. K. Perry, D. W. Dickson, L. A. Hansen, D. P. Salmon, J. Lowe, S. S. Mirra, E. J. Byrne, G. Lennox, N. P. Quinn, J. A. Edwardson, P. G. Ince, C. Bergeron, A. Burns, B. L. Miller, S. Lovestone, D. Collerton, E. N. Jansen, C. Ballard, R. A. de Vos, G. K. Wilcock, K. A. Jellinger, and R. H. Perry. 1996. Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): Report of the consortium on DLB international workshop. *Neurology* **47:** 1113–1124.
31. Adams, J. C. 1992. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J. Histochem. Cytochem.* **40:** 1457–1463.
32. Forno, L. S. 1996. Neuropathology of Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **55:** 259–272.
33. Paxinou, E., Q. Chen, M. Weisse, B. I. Giasson, E. H. Norris, S. M. Rueter, J. Q. Trojanowski, V. M. Lee, and H. Ischiropoulos. 2001. Induction of alpha-synuclein aggregation by intracellular nitrative insult. *J. Neurosci.* **21:** 8053–8061.
34. Bence, N. F., R. M. Sampat, and R. R. Kopito. 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* **292:** 1552–1555.
35. Okochi, M., J. Walter, A. Koyama, S. Nakajo, M. Baba, T. Iwatsubo, L. Meijer, P. J. Kahle, and C. Haass. 2000. Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. *J. Biol. Chem.* **275:** 390–397.
36. Nakamura, T., H. Yamashita, T. Takahashi, and S. Nakamura. 2001. Activated Fyn phosphorylates alpha-synuclein at tyrosine residue 125. *Biochem. Biophys. Res. Commun.* **280:** 1085–1092.
37. Ellis, C. E., P. L. Schwartzberg, T. L. Grider, D. W. Fink, and R. L. Nussbaum. 2001. alpha-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. *J. Biol. Chem.* **276:** 3879–3884.
38. Giasson, B. I., J. E. Duda, I. V. Murray, Q. Chen, J. M. Souza, H. I. Hurtig, H. Ischiropoulos, J. Q. Trojanowski, and V. M. Lee. 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* **290:** 985–989.
39. Souza, J. M., B. I. Giasson, Q. Chen, V. M. Lee, and H. Ischiropoulos. 2000. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J. Biol. Chem.* **275:** 18344–18349.
40. Good, P. F., A. Hsu, P. Werner, D. P. Perl, and C. W. Olanow. 1998. Protein nitration in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **57:** 338–342.
41. D'Andrea, M. R., S. Ilyin, and C. R. Plata-Salaman. 2001. Abnormal patterns of microtubule-associated protein-2 (MAP-2) immunolabeling in neuronal nuclei and Lewy bodies in Parkinson's disease substantia nigra brain tissues. *Neurosci. Lett.* **306:** 137–140.
42. Iseki, E., W. Marui, H. Akiyama, K. Ueda, and K. Kosaka. 2000. Degeneration process of Lewy bodies in the brains of patients with dementia with Lewy bodies using alpha-synuclein-immunohistochemistry. *Neurosci. Lett.* **286:** 69–73.
43. Togo, T., E. Iseki, W. Marui, H. Akiyama, K. Ueda, and K. Kosaka. 2001. Glial involvement in the degeneration process of Lewy body-bearing neurons and the degradation process of Lewy bodies in brains of dementia with Lewy bodies. *J. Neurol. Sci.* **184:** 71–75.
44. Wakabayashi, K., S. Engelender, M. Yoshimoto, S. Tsuji, C. A. Ross, and H. Takahashi. 2000. Synphilin-1 is present in Lewy bodies in Parkinson's disease. *Ann. Neurol.* **47:** 521–523.
45. Shimura, H., N. Hattori, S. Kubo, Y. Mizuno, S. Asakawa, S. Minoshima, N. Shimizu, K. Iwai, T. Chiba, K. Tanaka, and T. Suzuki. 2000. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25:** 302–305.