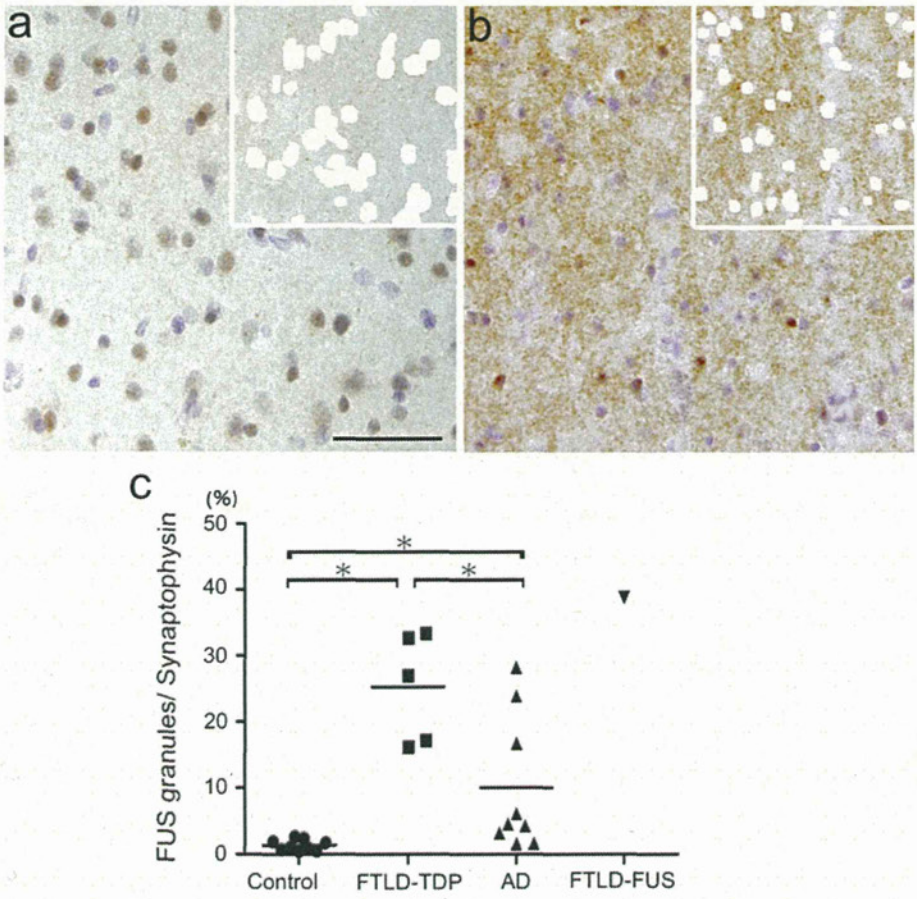

Fig. 7 Morphometric analyses of FUS-positive neuropil granules in the neocortex of the parahippocampal gyrus. Digital images were taken from FUS and haematoxylin-stained tissue sections of control subjects (**a**), FTLD-TDP patients Alzheimer's disease patients and an FTLD-FUS patient (**b**). Then, the nuclei were dissected out manually (**b** and **c**, *inset*) and the images were converted to a gray scale for quantitation. **c** FUS immunopositive pixels were counted and expressed as per cent of synaptophysin-positive pixels similarly counted in a nearby section. The results are shown as scatter plots. The *horizontal bars* indicate the mean values. $*p < 0.05$. The size of each image is 2,000 × 2,000 pixels. *Scale bar* 50 μm in **a**

degenerative change, in addition to its physiological functions. In a cell biologic study, FUS was shown to be recruited, in response to stress stimuli that lead to apoptosis, to stress granules, a form of cytoplasmic RNA granules that are composed of RNA-binding proteins and mRNA [8]. The increased dendritic FUS, therefore, might be associated with stress responses in neurodegenerative diseases. On the other hand, in a study with Drosophila models, cytoplasmic but not nuclear localization of FUS was linked to cellular degeneration [22]. The more pronounced increase in FTLD-TDP than in AD suggests its association with some abnormality in the dendritic RNA translation in which both FUS and TDP-43 are involved. In this study, only one FTLD-FUS case was available and, therefore, the significance of the result from this case remains uncertain. Nevertheless, an extreme increase of FUS granules in this case might support a notion that such a change is associated with the pathogenesis or pathophysiology of these RNA-binding protein-associated neurodegenerative diseases. Whether or not our observations are related to a recent finding that accumulation of FUS granules in the cytoplasm of spinal anterior horn cells in familial ALS with FUS mutations [25] is an issue of significant interest.

In conclusion, the immunohistochemical study of lightly fixed, free-floating sections of postmortem brains has revealed that FUS resides, not only in the nucleus, but also in the dendrites. At least, a portion of such FUS-immunoreactivity is located in the post-synapses. The specific neuroanatomical distribution, which is similar in mouse and human, indicates a physiological role, in particular, in the brainstem where the expression is mostly constitutive. The increase in the cerebral cortex in diseased brains infers that dendritic FUS is also related to some pathological processes.

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Familial ALS with *FUS* P525L mutation: two Japanese sisters with multiple systems involvement

Yoko Mochizuki [a,*], Eiji Isozaki [b], Masaki Takao [c], Tomoyo Hashimoto [d,e], Makoto Shibuya [a,f], Makoto Arai [g], Masato Hosokawa [g], Akihito Kawata [b], Kiyomitsu Oyanagi [d,e], Ban Mihara [h], Toshio Mizutani [a,i]

[a] Department of Pathology, Tokyo Metropolitan Neurological Hospital, Tokyo 183–0042, Japan
[b] Department of Neurology, Tokyo Metropolitan Neurological Hospital, Tokyo 183–0042, Japan
[c] Division of Neuropathology, Mihara Memorial Hospital, Gunma 372–0006, Japan
[d] Department of Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183–8526, Japan
[e] Division of Neuropathology, Department of Brain Disease Research, Shinshu University School of Medicine, Nagano, Japan
[f] Department of Pathology, Tokyo Medical University Ibaraki Medical Center, Ibaraki 300–0395, Japan
[g] Department of Neuropathology, Tokyo Metropolitan Institute of Medical Science, Tokyo 156–8506, Japan
[h] Department of Neurology, Mihara Memorial Hospital, Gunma 372–0006, Japan
[i] Fuchu Medical Center for the Disabled, Tokyo, Japan

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ABSTRACT

We evaluated the clinicopathological features of familial amyotrophic lateral sclerosis (ALS) with the *fused in sarcoma (FUS)* P525L mutation. Two sisters and their mother had a similar clinical course, which was characterized by the development of limb weakness at a young age with rapid disease progression. An elder sister, patient 1, progressed into a totally locked-in state requiring mechanical ventilation and died 26 years after the onset of the disease. In contrast, the younger sister, patient 2, died in the early stages of the disease. The patients had neuropathological findings that indicated a very active degeneration of motor neurons and multiple system degeneration, which led to marked brain and spinal cord atrophy in the long term clinical outcome. The multiple system degeneration included the frontal lobe, the basal ganglia and substantia nigra, cerebellum and related area. Compared with previously reported ALS cases, the severe degeneration of the frontal lobe and the striatum were the characteristic features in the patient 1 in this case study. The degeneration spread over multiple systems might be caused not only by the appearance of the FUS immunoreactive neuronal cytoplasmic inclusions but also by the degeneration of neuronal connections from the primary motor cortex and related areas.

1. Introduction

Sporadic amyotrophic lateral sclerosis (ALS) with basophilic cytoplasmic inclusions (BIs), characterized by an onset before 25 years of age with rapid progression, has been proposed to constitute a distinct clinical entity [1,2]. Bäumer et al. [3] demonstrated that the BIs in the cases of sporadic juvenile ALS were immunoreactive for fused in sarcoma (FUS), and two of their six patients had the *FUS* P525L mutation. Therefore, they proposed that juvenile ALS with BIs should be classified as ALS–FUS. Neuropathologically, Mackenzie et al. [4] divided patients with ALS–FUS in two groups: early-onset cases, including two with the *FUS* P525L mutation [3] and late-onset cases, including two with the *FUS* R521C mutation. Furthermore, they [4] suggested that patients with ALS–FUS and frontotemporal lobar degeneration (FTLD) with FUS-immunoreactive pathology (FTLD–FUS) were distinct because none of their ALS–FUS cases showed involvement of the broad range of neuroanatomical regions that occurs in the FTLD cases [5]. The neuropathological features of ALS with the *FUS* P525L mutation have been reported in only one other patient [6], who showed similar neuropathological features as to early-onset ALS–FUS [4]. This patient used non-invasive positive pressure ventilation during the last 4 months of her life; however, the other reported patients with the *FUS* P525L mutation [3,4] did not use respiratory assistance. Therefore, although it has been shown that an ALS case with the *FUS* R521C mutation, late-onset ALS–FUS showed no cerebral cortical involvement even in the prolonged stage with using mechanical ventilation [7], much is unknown about the clinicopathology of ALS with the *FUS* P525L mutation, early-onset ALS–FUS. Herein, we report the clinical and neuropathological findings in two autopsied sisters and their mother with Japanese familial ALS associated with the *FUS* P525L mutation. The two autopsied patients used mechanical ventilators: the elder sister used it for over 20 years; however, the younger sister only used it for approximately one year. Therefore, the difference between the clinicopathological

* Corresponding author at: Department of Pathology, Tokyo Metropolitan Neurological Hospital, 2-6-1 Musashidai Fuchu-shi, Tokyo 183–0042, Japan. Tel.: +82 423 23 5110.
E-mail address: mochi@nihon-u.ne.jp (Y. Mochizuki).

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findings of the elder sister at the late stage of the disease and those of the younger sister at the early stage of the disease are most likely indicative of the mechanism of disease progression. The significance of their neuroanatomical regions is discussed.

2. Case reports

2.1. Patient 1

The elder sister (proband, Subject III-1 in Fig. 1) exhibited a slight developmental delay in childhood. At the age of 13 years, she developed left and then right leg weakness, followed by quadriparesis. Her clinical feature was suggested to be a polyneuropathy. However, plasmapheresis was not effective for her. One year and 3 months after the onset, she was admitted to the Tokyo Metropolitan Neurological hospital because dysphagia, dyspnea, and dysarthria appeared. She was diagnosed as ALS and underwent a tracheostomy, with subsequent prolonged mechanical ventilation. Three years after the onset, she developed adduction paresis of the right eye, followed by ophthalmoparesis of both eyes. However, she was able to comprehend her situation. Head CT showed progressive brain atrophy (Fig. 2). Finally, all of her voluntary movement disappeared at the age of 23 years. Specifically, she progressed to a totally locked-in state that has been proposed [8] as one of the subgroups in the terminal condition of respirator-assisted long-survival ALS [9]. She died of pneumonia at the age of 40 years, 26 years after the onset.

2.2. Genetic analysis

In patient 1, DNA was extracted from the patient's leukocytes using a conventional method with informed consent. All of the coding regions and exon–intron boundaries of the *FUS* gene were examined by direct sequencing of polymerase chain reaction (PCR) products. Detailed information regarding the PCR amplification conditions is available from the authors upon request. Sequencing of the PCR products was performed using a BigDye Terminator Cycle Sequencing Reaction kit (Life Technologies Japan) and an ABI PRISM 3100 Genetic Analyzer (Life Technologies Japan).

The sequence analysis of the *FUS* gene identified a proline 525 to leucine (P525L) mutation. Therefore, we performed a deep resequencing analysis of the target gene and confirmed the presence of a rare heterozygous C-to-T transition at cDNA position 1574, resulting in a P525L missense mutation within the arginine-glycine-glycine motif of exon 15.

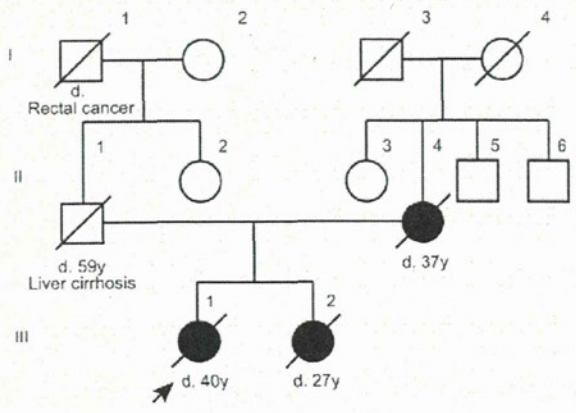

Fig. 1. Pedigree of the family. The arrow indicates the proband. The affected individuals are represented by the solid black symbols. I-4 lived to be more than 81 years old.

2.3. Patient 2

Patient 2 (Subject III-2 in Fig. 1) was the younger sister of patient 1. She developed right hand weakness at the age of 25 years. One year after, she showed gait disturbance, dysphagia, and respiratory failure. Subsequently, she was placed on mechanical ventilation and was transferred to the Mihara Memorial hospital and evaluated by a neurologist. Neurological examination at the age of 26 years showed quadriplegia with hypotonia and decreased tendon reflexes in the all extremities, while Babinski's sign appeared. She died of pneumonia at the age of 27 years.

2.4. Patient 3

The mother of patients 1 and 2 (Subject II-4 in Fig. 1) had developed left arm weakness and died of progressive bulbar palsy within 6 months of the disease duration at the age of 35 years.

3. Methods

3.1. Neuropathological study

The brain and spinal cord specimens were fixed with 20% buffered formalin and embedded in paraffin. Neuronal loss and/or fiber loss and gliosis was assessed in various regions of the nervous system using 10-μm-thick sections with hematoxylin and eosin (HE) and Klüver–Barrera (KB) stains. When necessary, Bodian, Nissl, periodic acid-Schiff (PAS) stain, luxol fast blue (LFB), cresyl violet, and Gallyas–Braak staining was performed.

3.2. Immunohistochemistry for BIs

For the immunohistochemistry, 6-μm-thick sections were prepared. Specimens of the frontal lobe, hippocampus with medial temporal lobe, and pons were immunostained for ubiquitin (DAKO, 1:600), 43-kDa TAR DNA binding protein-43 (TDP-43) (Polyclonal Protein Tech Group, 1:500), α-internexin (Cosmo Bio, 1:250), α-synuclein (Santa Cruz Biotechnology, 1:400), and phosphorylation-dependent τ (AT8; Innogenetics, 1:5000), and the specimens of each cerebral lobe, basal ganglia, cerebellum, brainstem, and spinal cord were immunostained for FUS (Sigma, 1: 100) using a labeled streptavidin–biotin method.

3.3. Electron microscopical study of BIs

Several pieces of formalin-fixed inferior frontal cortex and pontine nuclei of patient 1 were postfixed with 4% osmium tetroxide and conventionally processed for electron microscopy (Hitachi H-9000).

4. Results

4.1. Neuropathological findings in patient 1

The brain weighed 715 g. Marked cerebral atrophy was observed in the brainstem and cerebellar regions (Fig. 3A). The frontal white matter was marked with atrophy, the caudate nucleus was thin, and the putamen and globus pallidus were atrophic and brownish in color (Fig. 3B). The ventral lateral nucleus of the thalamus showed severe atrophy. However, the limbic system, including the medial temporal area, mammillary body, and cingulate gyrus were preserved.

The brainstem and spinal cord were markedly atrophic (Fig. 4A), and the anterior horn of the spinal cord (Fig. 4B) and all motor nuclei of the brainstem showed severe neuron loss and gliosis (Table 1). Although some neurons were observed in the intermediolateral nucleus, neurons in Clarke's nucleus were markedly decreased. The dorsal root ganglion cells were preserved. In the spinal cord, although the posterior column was preserved, almost all of the fibers from the other areas were lost

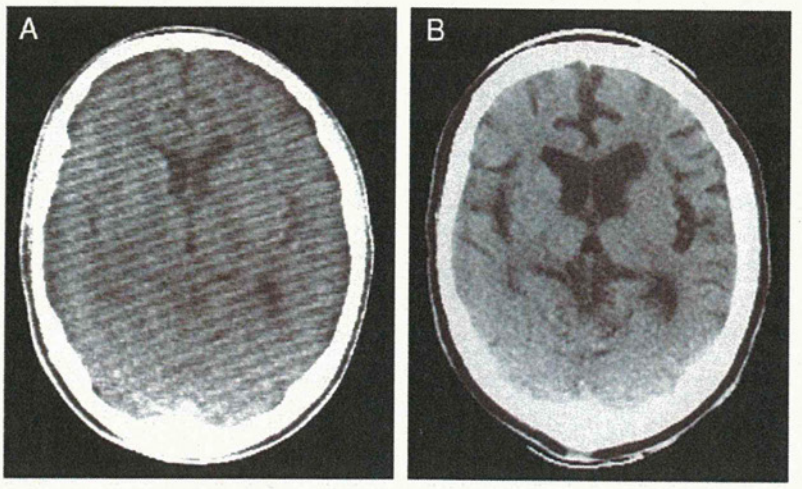

Fig. 2. Head CT of patient 1. (A) Mild frontal atrophy was observed at 2 years after the onset. (B) Moderate cerebral atrophy, particularly in the frontal lobe, and mild atrophy of the caudate nucleus were observed at 6 years after the onset.

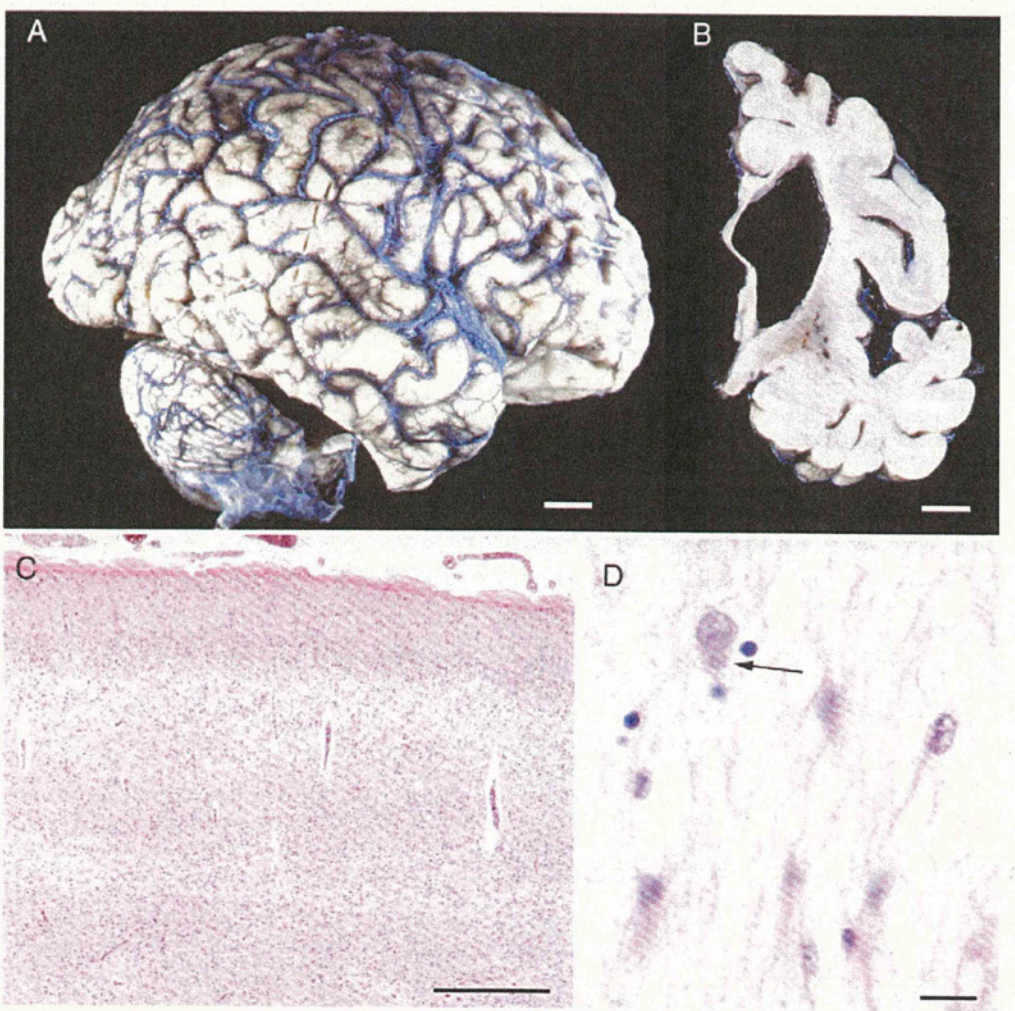

Fig. 3. Macroscopic findings and histology in patient 1. (A) Marked atrophy of the brain are observed, especially in the frontal lobe. (B) In the coronal section, enlargement of the anterior horn of the lateral ventricle, and atrophy of the striatum and frontal lobe with thinner corpus callosum are observed; however, the amygdala and temporal lobe are preserved. (A, B: bar = 1 cm). (C) Primary motor cortex showing neuronal loss with astroglial proliferation and spongiform changes in the upper cortical layers. (Bar = 500 μm). (D) Caudate nucleus showing marked neuronal loss with gliosis. The arrow indicates a basophilic cytoplasmic inclusion. (Bar = 50 μm, C, D: hematoxylin and eosin staining).

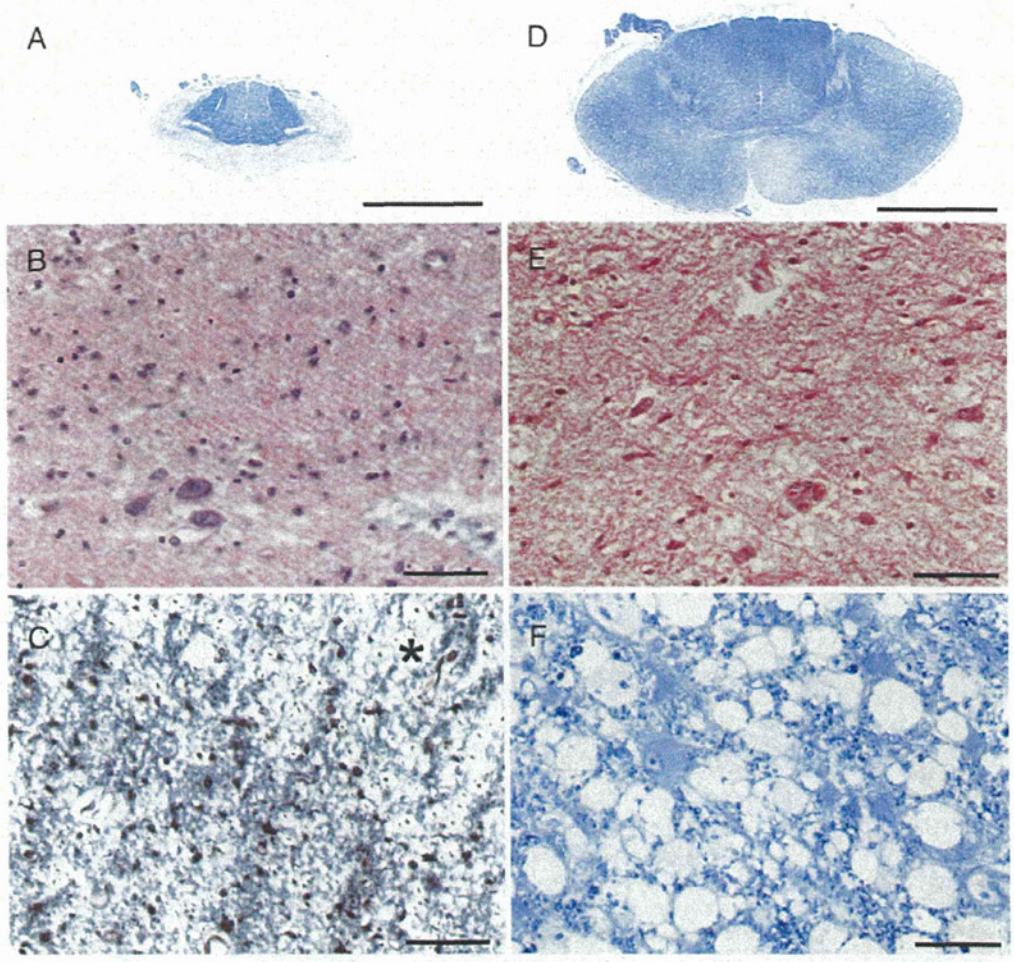

Fig. 4. Cervical spinal cord features. (A) Marked atrophy is observed at C7. The posterior column is relatively preserved while the numbers of other fibers were decreased. (A–C: patient 1) (B) Marked neuronal loss and gliosis of the anterior horn are observed at C7. (C) Marked fiber loss in both the lateral corticospinal tract and spinocerebellar tract (*) is observed at C7. (D) Degeneration in the anterior horn and lateral to the anterior column, particularly in pyramidal tract and preserved posterior column, are observed in the cervical spinal cord. (D–F: patient 2) (E) Marked neuronal loss and gliosis of the cervical anterior horn is observed. (F) Many macrophages and astroglial proliferation are observed in the lateral corticospinal tract. (A, D, F: Klüver–Barrera staining, bar = 0.5 cm, B, E: hematoxylin and eosin staining, C: Bodian staining, B, C, E, F: bar = 50 μm).

(Fig. 4A, C). Nerve fibers of the brainstem were barely observed except for relative preservation in the temporoparietooccipitopontine tract, medial lemniscus, pontocerebellar fibers and middle cerebellar peduncle. Only a few Betz cells were observed and these were atrophic and generalized astroglial proliferation in all layers were observed in the primary motor cortex. Furthermore, the primary motor cortex, primary somatosensory cortex, and frontal and parietal cortices showed neuronal loss with gliosis. In these cortices, the upper cortical layers showed spongiform changes, and the deep cortical layers showed a loss of the radiating myelinated fibers (Fig. 3C). Nerve fibers in the frontal white matter were markedly decreased in number. The following systems were severely affected by the disease (Table 1): the basal ganglia, which were severely affected in the striatum (Fig. 3D), subthalamic nucleus, and substantia nigra, and were mildly affected in the globus pallidus; the cerebellar dentate nucleus and efferent fibers and the red nucleus; the pontine nucleus, the inferior olive, and Purkinje cells.

The presence of BIs were difficult to establish in the lower motor neurons, the neurons of the substantia nigra and in the cerebellar dentate nucleus because of their marked neuronal loss (Table 1). The caudate nucleus showed severe degeneration, and many BIs were found there (Fig. 3D). In contrast, BIs were rarely found in the limbic area and occipital cortex. The BIs were round in shape and compactly or loosely packed (Fig. 5A), sometimes with distinct basophilic rims. The BIs were positive with Bodian staining, while negative with PAS, cresyl violet, LFB, or Gallya–Braak staining.

4.2. Neuropathological findings in patient 2

Limited anatomical regions were available for neuropathological analysis. The spinal cord atrophy was mild (Fig. 4D), and the anterior horn and hypoglossal nucleus showed mild atrophy in spite of marked neuronal loss with astroglial proliferation (Fig. 4E). Nerve fiber loss with numerous macrophages was observed in the corticospinal tract (Fig. 4F) and spinocerebellar tract. The corticospinal tract showed the same features as the internal capsule. However, the primary motor cortex, the hippocampus and the amygdala were not examined. Numerous BIs were observed in extensive areas with slight degeneration (Table 1). The shape of the BIs in patient 2 was similar to that in patient 1, especially in the anterior horn of the spinal cord and the pigmented neurons of the substantia nigra. However, some BIs in the globus pallidus, thalamus, pontine nucleus, inferior olivary nucleus, and dentate nucleus of

Table 1
Neuropathological findings.

	Patient 1			Patient 2		
	DEG	BI	NCI	DEG	BI	NCI
Motor neurons						
Primary motor cortex	++	2	2	n	n	n
Anterior horn of the spinal cord	+++	1	1	+++	2	3
Hypoglossal nucleus	+++	–	–	++	1	1
Oculomotor nucleus	+++	1	1	–	1	1
Frontal cortex	++	2	2	+	1	1
Basal ganglia and substantia nigra						
Caudate nucleus	+++	3	3	+	1	2
Putamen	+++	2	1	–	1	2
Globus pallidus	++	1	–	+	1	3
Substantia nigra	+++	–	–	+	1	3
Cerebellum and related area						
Purkinje cells	++	–	–	+	–	–
Cerebellar dentate nucleus	+++	1	–	+	2	3
Red nucleus	++	–	–	+	1	1
Inferior olivary nucleus	++	–	1	–	2	3
Pontine nucleus	++	2	2	–	1	2

DEG: degeneration assessed on the hematoxylin and eosin-, Klüver-Barrera-stained sections. The degeneration was indicated as absent (–), slight (+), mild (++) or severe (+++). BIs: basophilic inclusions. NCI: FUS-immunoreactive (ir) neuronal cytoplasmic inclusion. n: not evaluated (or not examined) The BIs and the FUS pathology was indicated as none (–), rare (1), occasional (2), frequently (3).

patient 2 were irregular in shape. Moreover, in patient 2, some cytoplasmic inclusions showed weak eosinophilia.

4.3. Immunohistochemistry of BIs

Both the BIs and the weak eosinophilic inclusions were strongly immunoreactive (ir) for FUS (Fig. 5B). The FUS-ir neuronal cytoplasmic inclusions (NCIs) were more numerous and widespread than NCIs identified with HE, KB, or Bodian staining (Table 1). A few FUS-ir neuronal intranuclear small round inclusions were observed in the cerebral cortex in patient 1 (Fig. 5C) and in the cerebellar dentate nucleus in patient 2. Some FUS-ir granular deposits were observed in the neuronal cytoplasm and neuritis (Fig. 5D). The BIs were slightly positive or negative for ubiquitin, while negative for TDP-43, α-internexin, AT8, and α-synuclein immunoreactivity. Some FUS-ir glial inclusions were observed in the cerebral cortex and the white matter in patient 1. The FUS-ir NCIs of the cerebral cortex in patient 1 appeared mainly in the frontal cortex, including the primary motor cortex and, to some extent, in the superior parietal lobe but rarely in the primary somatosensory cortex, temporal cortex or occipital cortex. There was no immunoreactivity in the granular neurons, while a few pyramidal neurons of the hippocampus were immunoreactive for FUS.

4.4. Electron microscopical findings of BIs

The BIs in patient 1 were loose clusters of filamentous structures associated with granules and had no limiting membrane (Fig. 5E). The BIs occasionally contained cytoplasmic organelles, such as mitochondria, but no neurofilaments with side arms or twisted tubules were found in them (Fig. 5F).

5. Discussion

All three patients in the family described in this case study developed limb weakness followed by bulbar palsy at a young age with a very rapid progression. In patient 1, the *FUS* P525L mutation was identified. Therefore, although a gene analysis was not conducted in the other two affected patients, it seems likely that they had the same *FUS* mutation. This clinical phenotype is similar to that of the already reported juvenile ALS patients (Table 2) [1–4,6,10–16]. Furthermore, similar to patient 1, some reported juvenile ALS patients had developmental delays [1,3,6,14], which suggests an early manifestation of the disease.

Despite the short disease duration in patient 2 and the juvenile ALS patients (Table 2) [1–4,6,12–16], their lower motor neuron degeneration was severe (Fig. 4D, E), which seems to be reflected in their very rapid progression of paralysis. Without obvious symptoms of upper motor neuron impairment, the corticospinal tract of patient 2 and patient 8 [6] in Table 2 showed very active mobilization of macrophages (Fig. 4F). These findings suggest that the lower motor neuron degeneration developed earlier than the upper motor neuron degeneration, and both of them were very active. Therefore, in patient 1, it is probable that the very severe degeneration of both the lower and upper motor neurons (Fig. 4A–C), including the primary motor cortex (Fig. 3C) came from those very active features in the early stages of the disease.

Furthermore, patient 1 showed severe involvement of multiple systems (Table 1). In all of the regions of patient 2, neuronal loss and gliosis were slight, whereas frequent BIs/FUS-ir NCIs appeared (Table 1). In contrast, the limbic system was preserved with rare BIs/FUS-ir NCIs. These findings suggest that the neuronal loss was preceded by the appearance of the BIs/FUS-ir NCIs. In the juvenile ALS patients (Table 2) [1–4,12,14], although various regions besides motor neurons had the BIs/FUS-ir NCIs, these regions did not showed neuronal loss. The disease duration of the juvenile ALS patients was shorter than patients of this case study because the patients of this case study used mechanical ventilation for more than one year. These results might indicate that the initiation of a neuronal loss needs some period of time from the appearance of the BIs/FUS-ir NCIs to occur.

Considering the neuroanatomical lesions, the marked fiber loss in the brainstem and spinal cord (Fig. 4A) of patient 1 appeared similar to respirator-assisted long-survival ALS patients, who progressed into a totally locked-in state [8,17–20]. However, their neuroanatomical lesions are various. Although all the patients in these previous studies [8,17–20] showed severe degeneration of the globus pallidus, substantia nigra, and subthalamic nucleus, degeneration of the striatum and the brain atrophy were not present or were mild [8,17–19], except in one patient [20]. This last patient showed the marked frontal lobe atrophy and the multiple system degeneration including the striatum that were also observed in patient 1. However, the patient from the previous study had widespread TDP-43-ir NCI [20]. Therefore, it was suggested that the degeneration of the striatum was correlated with the frontal atrophy of either FUS or TDP-43 pathology.

In view of the connections among neurons, the striatum is well known to be the main entry point from the motor-related cortex and connected by the radiating fibers, which come from the deep cortical layers. The loss of radiating fibers in patient 1 corresponded to this feature. The other degenerated lesions in the pontine nucleus and subthalamic nucleus were also connected with the motor-related cortex. Furthermore, in the upper cortical layer, degeneration was contributed to loss of the association fibers, which connected the cerebral cortex. Therefore, the loss of the radiating and associated fibers in the primary motor cortex and the related cortex might induce multiple system involvement, concomitantly with the cerebral white matter atrophy.

Regarding the respirator-assisted long-survival ALS with the other mutation in the FUS gene, there has been one ALS patient with the *FUS* R521C mutation. This patient became quadriplegic with ophthalmoparesis and neuropathologically showed multiple systems degeneration with the BIs/FUS-ir NCI and FUS-ir glial cytoplasmic inclusions [7]. Among the findings of the present case study, the feature of ganglion cell loss of the dorsal root ganglia posterior column degeneration was not observed. Another study has described a patient suggested to have the *FUS* R521C mutation, with similar findings [21]. Her disease duration was only three years without using mechanical ventilation. Therefore, posterior column degeneration is considered to be a characteristic of ALS with the

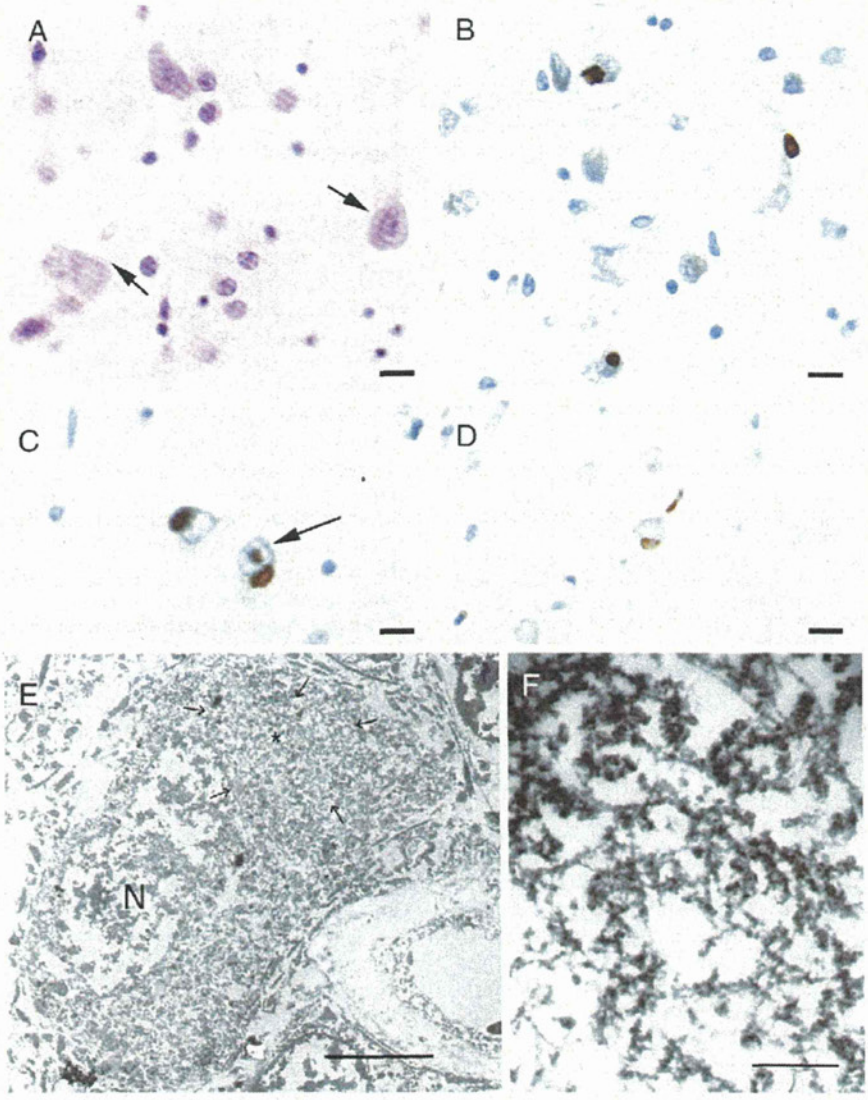

Fig. 5. Basophilic cytoplasmic inclusions (BIs). (A) BIs (arrows) are round (hematoxylin and eosin staining). (B) BIs are strongly immunoreactive (ir) for fused in sarcoma (FUS). (A, B: primary motor cortex of patient 1). (C) FUS-ir neuronal intranuclear inclusions are small and round. (D) FUS-ir deposits are rarely found in neurites. (C, D: parietal cortex of patient 1. A–D: bar = 10 μm). (E) Electron micrograph of a BI (*arrows*). Note its globular shape with no limiting membrane. (Pons of patient 1. N: nucleus, *BI, bar = 5 μm). (F) At higher magnification, the filamentous structures appeared to be straight or curved with a diameter of range from 15 to 25 nm and had no side arm or constriction. The granules were 20–30 nm in diameter. (Inferior frontal cortex of patient 1, bar = 500 nm, C, D: uranyl acetate and lead citrate staining.)

FUS R521C mutation. According to this feature, it is probable that there are some differences in the disease phenotype between ALS patients with the *FUS* P525L mutations and those with R521C mutations. Furthermore, the findings of ALS patients with the *FUS* R521C mutations are of interest because posterior column degeneration is known to be the characteristic of ALS patients with a mutation of the copper/zinc superoxide dismutase (*SOD1*) gene. A patient with the *SOD1* V118L mutation, who progressed into a totally locked-in state, showed posterior column degeneration [19]. Based on the finding that posterior column degeneration is characteristic in ALS patients with either *FUS* or *SOD1* gene mutations, the disease phenotype may have some factors besides gene mutation.

Although variability in the morphology and tinctorial characteristics of BIs has been noted [4], the ultrastructural profiles of BIs in the present patients were essentially similar to those of BIs in other diseases that were recently reported to have a FUS-ir pathology [22]. The diseases with BIs and a FUS-ir pathology include juvenile sporadic ALS [2,6,14], adult-onset sporadic ALS [23,24], and generalized variants of Pick's disease [25], which are now classified as basophilic inclusion body disease subtypes of FTLD-FUS [5,26]. Furthermore, the findings of striatum and frontal lobe atrophy with frequent FUS-ir NCIs [5,22–29] were the same as the findings observed in the present patients. However, the preserved amygdala and hippocampus and no vermiform FUS-ir neuronal intranuclear inclusions, a consistent feature of FTLD-FUS observed in the present patients were different from the findings of FTLD-FUS patients [5,26]. Therefore, ALS with the *FUS* P525L mutation might have a partially common pathology to FTLD-FUS.

In conclusion, we examined three members in a family with a very rapid progressive ALS with the *FUS* P525L mutation. In contrast to patient 1, who progressed into a totally locked-in state and showed multiple systems degeneration in addition to marked motor neuron degeneration, patient 2 died in the early stage of the disease and showed very active motor neuron degeneration and slight multiple systems degeneration with frequent BIs/FUS-ir NCIs. The clinicopathological findings of these patients indicate that the degeneration

Table 2
Clinical features and distribution of basophilic cytoplasmic inclusions of juvenile amyotrophic lateral sclerosis.

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	FUS P525L mutation							#	Sporadic juvenile ALS with basophilic cytoplasmic inclusions										
Family history	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Age at onset (years)	13	27	35	22	16–32	22	18	13	18	12	16	25	21	24	15	19	22	11	14
Gender	W	W	W	NR	2 W, 2 M	W	W	W	W	W	W	W	W	W	M	W	W	W	M
Duration *respiratory assist (months)	312 *294	28 * 14	6	6	<12 in 3, <24 in 1	10	11	20 * 4 (NIPPV)	6	12	12	37 * 27	4	7	18	32 *several	6	12	<12
Developmental delay	+	NR	–	NR	–	–	–	+	+	+	–	–	+	–	–	–	+	–	–
Initial symptom	LE	UE	UE	NR	UE, Bulbar	Hip	UE	LE	UE	UE	UE	UE	UE	UE	NR	UE	UE	UE	UE
Atypical symptoms as ALS	Ophthalmoparesis	–	NR	NR	–	–	–	–	–	–	Autonomic symptom	–	–	–	–	Dementia, ophthalmoparesis, autonomic symptom	–	–	–
Apearance of FUS-immunoreactive neuronal cytoplasmic inclusions or basophilic inclusions																			
Motor cortex	+	NR	No	No	No autopsy	+	+	+	+	+	+	+	+	+	+	Various locations	–	–	–
Lower motor neurons	+	+	autopsy	autopsy		+	+	+	+	+	+	+	+	+	+		+	+	+
Basal ganglia	+	+				+	–	–	–	–	+	–	–	–	–		–	–	–
Pontine nucleus	+	+				+	+	–	+	+	+	–	–	–	–		–	–	–
Cerebellar dentate nucleus	+	+				+	+	–	NR	+	+	–	–	–	–		–	–	–
Substantia nigra	–	+				+	+	–	NR	+	+	+	–	+	–		–	–	–
Reference		Present patients		[10]	[11]	[3,4]	[3,4]	[6]	[3,4]	[1]	[2]	[3]	[6]	[12]	[13]	[14]	[14]	[15]	[16]

W: woman, M: man, NIPPV: non – invasive positive pressure ventilation, NR: not recorded, LE: lower exteremities, UE: upper extremities.
Deletion mutation in exon 15 of the *FUS* gene.

spread over multiple systems during ALS with the *FUS* P525L mutation is caused by not only the appearance of the BIs/FUS-ir NCIs but also the degeneration of the neuronal connection from the primary motor cortex and related areas. The degeneration of the striatum might be considered to be the cause of frontal lobe degeneration in ALS patients.

Conflict of interest

The authors have no conflicts of interest to report.

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Co-occurrence of argyrophilic grain disease in sporadic amyotrophic lateral sclerosis

K. Soma*, Y.-J. Fu*, K. Wakabayashi§, O. Onodera†, A. Kakita‡ and H. Takahashi*

**Department of Pathology, and Departments of †Molecular Neuroscience and ‡Pathological Neuroscience, Center for Bioresources, Brain Research Institute, University of Niigata, Niigata, and §Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki, Japan*

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Co-occurrence of argyrophilic grain disease in sporadic amyotrophic lateral sclerosis

Aims: Phosphorylated TDP-43 (pTDP-43) is the pathological protein responsible for amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease. Recently, it has been reported that accumulation of pTDP-43 can occur in the brains of patients with argyrophilic grain disease (AGD), in which phosphorylated 4-repeat tau is the pathological protein. To elucidate the association of ALS with AGD, we examined the brains from 37 consecutively autopsied patients with sporadic ALS (age range 45–84 years, mean 71.5 ± 9.0 years). **Methods:** Sections from the frontotemporal lobe were stained with the Gallyas-Braak method and also immunostained with antibodies against phosphorylated tau, 4-repeat tau and pTDP-43. **Results:** Fourteen (38%) of the 37 ALS patients were found to have AGD. With regard to staging, 5 of these 14 cases were rated as I, 4 as II and 5 as III. pTDP-43 immunohistochemistry revealed the presence of positive neuronal and glial cytoplasmic inclusions in the affected medial temporal lobe in many cases (93% and 64%, respectively). On the other hand, pTDP-43-positive small structures corresponding to argyrophilic grains were observed only in one case. A significant correlation was found between AGD and the Braak stage for neurofibrillary pathology (stage range 0–V, mean 2.1). However, there were no significant correlations between AGD and any other clinicopathological features, including dementia. **Conclusions:** The present findings suggest that co-occurrence of AGD in ALS is not uncommon, and in fact comparable with that in a number of diseases belonging to the tauopathies or α-synucleinopathies.

Keywords: α-synuclein, amyotrophic lateral sclerosis, argyrophilic grain disease, dementia, tau, TDP-43

Introduction

Amyotrophic lateral sclerosis (ALS), most cases of which are sporadic, is now recognized as an adult-onset TDP-43 proteinopathy widely affecting the central nervous system, including the motor and non-motor neurone systems [1,2]. It is also known that involvement of the medial temporal lobe is a feature of some ALS cases [3,4], and that this may be related to dementia [2]. On the other hand, argyrophilic grain disease (AGD), which was first described by Braak and Braak as an adult-onset form of dementia [5,6], is a sporadic 4-repeat tauopathy affecting the medial temporal lobe [7,8].

Recently, Yokota *et al.* reported the co-occurrence of AGD in ALS in a 69-year-old man as a clinicopathologically rare and important case [9], with a review of similar cases reported previously [10,11]. Subsequently, Fujishiro *et al.* reported accumulation of phosphorylated TDP-43 (pTDP-43), with a frequency of 60% and a distribution pattern similar to that of argyrophilic grains, in the brains of patients with AGD [12]. Considering these reports, it

Correspondence: Hitoshi Takahashi, Department of Pathology, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan. Tel: +81 25 227 0633; Fax: +81 25 227 0817; E-mail: hitoshi@bri.niigata-u.ac.jp

now appears necessary to determine the actual frequency of AGD co-occurrence in patients with sporadic ALS.

Materials and methods

Subjects

In the present study, we examined 37 consecutively autopsied patients with sporadic ALS (25 men and 12 women) seen at our department between 2000 and 2008. In each case, the previous pathological diagnosis of ALS [13] was further confirmed by pTDP-43 immunohistochemistry [2]. The patients ranged in age from 45 to 84 years (mean 71.5 ± 9.0 years) and their disease durations ranged from 8 to 180 months (median, 22 months). Six patients had been receiving respirator support (duration 19–157 months). Mild to moderate dementia had been noted in 11 of the patients during the illness. Twenty-six cases showed pTDP-43-positive cytoplasmic inclusions in the hippocampal dentate granule cells, and these cases were classified as ALS with temporal lesions. No significant correlation was found between dementia and temporal lesions (Fisher's exact test). Moreover, with additional immunostaining procedures for diagnostic neuropathology, five patients were found to have had another distinct pathological process, including Alzheimer's disease (AD, Braak stage V/C [14,15]; one case), Parkinson's disease (PD, subclinical; three cases) and progressive supranuclear palsy (PSP, clinically suspected; one case). All but one case had Alzheimer neurofibrillary pathology (pretangles/tangles); the stages ranged from 0 to V (mean 2.1) [14,15]. No significant correlation was found between the Braak stage and dementia (Mann–Whitney *U*-Test).

Histopathological examination

In all cases, we newly prepared 4-μm-thick, paraffin-embedded sections of the frontotemporal lobe cut coronally at two levels: the amygdaloid and lateral geniculate body. These sections were stained with the Gallyas-Braak method and also immunostained by the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector, Burlingame, CA, USA), using mouse monoclonal antibodies against phosphorylated tau (pTau) (AT8; Innogenetics, Ghent, Belgium; 1:200), 4-repeat tau (RD4; Upstate, Charlottesville, VA, USA; 1:100) and pTDP-43 (pS409/410; Cosmo Bio Co., Ltd, Tokyo, Japan; 1:5000). Diaminobenzidine (DAB) was used as the chromogen. When the presence of argyrophilic grains and/or RD4-positive grain-like structures was confirmed in the individual cases examined, sections from additional brain regions were cut and similarly processed for histopathological examination to obtain the stage of AGD [16].

Statistical analysis

All the statistical analyses were conducted using SPSS software for Windows version 17.0 (SPSS Inc, Chicago, IL, USA). Student's *t*-test, Mann–Whitney *U*-Test, χ^2-test or Fisher's exact test was used for comparison of clinicopathological findings between ALS cases with and without AGD. The level of statistical significance was set at $P < 0.05$.

Results

Argyrophilic grain disease lesions [7,8] were detected in 14 (38%, 10 men and 4 women) of the 37 ALS patients; among these 14 patients, one (case 6) was also found to have AD (Braak stage V/C). Eleven (79%) of the 14 patients belonged to the subgroup with temporal lesions. With regard to the stage of AGD [16], 5 of the 14 cases were rated as I, 4 as II and 5 as III (mean 2.0). These clinical and pathological findings in the 14 cases of ALS with AGD are summarized in Table 1.

Histologically, in a case of ALS with most prominent and advanced AGD (case 11, stage III), for example, severe neuronal loss and gliosis were evident in the amygdala (Figure 1A), ambient gyrus (Figure 1B), and the anterior temporal, entorhinal and transentorhinal cortices. Many Gallyas-Braak-positive argyrophilic grains were also evident in the hippocampal CA1 (Figure 1C) and subiculum. Ballooned neurones were observed in the amygdala, ambient gyrus, anterior temporal cortex, insula (Figure 1D) and other areas.

Immunohistochemically, the presence of pTau-positive lesions, both neuronal (pretangles/tangles) and glial (coiled bodies and/or bush-like astrocytes), was evident in the affected medial temporal lobe in all 14 cases (Figure 2A,B). Ballooned neurones were often positive for pTau. RD4 immunohistochemistry clearly revealed the presence of small round-to-oval or comma-shaped structures corresponding to argyrophilic grains in the neuropil (Figure 2C). pTDP-43-positive neuronal and glial cytoplasmic inclusions (NCI and GCI) were also observed in

Table 1. Clinical and pathological findings in 14 cases of sporadic amyotrophic lateral sclerosis with argyrophilic grain disease

Case no.	*Age at death (years)*	*Gender*	*Disease duration (months)*	*Respirator (months)*	*Dementia*	*Brain weight (g)*	*NFP Braak stage*	*Temporal lesion*	*AGD Saito stage*	*Additional pathology*
1	45	Female	17	–	–	1270	I	–	I	–
2	82	Female	24	–	–	1100	III	+	I	–
3	70	Male	26	19	–	1550	III	+	II	–
4	74	Male	12	–	–	1350	III	+	III	–
5	76	Male	20	–	–	1350	II	+	I	–
6	79	Female	12	–	+	1150	V	–	III	AD
7	76	Male	156	141	–	1050	II	+	II	–
8	74	Male	17	–	–	1055	II	+	I	–
9	78	Female	22	–	–	1130	III	+	III	–
10	72	Male	120	–	–	1230	I	+	II	–
11	67	Male	26	–	+	1225	III	+	III	–
12	65	Male	26	–	–	1180	III	+	I	–
13	73	Male	9	–	+	1160	III	+	II	–
14	78	Male	12	–	+	1220	III	–	III	–

Temporal lesion, defined as occurrence of phosphorylated TDP-43-positive cytoplasmic inclusions in hippocampal dentate granule cells. NFP, neurofibrillary pathology [14,15]; AGD, argyrophilic grain disease [16]; AD, Alzheimer's disease; –, absent; +, present.

the medial temporal lobe, the former being observed in 13 (93%) of the 14 cases and the latter in 9 (64%) (Figure 2D,E). No pTDP-43-positive bush-like astrocytes were found, and pTDP-43-positive structures corresponding to argyrophilic grains were detected in only one case (case 13) (Figure 2F). These neuropathological findings in the 14 cases of ALS with AGD are summarized in Table 2.

A significant correlation was found between AGD and the Braak stage for neurofibrillary pathology (Mann–Whitney *U*-Test, $P = 0.01$). However, there were no significant correlations between AGD and any other clinicopathological factors, including age at death, gender, disease duration, artificial respiratory support, dementia or temporal lesions. There was also no evident significant correlation between the presence of temporal lesions/AGD (11 cases) and dementia.

Discussion

It has been recognized that AGD is an age-associated disease, with or without dementia [7,8,16–18]. Knopman *et al.* reported that 12 (31%) of 39 selected cognitively normal elderly individuals (age range 74–95, median 85 years) had AGD [17]. Saito *et al.* also reported 449 (36%) of 1241 serial autopsy cases (age range 48–104, mean 86.0 ± 8.9 years) from a geriatric hospital had AGD [16]. However, its presence has been estimated at 5–9% in non-selected consecutive adult autopsy series [19–21]. Recently, in a review article, Ferrer *et al.* [8] mentioned that in their series of 1000 consecutive autopsy cases from an adult general hospital, the incidence of AGD was 4%, and that its age distribution was: <60 years, 10%; 61–≤70 years, 17%; 71–≤80 years, 30%; and >80 years, 43%.

Argyrophilic grain disease has also been reported in a number of neurodegenerative disorders, including both tauopathies and α-synucleinopathies [7,8]. Togo *et al.* found AGD in 4 (3%) of 144 AD cases in a single series [22], and in 10 (3%) of 354 AD cases in another [23]. On the other hand, Fujino *et al.* studied 239 AD cases (Braak stage IV–VI, mean 5.4 ± 0.0) using a specific antibody against 4-repeat tau and found AGD in 61 cases (26%), indicating a significantly higher incidence of AGD in AD [24]. Recently, Saito *et al.* also found AGD in 30 (29%) of 105 AD cases; however, the incidence was not significantly higher than in the background population of their study, which was based on 1241 cases [16]. With regard to PSP and corticobasal degeneration (CBD), Togo *et al.* found AGD in 22 (19%) of 177 PSP cases and in 7 (41%) of 17 CBD cases [23]; these two incidences were reported to be significantly higher (χ^2-test) than those reported previously in studies of large series of non-selected autopsy cases [19–21].

Wakabayashi *et al.* found AGD in 5 (19%) of 26 cases of multiple system atrophy [25], the incidence being significantly higher (χ^2-test) than that in the same age group studied by Braak and Braak [21]. Saito *et al.* found AGD

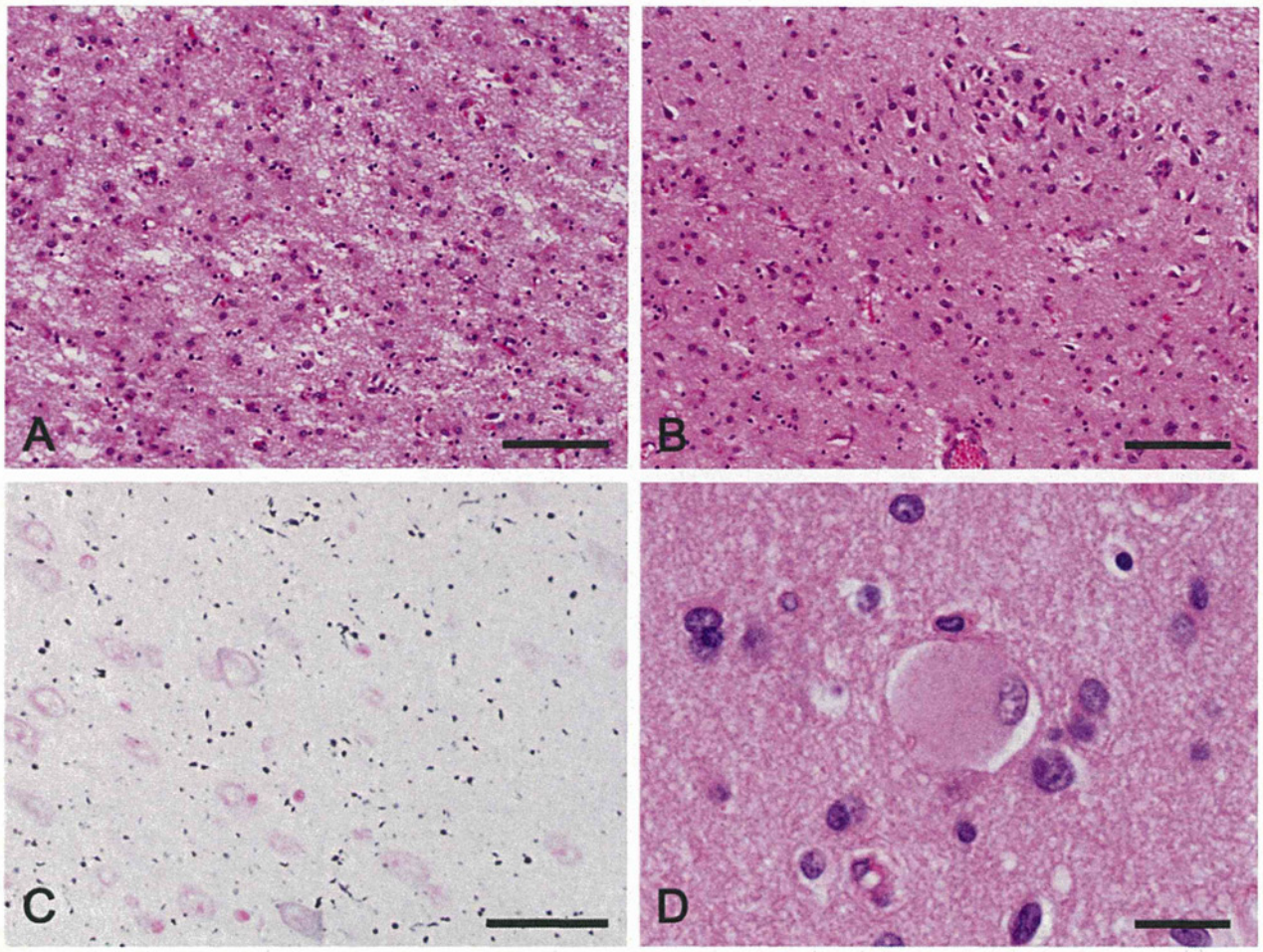

Figure 1. Histological findings obtained from case 11. Sections stained with haematoxylin and eosin (A, B, D), and the Gallyas-Braak method (C). (A, B) Neuronal loss and gliosis are evident in the amygdala (A) and ambient gyrus (B). Bars = 100 μm. (C) Many argyrophilic grains are evident in the hippocampal CA1 area. Bar = 50 μm. (D) A ballooned neurone observed in the insular cortex. Bar = 20 μm.

in 22 (65%) of 34 cases of dementia with Lewy bodies, the incidence being significantly higher than in the background population of their study; in this connection, they found AGD in 2 (20%) of 10 cases of PD [16].

With regard to the co-occurrence of AGD in ALS, there have so far been no studies of large case series. Martinez-Lage and Munoz, and Saito *et al.* reported an incidence of 8% (1 of 13 cases) in motor neurone disease and of 22% (2 of 9 cases) in ALS. In the present study, we detected AGD in 14 (38%) of 37 ALS cases. One finding of interest was the presence of AGD in a 45-year-old patient with ALS. Two previous studies of large series of non-selected adult autopsy cases revealed no AGD in individuals aged ≤50 years [20,21]. In the present study, it was also noteworthy that the incidence of AGD in the 71–≤ 80 year age group was 50% (9 of the 18 ALS cases), which was much higher than the incidence of 30% found in the same age group by Ferrer *et al.* [8] and significantly higher (χ^2-test, $P < 0.001$) than the 7% (45/605 cases) recorded for the same age group by Braak and Braak [21].

In the present study, pTDP-43-positive NCI and GCI were observed in the medial temporal lobe in many cases of ALS with AGD; however, pTDP-43-positive structures corresponding to argyrophilic grains were found in only one case (case 13). It was actually impossible to determine whether these pTDP-43-positive NCI and/or GCI (only oligodendrocytic coiled bodies being evident) were attributable to ALS or to AGD. Such pTDP-43-positive neuronal and glial lesions have also been observed in cases of ALS

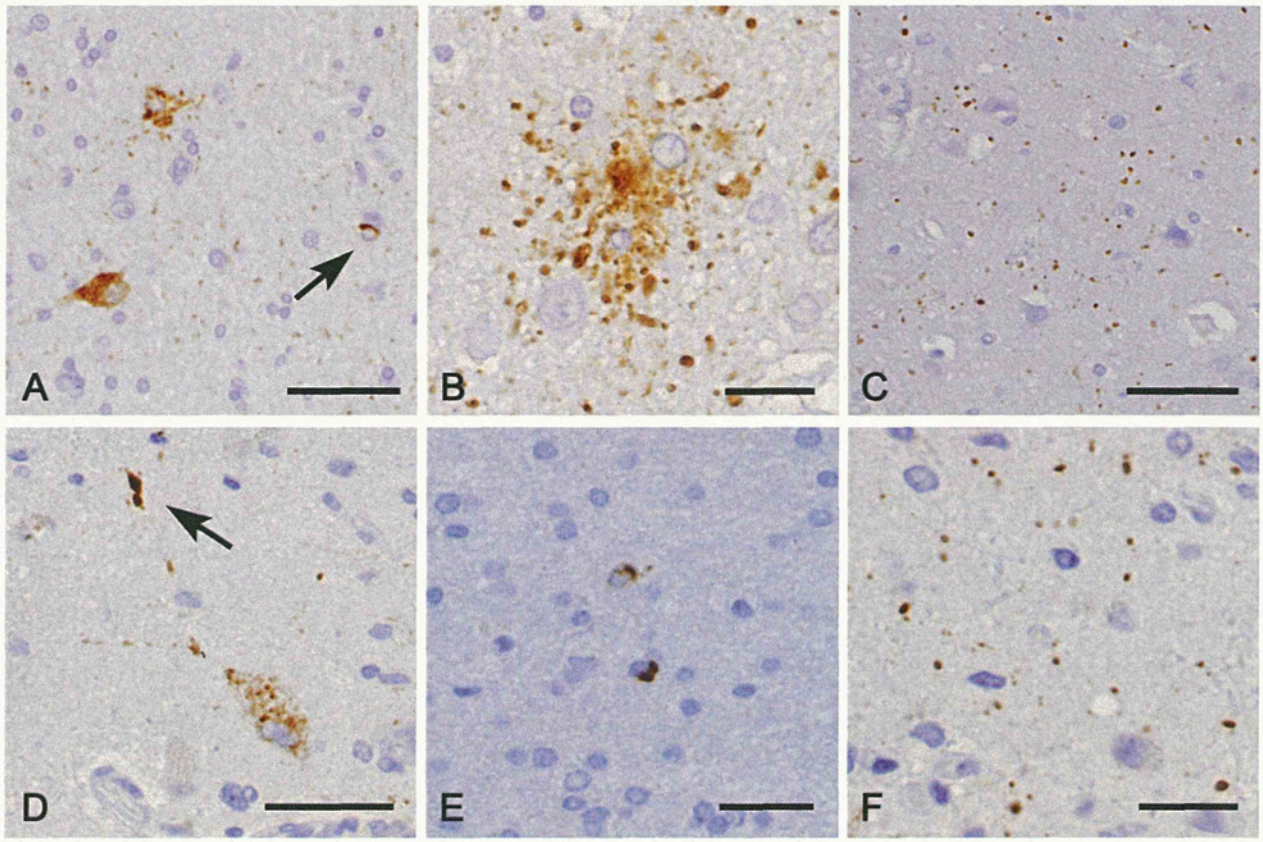

Figure 2. Immunohistochemical findings obtained from case 13. Sections stained with AT8 (A, B), RD4 (C) and pS409/410 (D, E, F). (A) pTau-positive structures, two pretangles and one coiled body (arrow), observed in the amygdala. Bar = 50 μm. (B) Two pTau-positive neighbouring bush-like astrocytes observed in the hippocampal CA1 area. Bar = 20 μm. (C) Many 4-repeat tau-positive small structures corresponding to argyrophilic grains observed in the hippocampal CA1. Bar = 50 μm. (D) A neurone possessing pTDP-43-positive granular cytoplasmic inclusions observed in the amygdala; arrow indicates grain-like structures. Bar = 40 μm. (E) Two oligodendrocytes possessing pTDP-43-positive cytoplasmic inclusions observed in the white matter near the amygdala. Bar = 25 μm. (F) Many pTDP-43-positive small structures corresponding to argyrophilic grains observed in the amygdala. Bar = 20 μm.

with temporal lesions [2]. In addition to pTDP-43-positive grains (case 13), it could only be considered that the pTDP-43-positive NCI observed in two cases of ALS without temporal lesions (cases 6 and 14) were a manifestation of AGD (Table 2).

In the present study, a significant correlation was found between AGD and the Braak stage for neurofibrillary pathology (stage range 0–V, mean 2.1). This finding would simply imply that neurofibrillary pathology, especially the pretangles, is a constant feature in the affected medial temporal lobe in cases of AGD [7,8].

In conclusion, we have shown that the co-occurrence of AGD in ALS is not uncommon, and in fact is comparable to that reported previously in tauopathies, including AD, PSP, CBD, as well as in α-synucleinopathies, including multiple system atrophy and dementia with Lewy bodies. The present findings, together with those of our previous study employing a large series of autopsy cases of ALS [13], strongly suggest a recent increase in the frequency of occurrence of ALS in aged individuals, in whom ALS pathology with temporal lesions or AGD, or with both, may not be uncommon, irrespective of the presence of dementia. Further studies on the association of ALS with AGD, in which many cases have been reported to show pTDP-43-positive NCI and GCI in the affected medial temporal lobe [12], are needed not only for broadening the picture of conditions encountered in neurology and neuropathology [9–11] but also for gaining a better understanding of ALS in relation to other TDP-43 proteinopathies [26,27].

Table 2. Pathological findings in the medial temporal lobe in 14 cases of sporadic amyotrophic lateral sclerosis with argyrophilic grain disease

Case no.	*Neuronal loss*	*Grains*	*Ballooned neurones*	*Coiled bodies*	*Bush-like astrocytes*	*Pretangles*	*Phosphorylated TDP-43*			
							NCI	*DN*	*GCI*	*Grains*
1	+ (−, −)	+ (+, −)	+ (−, −)	+ (+, −)	+ (−, −)	+ (+, +)	−	−	−	−
2	+ (−, +)	+ (+, +)	+ (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (−, +)	+ (−, −)	+ (−, +)	−
3	+ (−, −)	+ (+, +)	+ (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (+, +)	−	−	−
4	+ (−, +)	+ (++, ++)	+ (−, −)	+ (+, ++)	+ (−, −)	+ (+, +)	+ (+, +)	+ (−, −)	+ (−, +)	−
5	+ (+, +)	+ (+, −)	+ (−, −)	− (−, −)	+ (−, −)	+ (+, −)	+ (+, +)	−	+ (+, +)	−
6	+ (+, +)	+ (++, ++)	+ (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (−, −)	−	−	−
7	+ (−, −)	+ (+, +)	+ (−, −)	+ (−, −)	+ (−, −)	+ (+, +)	+ (−, +)	−	−	−
8	+ (−, −)	+ (+, −)	+ (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (+, +)	−	+ (−, +)	−
9	+ (+, +)	+ (+++, +)	+ (−, −)	+ (+, +)	+ (−, −)	+ (+, +)	+ (+, +)	−	+ (+, −)	−
10	+ (+, ++)	+ (+, +)	+ (−, −)	+ (+, +)	+ (−, +)	+ (+, +)	+ (+, +)	−	+ (+, +)	−
11	+ (+, +)	+ (+++, +++)	+ (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (+, +)	−	+ (+, +)	−
12	+ (−, −)	+ (+, −)	− (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (+, +)	−	+ (+, +)	−
13	+ (+, ++)	+ (++, +)	+ (−, −)	+ (+, +)	+ (+, +)	+ (+, +)	+ (+, +)	−	+ (+, +)	+ (+, −)
14	+ (−, +)	+ (+, ++)	+ (−, −)	+ (+, +)	+ (−, +)	+ (+, +)	+ (+, +)	−	−	−

Pathological changes in the hippocampal CA1 (left) and subiculum (right) are shown in the parentheses; −, absent; +, mild; ++, moderate; +++, severe.
NCI, neuronal cytoplasmic inclusions; DN, dystrophic neurites; GCI, glial cytoplasmic inclusions; −, absent; +, present.

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Original Article

Primary lateral sclerosis: Upper-motor-predominant amyotrophic lateral sclerosis with frontotemporal lobar degeneration – immunohistochemical and biochemical analyses of TDP-43

Takayuki Kosaka,[1] Yong-Juan Fu,[1] Atsushi Shiga,[1] Haruka Ishidaira,[2] Chun-Feng Tan,[1] Takashi Tani,[5] Ryoko Koike,[5] Osamu Onodera,[3] Masatoyo Nishizawa,[2] Akiyoshi Kakita[4] and Hitoshi Takahashi[1]

Departments of [1]Pathology and [2]Neurology, [3]Molecular Neuroscience and [4]Pathological Neuroscience (Center for Bioresources), Brain Research Institute, University of Niigata, and [5]Department of Neurology, Nishi-Niigata Chuo National Hospital, Niigata, Japan

Primary lateral sclerosis (PLS) is clinically defined as a disorder selectively affecting the upper motor neuron (UMN) system. However, recently it has also been considered that PLS is heterogeneous in its clinical presentation. To elucidate the association of PLS, or disorders mimicking PLS, with 43-kDa TAR DNA-binding protein (TDP-43) abnormality, we examined two adult patients with motor neuron disease, which clinically was limited almost entirely to the UMN system, and was followed by progressive frontotemporal atrophy. In the present study, the distribution and severity, and biochemical profile of phosphorylated TDP-43 (pTDP-43) in the brains and spinal cords were examined immunohistochemically and biochemically. Pathologically, in both cases, frontotemporal lobar degeneration with ubiquitin inclusions (FTLD-U) was evident, with the most severe degeneration in the motor cortex. An important feature in both cases was the presence of Bunina bodies and/or ubiquitin inclusions, albeit very rarely, in the well preserved lower motor neurons. The amygdala and neostriatum were also affected. pTDP-43 immunohistochemistry revealed the presence of many positively stained neuronal cytoplamic inclusions (NCIs) and dystrophic neurites/neuropil threads in the affected frontotemporal cortex and subcortical gray matter. By contrast, such pTDP-43 lesions, including NCIs, were observed in only a few lower motor neurons. pTDP-43 immunoblotting revealed that fragments of ~25-kDa were present in the cortices, but not in the spinal cord in both cases. Genetically, neither of the patients had any mutation in the *TDP-43* gene. In conclusion, we consider that although PLS may be a clinically significant disease entity, at autopsy, the majority of such clinical cases would present as upper-motor-predominant amyotrophic lateral sclerosis with FTLD-TDP.

Key words: amyotrophic lateral sclerosis, Bunina body, frontotemporal lobar degeneration, primary lateral sclerosis, TDP-43.

INTRODUCTION

Primary lateral sclerosis (PLS) has been considered a rare form of motor neuron disease (MND) that selectively affects the upper motor neuron (UMN) system.[1–3] However, the issue of whether or not PLS represents a nosological entity appears to be problematic;[4,5] PLS may represent one end of a continuous spectrum of amyotrophic lateral sclerosis (ALS), which is a common from of MND that affects both the upper and lower motor neuron systems.[6] In recent years, new diagnostic categories of UMN-predominant MND[7] and diagnostic criteria for PLS[8] have been proposed, the goals of which appear to be distinction between PLS and ALS. Although neurologists have emphasized the substantial merits of establishing, a

Correspondence: Hitoshi Takahashi, MD, Department of Pathology, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan. Email: hitoshi@bri.niigata-u.ac.jp

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clinical diagnosis of PLS,[7–11] ubiquitin immunohistochemistry of autopsy cases of PLS, or disorders mimicking it, have failed to demonstrate a phenotype that represents pathologically pure PLS.[8,12–14] In the established diagnostic categories of UMN-predominant MND (2006),[7] autopsy-proven PLS is defined as clinically diagnosed PLS with degeneration of the motor cortex and corticospinal tracts, lack of motor neuron loss, lack of gliosis in anterior horn cells and lack of Bunina bodies or ubiquitinated inclusions. Again, to our knowledge, there have so far been no such clinicopathological examples of PLS.

On the other hand, a recent review article has pointed out that PLS is heterogeneous in its clinical presentation, and that degeneration is not restricted to the UMN system;[12] dementia (frontotemporal lobar degeneration with ubiquitin inclusions: FTLD-U)[14,15] or parkinsonian symptoms[16,17] may be associated with PLS. Moreover, in 2006, a major breakthrough in our understanding of the pathogenesis of neurodegeneration was reported: a nuclear protein, 43-kDa TAR DNA-binding protein (TDP-43), was identified as the pathological protein responsible for FTLD-U and ALS.[18,19] In 2007, PLS and FTLD/PLS were first subjected to TDP-43 immunohistochemical investigation by Dickson and colleagues.[20,21]

In the present study, we analyzed the clinical and pathological features, as well as the distribution and severity, and biochemical profile of pathological TDP-43, that is, phosphorylated TDP-43 (pTDP-43), in two autopsied patients showing clinical features of PLS, or disorders mimicking it.[7–9,12,14,15] One has already been reported with ubiquitin pathology in 2003[14] and the other was a new patient who we recently encountered (case 1 in the present study). The results, both immunohistochemical and biochemical, appear to be of great interest when considering the patients' disease conditions.

MATERIALS AND METHODS

Patients

Two Japanese female patients were studied. The patients died aged 60 years and 82 years after disease durations of 6 years and 4 months, and 7 years and 4 months, respectively. Neither had a family history of ALS (or MND), dementia or other neurological disease. The clinical features are described below as case reports (cases 1 and 2, respectively).

Neuropathological examination

Diagnostic neuropathology

As mentioned above, the neuropathological findings in case 2 have been previously reported.[14] In case 1, similar procedures were employed for diagnostic neuropathology. The brain and spinal cord were fixed with 20% buffered formalin, and multiple tissue blocks were embedded in paraffin. Histological examinations were performed on 4-μm-thick sections using several stains, including HE, KB and Holzer. Selected sections were also immunostained by the avidin-biotin-peroxidase complex (ABC) method using a Vectastain ABC kit (Vector, Burlingame, CA, USA) with rabbit polyclonal antibodies against ubiquitin (Dako, Glostrup, Denmark; 1:800) and cystatin C (Dako; 1:800), and mouse monoclonal antibodies against phosphorylated tau (AT8; Innogenetics, Ghent, Belgium; 1:200), phosphorylated α-synuclein (no. 64; Wako, Osaka, Japan; 1:10 000) and phosphorylated neurofilament protein (SMI31; Sternberger Monoclonals, Baltimore, MD, USA; 1:1000). Diaminobenzidine was used as the chromogen.

Immunohistocemical study for TDP43

In both cases, the distribution and severity of pTDP-43 lesions were investigated. In each case, we newly prepared 4-μm-thick sections from the original blocks of various brain and spinal cord regions, as well as those from the dorsal root (cervical and/or lumbar) and parasympathetic (paravertebral and/or celiac) ganglia. These sections were immunostained similarly with a mouse monoclonal antibody against phosphoserines 409 and 410 of TDP-43 (pS409/410; Cosmo Bio Co., Ltd, Tokyo, Japan; 1:5000). In addition, several selected sections were immunostained with a rabbit polyclonal antibody against TDP-43 (10782-1-AP; Protein Tech Group Inc., Chicago, IL, USA; 1:4000) for comparison.

Fractionation of frozen human tissues and TDP-43 immunoblotting

Proteins from the temporal and motor cortices and spinal cords of one case of sporadic, typical ALS and the present two cases were extracted, as previously described.[22] Briefly, frozen tissues were homogenized in buffer A [10 mmol Tris-HCl (pH 7.5), 1 mmol ethylene glycol-bis[-β- aminoethylether]-tetra-acetic acid, 1 mmol dithiothreitol, 10% sucrose] and centrifuged at 25 000×*g* for 30 min at 4°C. The resulting pellets were then extracted in buffer A containing 1% Triton X-100 and centrifuged at 180 000×*g* for 30 min at 4°C. These pellets were subsequently homogenized in buffer A containing sarkosyl, incubated for 1 h with gentle agitation, and centrifuged at 180 000×*g* for 30 min at 22°C. The sarkosyl-insoluble pellets were solubilized in 8 mol urea buffer. After centrifugation at 25 000×*g* for 30 min at 22°C, the urea-soluble fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting with the rabbit polyclonal anti-TDP-43 antibody (10782-1-AP; 1:1000) and mouse