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CASE REPORT

Sporadic ALS with compound heterozygous mutations in the *SQSTM1* gene

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Abstract Accumulating evidence suggests that heterozygous mutations in the *SQSTM1* gene, which encodes p62 protein, are associated with amyotrophic lateral sclerosis (ALS). Here, we report a Japanese patient with sporadic, late-onset ALS who harbored compound heterozygous *SQSTM1* mutations (p.[Val90Met];[Val153Ile]). Autopsy examination revealed that although TDP-43 pathology was rather widespread, the selective occurrence of p62-positive/TDP-43-negative cytoplasmic inclusions in the lower motor neurons (LMNs) was a characteristic feature. No Bunina bodies were found. Ultrastructurally, p62-positive cytoplasmic inclusions observed in the spinal anterior horn cells were composed of aggregates of ribosome-like granules and intermingled bundles of filamentous structures. Another feature of interest was concomitant Lewy body pathology. The occurrence of distinct p62 pathology in the LMNs in this patient indicates the pathogenic role of *SQSTM1* mutations in the development of a subset of ALS.

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Introduction

A number of genes, including *SOD1* [4, 16], *TARDBP* (*TDP-43*) [18, 22], *FUS* [9, 21], *UBQLN2* [5], and *C9Orf72* [3, 15] have been identified as the causative genes for familial or sporadic amyotrophic lateral sclerosis (ALS). Recently heterozygous mutations in the sequestosome 1 (*SQSTM1*) gene, which encodes p62 and is one of the causative genes for Paget disease of bone (OMIM#602080), have been reported to be associated with ALS and frontotemporal lobar degeneration (FTLD) [6, 7, 17]. Very recently, autopsy findings of three patients with heterozygous mutations of *SQSTM1* gene have also been reported [20]. However, rare variants of *SQSTM1* have also been observed in normal control individuals [6, 17, 20]. Therefore, it remains obscure whether the *SQSTM1* mutations found in patients with ALS/FTLD are causative of ALS/FTLD [6, 7, 17, 20].

Here, we report the clinicopathological features of a 75-year-old Japanese man with sporadic, late-onset ALS without dementia who harbored compound heterozygous mutations in the *SQSTM1* gene. The distinct p62 pathology observed in the lower motor neurons (LMNs) appeared to be important when considering the role of *SQSTM1* mutations in the development of ALS.

Patient and methods

The present study was conducted in the frame of a study "Neuropathological and Molecular-Genetic investigation of

CNS Degenerative Diseases" approved by the Institutional Review Board of the University of Niigata.

Patient

The patient became aware of weakness of both hands at the age of 72 years. Over the next 6 months, he developed muscle weakness, atrophy and fasciculation in the trunk and all four extremities. On examination, deep tendon reflexes were decreased. Electromyography showed neurogenic changes in the muscles of the upper and lower extremities as well as the tongue. He was then diagnosed as having ALS. His serum IgG level was 1,754 mg/dl (normal value 870–1,700 mg/dl), soluble interleukin 2 receptor (sIL-2R) level was over 5,500 U/ml (normal value 190–650 U/ml), and the presence of M-protein was noted, leading to a diagnosis of concomitant multiple myeloma of the IgG kappa type. The weakness progressed slowly and steadily; even in the late stage, although dyspnea became evident, the patient was able to walk a short distance. Moreover, bulbar symptoms, such as dysphagia, were not evident. He died of respiratory failure at the age of 75. Babinski reflex was first elicited 1 month before death. Dementia, extrapyramidal signs, or autonomic failure were not noted throughout the illness. There were no symptoms or laboratory findings suggestive of Paget disease of bone. There was no history of similar neurological disorders or dementia in the patient's family members, including the parents and five siblings. A general autopsy was performed 1 h after death, at which time the brain weighed 1,315 g. Both lungs showed mild bronchopneumonia.

Neuropathological examination

The brain and spinal cord were fixed with 20 % buffered formalin for 40 days, and multiple tissue blocks were embedded in paraffin. Histological examinations were performed on 4-μm thick sections using several stains, including hematoxylin and eosin and Klüver–Barrera. In each anatomical region, the severity of neuron loss was graded according to the percentage of the lost neurons: −, none; +, less than 40 %; ++, 40–70 %; +++, more than 70 %.

For immunohistochemistry, 4-μm thick selected sections were incubated overnight at 4 °C with one of the primary antibodies listed in Table 1. Pretreatment, if necessary, was performed by heat/autoclaving for 10 min at 121 °C or in a microwave oven for 30 min at 90 °C (both in 10 mM sodium citrate buffer), or in formic acid for 5 min. Bound antibodies were visualized by the peroxidase-polymer-based method using a Histofine Simple Stain MAX-PO kit (Nichirei, Tokyo, Japan) with diaminobenzidine as the chromogen. Immunostained sections were counterstained with hematoxylin. In each anatomical region, the numbers of neuronal cytoplasmic inclusions (NCIs) and glial cytoplasmic inclusions (GCIs), as well as α-synuclein-positive Lewy bodies, were counted per ×100 power field, and graded as follows: −, absent; +, ~2 inclusions; ++, 3–5 inclusions; +++, more than 5 inclusions. In addition, some selected p62-immunostained sections were recycled for conventional electron microscopy.

For double-labeling immunofluorescence study, selected sections were similarly pretreated in a microwave oven and immunostained with monoclonal anti-p62 (1:500), polyclonal anti-ubiquitin (1:400) or anti-TDP-43 (1:2,000). The second antibodies used were Alexa Fluor 555 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA; 1:1,000) and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes; 1:1,000). Slides were treated with an Autofluorescence Eliminator Reagent (Millipore, Billerica, MA, USA), mounted with glass coverslips using VECTAshield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories, Burlingame, CA, USA), and analyzed using a confocal laser-scanning microscope.

Table 1 List of antibodies

Primary antibodies	Host	Dilution	Pretreatment	Source
p62 (3/p62 LCK LIGAND)	m (mc)	1:1,000	Microwave	BD Biosciences, San Jose, CA, USA
TDP-43 (10782-1-AP)	r (pc)	1:4,000	Heat/autoclaving	ProteinTec Group Inc., Chicago, IL, USA
pTDP-43 (pS409/410)	m (mc)	1:3,000	Heat/autoclaving	Cosmo Bio Co., Ltd, Tokyo, Japan
Ubiquitin	r (pc)	1:800	–	Dako, Glostrup, Denmark
Ubiquilin 2 (5F5)	m (mc)	1:10,000	Heat/autoclaving	Novus Biologicals, Litlleton, CO, USA
Phosphorylated α-synuclein (#64)	m (mc)	1:10,000	Formic acid	Wako, Osaka, Japan

m (mc), mouse (monoclonal); r (pc) rabbit (polyclonal)

Mutational analysis of SQSTM1

High molecular weight genomic DNA was extracted after obtaining informed consent from the patient's family members. We amplified all the exons of *SQSTM1* (NM_003900.4) using a series of primers, followed by sequence reaction. In 189 unrelated healthy control subjects, we assessed the presence or absence of the two substitutions in *SQSTM1* identified in the present case, using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA).

Results

Neuropathological findings

The brain appeared normal in external appearance. In sections, mild depigmentation of the substantia nigra and locus ceruleus was noted. The spinal cord and anterior roots were atrophic.

Histological examination revealed that the patient had LMN-predominant ALS. The lateral corticospinal tracts of the spinal cord showed mild degeneration (Fig. 1a). The motor cortex also showed mild loss of Betz cells, as evidenced by the presence of a few Betz cell-sized holes containing lipofuscin-laden macrophages. In the spinal cord and brainstem, varying degrees of LMN loss were observed, being severe in the cervical anterior horns, moderate in the lumbar anterior horns (Fig. 1b), and much milder in the hypoglossal and facial nerve motor nuclei (Fig. 1c). No Bunina bodies were found. The diaphragm showed severe neurogenic muscular atrophy.

Immunohistochemically, large p62-positive NCIs were observed in the LMNs (Fig. 1d). p62-positive "skein-like" NCIs were extremely rare (Fig. 1e). Although less frequently, p62-positive inclusions were also observed in the swollen cell processes (dystrophic neurites) of LMNs, as well as the cytoplasm of glial (oligodendrocytic) cells (GCIs) (Fig. 1f). However, in the LMN nuclei, including the spinal anterior horns, no NCIs or GCIs positive for TDP-43 or phosphorylated TDP-43 (pTDP-43) were evident. Moreover, the LMNs retained endogenous nuclear staining for TDP-43. Immunostaining performed on serial sections confirmed that p62-positive NCIs in the LMNs were negative for TDP-43 (Fig. 1g), pTDP-43, and ubiquilin 2. Double-labeling immunofluorescence also demonstrated that p62-positive NCIs in LMNs were negative for ubiquitin (Fig. 1h) and TDP-43 (Fig. 1i). Ultrastructurally, p62-positive NCIs appeared as aggregates of ribosome-like granules containing scattered bundles of filamentous structures (Fig. 1j). p62-positive NCIs were also present in non-motor neurons. In the pontine, cerebellar dentate and subthalamic nuclei, where no neuronal loss was evident, a few p62-positive NCIs and/or GCIs were found (Fig. 1k). Immunostaining for TDP-43 or ubiquitin showed no positive NCIs or GCIs in these regions.

In the other CNS regions, however, small numbers of TDP-43-positive NCIs, dystrophic neurites and GCIs were encountered (Fig. 1l). The hippocampal dentate granule cells showed no such lesions, and the distribution pattern of TDP-43-positive NCIs was reminiscent of that in ALS (Type 1), except for virtual sparing of LMNs [14]. Although less frequent, p62-positive inclusions similar in morphology and distribution to the TDP-43-positive inclusions were also detected, whereas no such inclusions were detectable by ubiquitin immunohistochemistry.

Cerebral cortical TDP-43 pathology was mild, some NCIs and fewer dystrophic neurites being evident in the frontal lobe, and classifiable as 'Type B' [10]. The hippocampal and cerebellar p62/ubiquilin 2 pathology seen in ALS with *C9orf72* mutations [1, 2, 13] was not evident in the present patient.

In addition, concomitant Lewy body pathology was also present; the patient was considered to have Parkinson's disease (PD). Neuronal loss and gliosis were noted in the substantia nigra, locus ceruleus, and dorsal vagal nucleus. Lewy bodies positive for α-synuclein, ubiquitin and p62 were widespread in the CNS, including the above-mentioned regions (Fig. 1m–o); the α-synuclein pathology was classified as 'limbic type' [11].

The histological and immunohistochemical findings are summarized in Table 2 [22].

Mutation detection

Because of the distinct pathological features revealed by p62 immunostaining, we performed sequence analysis of the *SQSTM1* gene and found two single base-pair substitutions: one at position 268 from G to A (c.268G>A) in exon 2, which resulted in a Val-to-Met substitution at position 90 (p.Val90Met) in the PB1 domain (Fig. 2a), and the other at position 457 from G to A (c.457G>A) in exon 3, which resulted in a Val-to-Ile substitution at position 153 (p.Val153Ile) in the ZZ-type zinc finger domain (Fig. 2b). Sequence analysis of the PCR fragments spanning exons 2 and 3 revealed that these substitutions were located in different alleles. We confirmed that neither p.Val90Met nor p.Val153Ile mutation of *SQSTM1* was present in any of 189 unrelated healthy individuals (378 chromosomes). PolyPhen2 predicted that the p.Val90Met and p.Val153Ile substitutions were possibly damaging and benign, respectively. No mutations were found in other genes associated with ALS, including *TDP-43*, *VCP*, *C9Orf72* or *CHMP2B*.

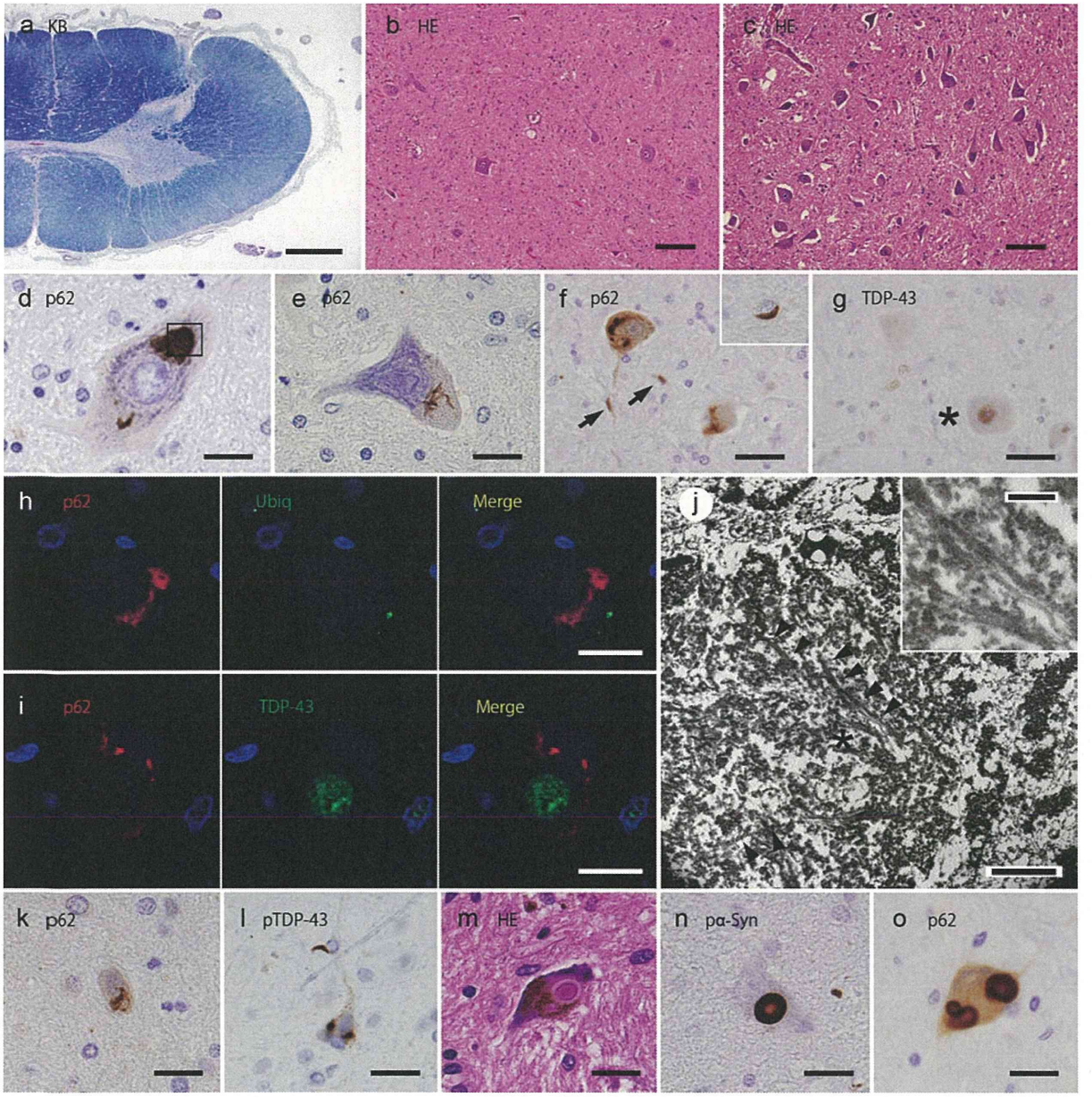

Fig. 1 **a** The cervical cord (C4), showing evident atrophy of the gray matter. Degeneration of the corticospinal tracts (both anterior and lateral) is not evident. **b** The lumbar anterior horn, showing several remaining lower motor neurons (LMNs). **c** The facial motor nucleus, showing relatively well-preserved LMNs. **d** Two p62-positive cytoplasmic inclusions are evident in a lumbar LMN: large (*upper right*) and small ones (*lower left*). **e** p62-positive skein-like inclusions are evident in a lumbar LMN. Two serial sections containing the facial motor nucleus, showing that p62-positive cytoplasmic inclusions in two LMNs (**f**) are negative for TDP-43 (**g**). Two p62-positive swollen cell processes (*arrows*), presumably those of LMNs, are evident (**f**). A glial cell possessing a p62-positive cytoplasmic inclusion is also shown (**f**, *inset*). Note that a LMN bearing p62-positive inclusions (**f**, *lower right*) retains endogenous TDP-43 nuclear staining (**g**, *asterisk*). Double-labeling immunofluorescence. p62-positive NCIs in a hypoglossal nucleus neuron are negative for ubiquitin (**h**). p62-positive NCIs in a neuron in the lumbar anterior horn are negative for TDP-43; note the preservation of endogenous TDP-43 nuclear staining (**i**). Nuclei were stained with DAPI (*blue*) (**h**, **i**). **j** Recycled electron microscopy specimen of the area indicated by the *upper right open square* in **d**, demonstrating aggregates of ribosome-like granules and intermingled bundles of filamentous structures (*arrowheads*). Higher-magnification view of the area indicated by the *asterisk*, showing the filamentous structures (*inset*). k p62-positive NCIs are evident in a subthalamic nucleus neuron. **l** A neuron with pTDP-43-positive inclusions (*lower*) and a glial cell with a cytoplasmic inclusion (*upper*) observed in the frontal cortex. **m** A typical Lewy body with an eosinophilic core and a peripheral halo in a substantia nigra pigmented neuron. **n** A phosphorylated α-synuclein-positive Lewy body in a neuron in the midbrain reticular formation. **o** p62-positive Lewy bodies in a neuron in the medullary reticular formation. *KB* Klüver–Barrera, *HE* hematoxylin and eosin. *Bars* 1 mm for **a**; 100 μm for **b**, **c**; 20 μm for **d**, **e**, **h**, **i**, **k**–**o**; 40 μm for **f**, **g**; 2 μm for **j**; 500 nm for **j** (*inset*)

Table 2 Summary of neuropathological findings

	Neuron loss	p62		pTDP-43		Ubiquitin		pα-Syn
		NCIs	GCIs	NCIs	GCIs	NCIs	GCIs	LBs
Cerebral cortex								
Frontal	−	+	+	+	+	+ (*LBs)	−	+
Motor	+	+	+	++	++	−	−	+
Insular	−	+ (*LBs)	+	+	+	+ (*LBs)	−	+
Temporal	−	+	+	+	+	+ (*LBs)	−	+
Occipital	−	−	−	−	−	−	−	−
Subcortical area								
Ammon	−	+	+	+	+	−	−	−
Dentate gyrus	−	−	−	−	−	−	−	−
Amygdaloid nuclei	−	+ (*LBs)	+	−	+	+ (*LBs)	−	++
Putamen	−	+	+	+	+	−	−	−
Globus pallidus	−	+	+	+	−	−	−	−
Thalamus	−	+	+	++	+	+ (*LBs)	−	+
Subthalamic nucleus	−	+	+	−	−	−	−	−
Basal nucleus of Meynert	−	++ (*LBs)	−	−	−	++ (*LBs)	−	+++
Brainstem								
Superior colliculus	−	−	−	+	+	−	−	−
Oculomotor nucleus	−	−	−	−	−	−	−	−
Red nucleus	−	+	++	+	++	−	−	−
Substantia nigra	+	+	+	+	+	+ (*LBs)	−	+
Facial nucleus	+	++	+	−	−	−	−	−
Pontine nucleus	−	+	−	−	−	−	−	−
Locus ceruleus	++	+ (*LBs)	−	−	−	+ (*LBs)	−	++
Hypoglossal nucleus	+	+	−	−	−	−	−	−
Dorsal vagal nucleus	+	++ (*LBs)	−	−	−	++ (*LBs)	−	+++
Ambiguus nucleus	−	−	−	−	−	−	−	−
Medullary reticular formation	−	+	+	+	+	+ (*LBs)	−	++
Inferior olivary nucleus	−	+	+	+	+	+	−	−
Cerebellum								
Cerebellar cortex	−	−	−	−	−	−	−	−
Dentate nucleus	−	+	−	−	−	−	−	−
Spinal cord								
Cervical anterior horn	+++	++	+	−	−	−	−	−
Lumbar anterior horn	++	++	+	−	−	−	−	−
Intermediate lateral nucleus	−	+ (*LBs)	−	−	−	−	−	+
Clarke nucleus	−	−	−	−	−	−	−	−

pTDP-43 phosphorylated TDP-43, *pα-Syn* phosphorylated α-Synuclein, *NCIs* neuronal cytoplasmic inclusions, *GCIs* glial cytoplasmic inclusions, *LBs* Lewy bodies

* LBs, p62- or ubiquitin-positive NCIs were mostly Lewy bodies. See "Neuropathological examination" for the grading of neuron loss and inclusions

Discussion

We have described a sporadic, late-onset, pathologically LMN-predominant ALS, carrying compound heterozygous mutations in the *SQSTM1* gene. We have also presented several lines of evidence suggesting that the *SQSTM1* mutations may be pathogenic for ALS. First, both mutations were absent in 378 chromosomes among Japanese controls, as well as in all controls studied previously [6, 7, 17, 20]. Second, p.Val153Ile mutation has been identified previously in two ALS patients [6]. The other mutation, p.Val90Met, is a novel mutation, and in

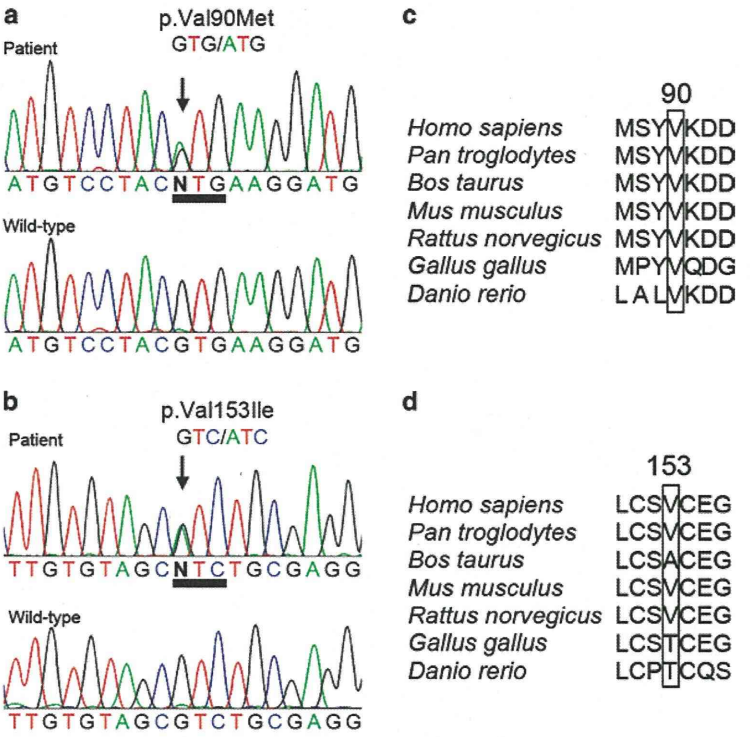

Fig. 2 Two heterozygous mutations in the *SQSTM1* gene detected in the present patient. **a** One mutation located in exon 2 (c.268G>A) results in a Val-to-Met substitution at position 90 (p.Val90Met). **b** The other mutation located in exon 3 (c.457G>A) results in a Val-to-Ile substitution at codon 153 (p.Val153Ile). The valine residues at positions 90 (**c**) and 153 (**d**) are well and relatively well conserved, respectively, in several species

silico analysis predicted that this mutation may be harmful. Third, Val residues at positions 90 and 153 are well and relatively well conserved evolutionally, respectively (Fig. 2c, d). Finally and most importantly, the neuropathological features of the LMNs in the present case were associated with p62 and quite distinct from those of sporadic ALS.

In sporadic ALS, it is well known that NCIs positive for ubiquitin, p62 and TDP-43 [8, 14], as well as Bunina bodies, are a feature of the remaining LMNs [12]. During the preparation of this manuscript, a related study was published, showing that all of the present cellular pathological alterations were observed in LMNs of three autopsied ALS patients with heterozygous *SQSTM1* mutations [20]. However, in the LMNs of the present ALS patient with compound heterozygous *SQSTM1* mutations, NCIs positive for only p62 were observed, and Bunina bodies were absent. These results suggest that the neuropathological features of ALS with *SQSTM1* mutations might be heterogeneous. However, it is noteworthy that there is a significant positive correlation between the occurrence of Bunina bodies and that of TDP-43-positive NCIs [12]. In the present patient, the lack of Bunina bodies was considered concordant with the sparse nature of TDP-43 deposits.

On the other hand, the present patient showed rather widespread TDP-43 pathology in the CNS non-motor neurons; although mild, the presence of cerebral cortical TDP-43 pathology suggests possible overlap between motor neuron disease and FTLD [20]. p62, a multifunctional protein related to protein degradation via the proteasome and autophagy, has been shown to physiologically bind to TDP-43, and is likely to be involved in the degradation of fragmented TDP-43 [19]. Disruption of this TDP-43–p62 interaction and subsequent aggregation of pTDP-43 could be a plausible pathomechanism underlying usual sporadic ALS [19], as well as ALS with heterozygous *SQSTM1* mutations [20]. In the present patient, however, such an explanation would not have been applicable to the LMNs. At present, it is tempting to speculate that the compound heterozygous *SQSTM1* mutations could have caused ALS with the different topographical distributions of p62 and TDP-43 described above. However, whether or not such a pathological picture would occur in all patients with compound heterozygous, or homozygous *SQSTM1* mutations awaits further studies.

Parkinsonism has been observed in 2 out of 14 ALS patients with *SQSTM1* heterozygous mutations [6]. In the autopsied patients mentioned above, neuronal loss was evident in the substantia nigra, although no Lewy bodies were found [20]. At present, we consider the Lewy body pathology in the present patient to have been coincidental.

In conclusion, the present patient with ALS is the first reported to have demonstrated compound heterozygous mutations in the *SQSTM1* gene, and in whom the selective occurrence of NCIs positive for only p62 in the affected LMNs was a characteristic feature. Further autopsy studies will be necessary to explore the molecular pathogenesis of ALS with *SQSTM1* mutations.

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Conflict of interest The authors declare that they have no conflict of interest.

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Case Report

Bunina bodies in motor and non-motor neurons revisited: A pathological study of an ALS patient after long-term survival on a respirator

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Bunina bodies (BBs) are small eosinophilic neuronal cytoplasmic inclusions (NCIs) found in the remaining lower motor neurons (LMNs) of patients with sporadic amyotrophic lateral sclerosis (SALS), being a specific feature of the cellular pathology. We examined a case of SALS, unassociated with *TDP-43* or *C9ORF72* mutation, of 12 years duration in a 75-year-old man, who had received artificial respiratory support for 9 years, and showed widespread multisystem degeneration with TDP-43 pathology. Interestingly, in this patient, many NCIs reminiscent of BBs were observed in the oculomotor nucleus, medullary reticular formation and cerebellar dentate nucleus. As BBs in the cerebellar dentate nucleus have not been previously described, we performed ultrastructural and immunohistochemical studies of these NCIs to gain further insight into the nature of BBs. In each region, the ultrastructural features of these NCIs were shown to be identical to those of BBs previously described in LMNs. These three regions and the relatively well preserved sacral anterior horns (S1 and S2) and facial motor nucleus were immunostained with antibodies against cystatin C (CC) and TDP-43. Importantly, it was revealed that BBs exhibiting immunoreactivity for CC were a feature of LMNs, but not of non-motor neurons, and that in the cerebellar dentate nucleus, the ratio of neurons with BBs and TDP-43 inclusions/neurons with BBs was significantly lower than in other regions. These findings suggest that the occurrence of BBs with CC immunoreactivity is intrinsically associated with the particular cellular properties of LMNs, and that the mechanism responsible for the formation of BBs is distinct from that for TDP-43 inclusions.

Key words: amyotrophic lateral sclerosis, Bunina body, cystatin C, non-motor neuron, TDP-43.

INTRODUCTION

Bunina bodies (BBs), which are small eosinophilic neuronal cytoplasmic inclusions (NCIs), are considered to be a specific feature of the cellular pathology in sporadic amyotrophic lateral sclerosis (SALS). BBs are found in lower motor neurons (LMNs) in the spinal cord and brainstem;[1] Piao *et al.* reported that they were observed in 88 (86.3%) of 102 cases of SALS.[2] However, BBs are very rare in the brainstem and in sacral LMNs innervating the striated muscles of the eye and the rectum and urethral sphincter.[1,3,4] Electron microscopy and immunohistochemical studies are important for identifying BBs in patients with SALS: they consist of electron-dense amorphous material often with inner clear areas containing cell organelles, such as filaments (neurofilaments) and vesicles,[1,2] and are immunoreactive for cystatin C (CC), a protein inhibitor of lysosomal cysteine proteases.[1,5]

In SALS, NCIs indistinguishable from BBs may also occur in non-motor neurons,[1] including those in the medullary reticular formation.[6] The ultrastructural features of such NCIs in non-motor neurons have been shown to be identical to those of BBs seen in LMNs.[1,6] However, no reported studies have yet investigated the immunoreactivity of BBs for CC or their relationship to trans-activation response DNA protein 43 (TDP-43) inclusions.

Recently, we encountered a patient with SALS who had survived for a long period on respirator support. In this patient, many small eosinophilic NCIs reminiscent of BBs,

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which were confirmed in the affected LMNs (described below), were observed in the oculomotor nucleus, medullary reticular formation and cerebellar dentate nucleus. Therefore we performed ultrastructural and immunohistochemical studies of these NCIs to gain further insight into the nature of BBs. Here we describe the clinicopathological features of this patient with new observations on Bunina bodies.

CASE REPORT

The present study was conducted with approval from the Institutional Review Board of the University of Niigata. Written informed consent was obtained from the patient's family prior to these genetic studies of the *TDP-43* and *C9ORF72* genes.

Clinical summary and pathological findings

A 63-year-old man became aware of muscle weakness in the right hand, and over the next 2 years, the muscle weakness extended to all of his extremities. On examination, fasciculation was evident in the tongue and deep tendon reflexes were increased; on this basis he was diagnosed as having ALS. About 3 years after onset, at the age of 66 years, he became bedridden with dysphagia and dyspnea, necessitating tube feeding and artificial respiratory support. Thereafter, ocular movement became limited in all directions, making communication impossible. The patient died of bronchopneumonia at the age of 75 years, about 12 years after disease onset. A general autopsy was performed 3 h after death, at which time the brain weighed 830 g, showing marked frontotemporal atrophy (frontal > temporal) (Fig. 1A).

The brain and spinal cord were fixed in 20% buffered formalin and multiple tissue blocks were embedded in paraffin. Histological examination was performed on 4-μm-thick sections using several stains, including HE, KB and Holzer. Selected sections were also immunostained with antibodies against phosphorylated TDP-43 (pTDP-43) (monoclonal, clone S409/410; Cosmo Bio, Tokyo, Japan; 1:3000, heat/autoclaving) and cystatin C (polyclonal, Dako, Glostrup, Denmark; 1:3000).

The entire spinal cord was markedly atrophic (Fig. 1B) and there was severe wasting in the anterior nerve roots. Histopathological examination revealed that except for the absence of Lewy body-like hyaline inclusions, the entire pathological picture was very similar to that shown in a case of SALS in a 71-year-old woman after long-term survival on a respirator, which we had previously reported.[7] With regard to the motor neuron system, almost complete loss of LMNs was observed in the spinal anterior horns at the levels of the cervical, thoracic and lumbar segments. The sacral anterior horns (S1 and S2), including Onuf's nucleus, contained a number of LMNs (Fig. 1C). In the brainstem, almost complete loss of LMNs was evident in the hypoglossal nucleus. The facial motor nucleus and oculomotor nucleus were relatively well preserved. BBs were found in the remaining LMNs in the sacral anterior horns, including Onuf's nucleus and the facial motor nucleus (Fig. 1D); immunostaining revealed that these BBs were

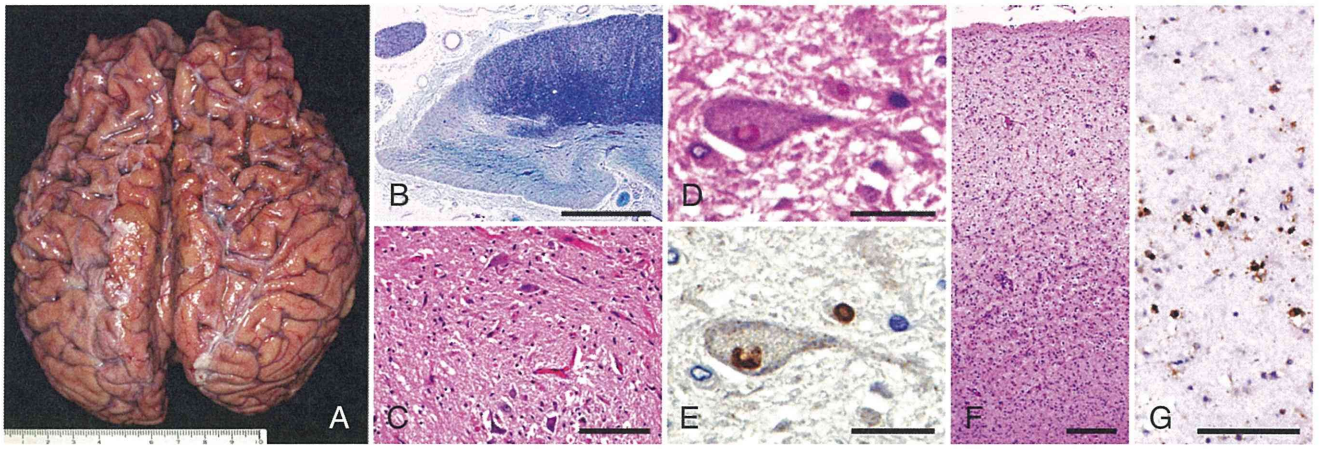

Fig. 1 Neuropathological findings in the brain and spinal cord. Sections stained by the KB method (B), HE (C,D,F) and immunostained with antibodies against cystatin C (CC) (E) and phosphorylated trans-activation response DNA protein 43 (pTDP43) (G). (A) Marked atrophy is evident in the frontal lobe, including the precentral gyrus. (B) The thoracic segment (T2), showing myelin pallor in the white matter except for the posterior columns. (C) Loss of lower motor neurons (LMNs) with gliosis is evident in the sacral (S1) anterior horn. Note that Onuf's nucleus contains a number of LMNs (lower). (D,E) Sequential staining of the same section, showing two facial motor neurons with Bunina bodies (BBs) (D) positive for CC (E). (F) Severe neuronal loss with gliosis is evident in the motor cortex. (G) Here, pTDP-43-positive neuronal cytoplasmic inclusions (NCIs) in layers II-III are shown. Scale bars = 1 mm for (B), 100 μm for (C,G), 20 μm for (D,E) and 200 μm for (F).