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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

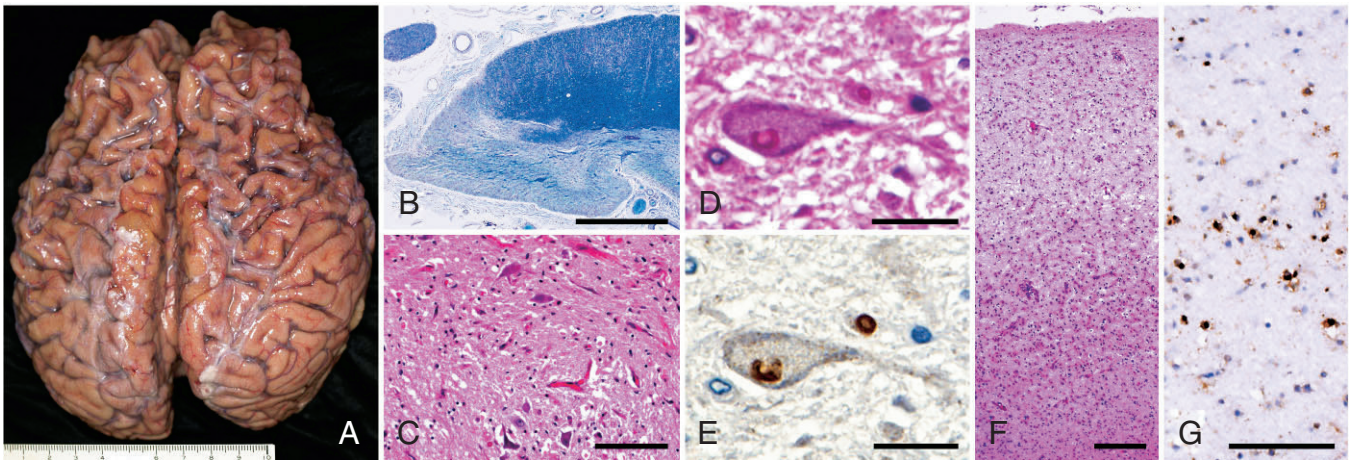

Fig. 1 Neuropathological findings in the brain and spinal cord. Sections stained by the KB method (B), HE (C,D,F) and immunostained with antibodies against cystatin C (CC) (E) and phosphorylated trans-activation response DNA protein 43 (pTDP43) (G). (A) Marked atrophy is evident in the frontal lobe, 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).

positive for CC (Fig. 1E). In the motor cortex, severe neuronal loss was also evident and no Betz cells were found (Fig. 1F); immunostaining revealed pTDP-43-positive NCIs mainly in layers II-III and V-VI (Fig. 1G). The histological findings are summarized in Table 1. Diffuse loss of cerebellar Purkinje cells appeared to be attributable to brain ischemia (Table 1).

Table 1 Pathological findings in the present case

Regions	Loss of neuron	pTDP-43-positive NCIs
Cerebral cortex		
Frontal	+++	+++
Motor	+++	+++
Parietal	++	+++
Cingulate	+++	+++
Insular	+++	+++
Entorhinal	++	+++
Hippocampus (DG/Sub)	+/+++	+++/++
Subcortical area		
Amygdala	++	+++
Basal nucleus of Mynert	+	+
Caudate nuclei	+++/+++	+++/+++
Globus pallidus	+	+++
Thalamus (medial/lateral)	++/+++	++/++
Subthalamic nucleus	nd	nd
Midbrain		
Midbrain tectum	+++	+++
Reticular formation	+++	+++
Oculomotor nucleus	+	+
Red nucleus	+	+
Substance nigra	+++	+
Pons		
Locus celreus	++	+
Reticular formation	++	+++
Facial nucleus (motor)	+	++
Vestibular nucleus	+	+
Pontine nucleus	+	++
Superior olivary nucleus	–	–
Medulla oblongata		
Hypoglossal nucleus	+++	–
Dorsal vagal nucleus	+	++
Reticular formation	++	+++
Nucleus ambiguus	nd	nd
Inferior olivary nucleus	+	+
Cerebellum		
Purkinje cell	+++	–
Granule cell	–	–
Dentate nucleus	+	++
Spinal cord		
Anterior horn	+++	+
Intermediate lateral nucleus	++	++
Clarke's nucleus	+++	–
Posterior horn	++	++
Anterior olfactory nucleus	++	++
Dorsal root ganglia	+	+

Loss of neurons: +, mild; ++, moderate; +++, severe. The numbers of pTDP-43-positive neuronal cytoplasmic inclusions (NCIs) were assessed using a semi-quantitative rating scale: –, absent or nearly absent; +, sparse; ++, moderate; +++, numerous. Hippocampus: DG, dentate gyrus (granule cells); Sub, subiculum. nd, not determined.

TDP-43 mutation and *C9ORF72* repeat expansion analyses

Genomic DNA was prepared from a frozen sample of cerebral cortex from the patient, and then examinations for *TDP-43* mutation and *C9ORF72* repeat expansion were carried out as previously described;[8,9] however, neither of these features was found to be present.

Bunina bodies in motor and non-motor neurons

In addition, the occurrence of many eosinophilic NCIs indistinguishable from BBs in the oculomotor nucleus, medullary reticular formation and cerebellar dentate nucleus was a feature of the present patient. Some representative inclusions in the oculomotor nucleus and medullary reticular formation were recycled for electron microscopy, and small tissue blocks from the formalin-fixed cerebellar dentate nucleus were also processed for ordinary electron microscopy. All of the studied NCIs, 2–3 in each region (Fig. 2A–C), were identified as BBs from their characteristic ultrastructural features (Fig. 2D–F). In the medullary reticular formation, the BB-containing neurons were distributed more widely than previously recorded.[6]

We then investigated the presence or absence of CC immunoreactivity in the BBs, as well as the correlation between the occurrence of BBs and that of pTDP-43-positive inclusions. Four-micrometer-thick paraffin sections that contained the bilateral oculomotor nuclei and medullary reticular formation, and unilateral cerebellar dentate nucleus were prepared, and then stained with HE, observed and photographed (Fig. 3A–C,G–I). They were then destained in absolute ethanol and finally immunostained for CC (Fig. 3D–F) or pTDP-43 (Fig. 3J–L). For comparison, the bilateral sacral anterior horns (S1 and S2) and facial motor nuclei were also similarly examined. The degrees of cytoplasmic staining intensity for CC were generally decreased in the LMNs containing BBs (Fig. 1E,3D–F). pTDP-43-positive NCIs appeared as fine to coarse granular (Fig. 3J), linear wisp-like, large irregular (Fig. 3K) or small round-to-oval inclusions (Fig. 3L); the small round-to-oval inclusions were often observed in neurons in the cerebellar dentate nucleus (Fig. 3L). In each region, the ratio of neurons containing CC-positive BBs to the total cell count of neurons containing BBs was calculated in one section. Similarly, the ratio of neurons containing both BBs and pTDP-43-positive inclusions to the total cell count of neurons containing BBs was calculated in one section. The results obtained are shown in Table 2.

DISCUSSION

Based on the distribution and severity of neuron loss and TDP-43 inclusions, the present case was considered to be

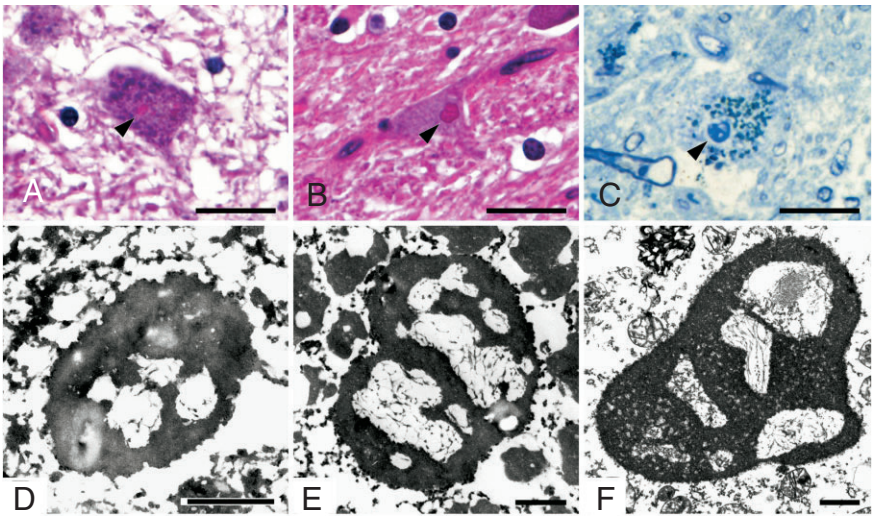

Fig. 2 Ultrastructural profiles of Bunina bodies (BBs) in neurons from the oculomotor nucleus (A), medullary reticular formation (B) and cerebellar dentate nucleus (C). Two paraffin sections stained with HE (A,B) and one Epon section stained with toluidine blue (C). Electron microscopy shows that all the BBs (A–C; arrowheads) have essentially the same ultrastructural profiles, appearing as electron-dense amorphous material with inner clear areas, in which filamentous structures are evident (D–F). In a Bunina body shown in (C), some of the filamentous structures can be identified as neurofilaments, or short fragments of the rough endoplasmic reticulum (F). Scale bars = 20 μm for A–C and 1 μm for D–F.

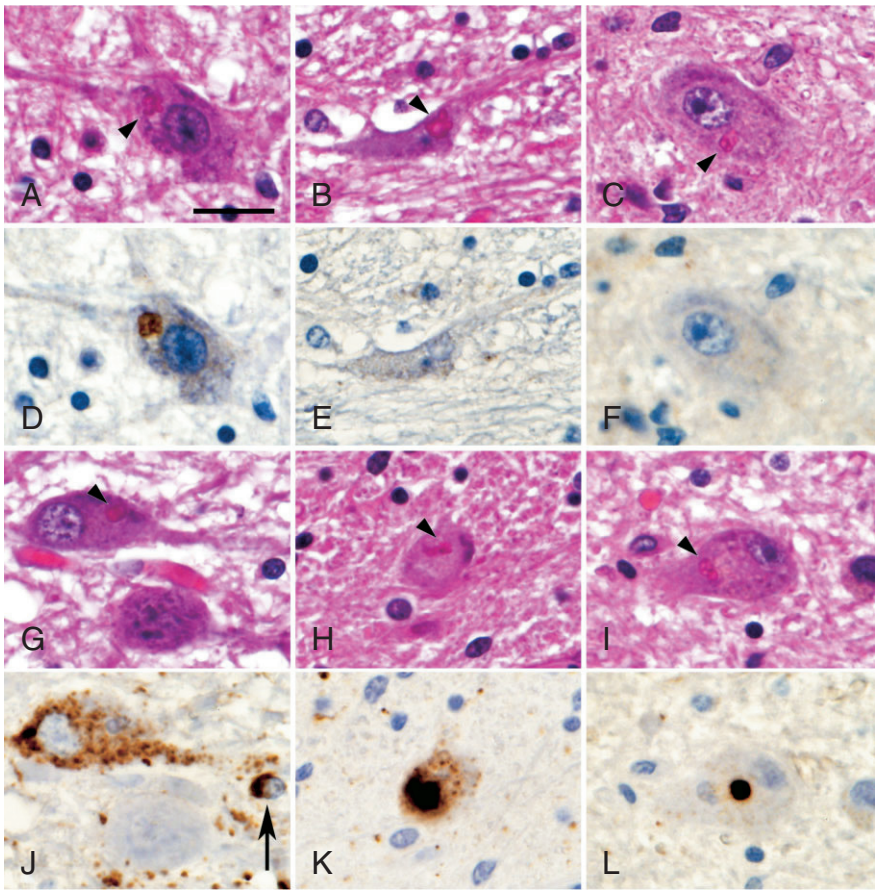

Fig. 3 Immunohistochemical profiles of Bunina bodies (BBs) in neurons from the oculomotor nucleus (A,G), medullary reticular formation (B,H) and cerebellar dentate nucleus (C,I). Sequential staining of the same sections with HE (A–C) and anti-cystatin C (CC) antibody (D–F), as well as with HE (G–I) and anti-phosphorylated trans-activation response DNA protein 43 (pTDP43) antibody (J–L). (A–F) BBs (arrowheads) seen in one lower motor neuron (A) and two non-motor neurons (B,C) are positive (D) and negative (E,F) for CC, respectively. (G–L) In all of the neurons, coexistence of BBs (arrowheads) and pTDP-43-positive neuronal cytoplasmic inclusions (NCIs) is evident; BBs themselves are negative for pTDP-43 (G,J; H,K; I,L). Arrow indicates cytoplasm of a glial cell positive for pTDP-43 (J). Scale bar = 20 μm for (A–L).

an additional example of SALS whose course had been extended by artificial respiratory support, showing widespread multisystem degeneration with TDP-43 pathology (Table 1) (Nishihira *et al*., Type 2;[10] frontotemporal lobar degeneration – TDP pathology, Type B[11]). We reviewed seven cases in which artificial respiratory support had been used (disease duration, >10 years; Type 1 = 5, Type 2 = 2[10]) and found no NCIs indistinguishable from BBs in the oculomotor nucleus, medullary reticular formation or cerebellar dentate nucleus. In the case (disease duration = $8^2/_3$ years) reported by Nishihira *et al*.,[7] only one BB, which was confirmed by electron microscopy of recycled material, was found in the medullary reticular formation (data not shown). Therefore, the present case, which lacked *TDP-43*

Table 2 Summary of pathological findings for Bunina bodies (BBs)

Region	Ratio (cystatin C)	Ratio (pTDP-43)
Sacral anterior horn	0.88 (7/8)	1.00 (5/5)
Facial motor nucleus	1.00 (8/8)	0.90 (9/10)
Oculomotor nucleus	1.00 (10/10)	1.00 (13/13)
Medullary reticular formation	0.17 (2*/12)†	0.77 (10/13)
Cerebellar dentate nucleus	0.00 (0/36)†	0.33 (12/ /36)††

Ratio (cystatin C): neurons with cystatin C-positive BBs/neurons with BBs; Ratio (pTDP-43): neurons with BBs and pTDP-43-positive inclusions/neurons with BBs. *Regarded as weakly positive. †$P < 0.01$ versus. sacral anterior horn, facial motor nucleus or oculomotor nucleus. ††$P < 0.05$ versus sacral anterior horn, and $P < 0.01$ versus facial motor nucleus, versus oculomotor nucleus or versus medullary reticular formation. Statistical analyses were performed by Ryan's multiple comparison tests using R software (http://www.r-project.org/).

or *C9OLF72* mutation, appeared to be very unusual in terms of the occurrence of BBs even among cases of SALS whose course had been extended by artificial respiratory support.

At present, TDP-43 is widely recognized to be the pathological protein in SALS.[10,12] BBs have been reported to be negative for TDP-43,[12] which was also confirmed in the present study using a monoclonal antibody against pTDP-43. However, the presence of both BBs and TDP-43-positive NCIs has also been shown to be a characteristic feature of ALS with *TDP-43* mutations,[8,12] emphasizing anew the significance of BBs as a specific feature of the cellular pathology of ALS.

Importantly, the present case is the first reported example in which the presence of BBs exhibiting immunoreactivity for CC was a feature of LMNs, but not of non-motor neurons (Table 2). At the ultrastructural level, it is noteworthy that in LMNs, the electron-dense material considered to represent BBs themselves is negative for CC;[5,13] it has been reported that CC immunoreactivity is markedly decreased in the spinal LMNs in SALS, and that the formation of TDP-43 inclusions, but not BBs, may be linked to the CC content of these LMNs.[13] Based on the present findings, we consider that the occurrence of BBs showing CC immunoreactivity is a phenomenon confined almost exclusively to LMNs, and that this must be associated with the particular cellular properties that characterize the LMNs themselves.

The present case is also the first reported to have demonstrated BBs in neurons in the cerebellar dentate nucleus. It has been reported that there is a significant positive correlation between the occurrence of BBs and that of TDP-43 inclusions in spinal and brainstem LMNs.[14,15] This also appears to be the case in the medullary reticular formation (Table 2). However, the ratio (pTDP-43) was significantly lower in the cerebellar dentate nucleus than in other regions (Table 2), indicating that the mechanism responsible for the formation of BBs is distinct from that for TDP-43 inclusions.

Finally, even though the present study involved only a single case and revealed negativity for BBs, as in other similar cases of SALS mentioned above, the results obtained are of considerable interest. In conclusion, the nature and origin of BBs still remain uncertain. When considering why LMNs are generally most vulnerable in ALS, further studies on the formation of BBs in association with the cellular molecular properties of LMNs are needed to elucidate the pathomechanism underlying the disease.

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