

## Original Article

# Reduced expression of BTBD10 in anterior horn cells with Golgi fragmentation and pTDP-43-positive inclusions in patients with sporadic amyotrophic lateral sclerosis

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**Overexpression of BTBD10 (BTB/POZ domain-containing protein 10) suppresses G93A-superoxide dismutase 1 (SOD1)-induced motor neuron death in a cell-based amyotrophic lateral sclerosis (ALS) model. In the present study, paraffin sections of spinal cords from 13 patients with sporadic ALS and 10 with non-ALS disorders were immunostained using a polyclonal anti-BTBD10 antibody. Reduced BTBD10 expression in the anterior horn cells was more frequent in spinal cords from ALS patients than in cords from patients with non-ALS disorders. We further investigated the relationship between the level of BTBD10 immunoreactivity and the morphology of the Golgi apparatus (GA) and the presence of phosphorylated TAR-DNA-binding protein 43 (pTDP-43). Mirror sections of spinal cords from five sporadic ALS cases were immunostained with antibodies against BTBD10 and trans-Golgi-network (TGN)-46 or pTDP-43. Whereas 89.7–96.5% of the neurons with normal BTBD10 immunoreactivity showed normal GA morphology and no pTDP-43 cytoplasmic aggregates, 86.2–94.3% of the neurons with reduced BTBD10 expression showed GA fragmentation and abnormal pTDP-43 aggregates. These findings suggest that reduced BTBD10 expression is closely linked to the pathogenesis of sporadic ALS.**

**Key words:** ALS, BTBD10, Golgi apparatus, pathology, pTDP-43.

## INTRODUCTION

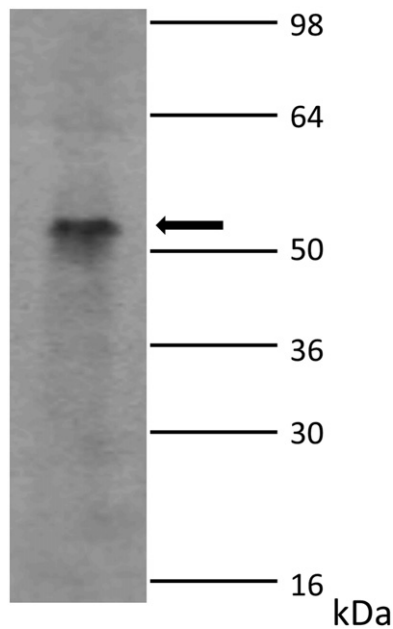
BTBD10 (BTB/POZ domain-containing protein 10) activates Akt by inhibiting the protein phosphatase 2A-mediated dephosphorylation and inactivation of Akt.<sup>1,2</sup> Akt family proteins, which are composed of Akt1, Akt2 and Akt3 in mammalian cells, mediate the primary intracellular critical prosurvival signal by phosphorylating many target proteins. They play a central role in the control of cell survival, growth, proliferation, and apoptosis throughout the body. Akt malfunction can cause a variety of complex diseases, including type-2 diabetes and cancer.<sup>3,4</sup> BTBD10 was first cloned by Chen *et al.*<sup>5</sup> It was found to be downregulated in gliomas<sup>5</sup> and identified as a molecule that interacts with Akt3.<sup>1,2</sup> BTBD10 equally binds to any Akt.<sup>1</sup>

The autosomal-recessive familial amyotrophic lateral sclerosis (ALS)-linked gene 2 (ALS2) product, alsin long form (LF), is involved in intracellular vesicle/membrane trafficking and endosome dynamics. Loss of alsin LF function contributes to motor neuron death.<sup>6</sup> In addition, alsin LF inhibits familial ALS-related Cu/Zn superoxide dismutase 1 (SOD1) mutant-induced neurotoxicity through activation of the prosurvival signal cascade mediated by Rac1/PI3K/Akt3. Akt3 is the major downstream mediator for alsin LF-mediated neuroprotection.<sup>7</sup>

Overexpression of BTBD10 inhibits G93A-SOD1-induced motor neuron death. In agreement with BTBD10-mediated upregulation of the Akt phosphorylation levels, enforced expression of BTBD10 leads to the suppression of mutant SOD-1-induced neuronal death *in vitro*.<sup>1</sup> In a recent study, Nawa *et al.* further suggested that BTBD10 expression is reduced in the anterior horn cells of the

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**Fig. 1** Anti-BTBD10 (BTB/POZ domain-containing protein 10) antibody was used in immunoblot analysis of lysates from whole normal human brain. The antibody revealed an immunoreactive band around 54 kDa (arrow).

spinal cord in sporadic ALS patients.<sup>8</sup> These findings suggest that BTBD10 is closely linked to motor neuron death. However, whether the reduced expression of BTBD10 is linked to human ALS pathogenesis remains insufficiently defined.

The Golgi apparatus (GA) is frequently fragmented in the anterior horn cells of patients with sporadic ALS; that is, the GA loses its normal network-like configuration, which is replaced by disconnected small elements.<sup>9,10</sup> In addition, TAR-DNA-binding protein 43 (TDP-43) is a major disease marker in ALS and frontotemporal lobar degeneration with TDP-43.<sup>11–13</sup> Using specific antibodies to phosphorylated TDP-43 (pTDP-43), Hasegawa *et al.* found

immunoreactivity in abnormal inclusions, but not nuclei, which are the normal physiological site for TDP-43 localization.<sup>14</sup>

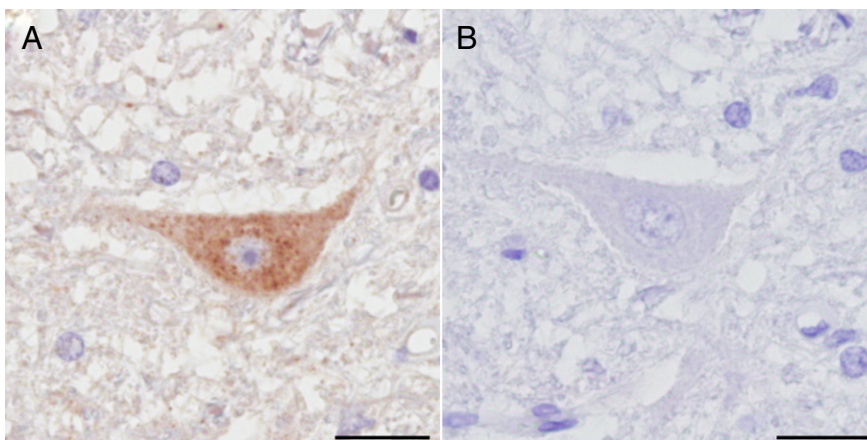
In the present study, we used immunohistological methods to investigate the relationship between the reduced BTBD10 expression and fragmentation of the GA, and the presence of pTDP-43 aggregates in the anterior horn cells of spinal cords from patients with sporadic ALS.

## MATERIALS AND METHODS

We examined the spinal cords of 13 patients with sporadic ALS (age at death: 56–75 years, mean: 64.3 years; 7 men and 6 women) and 10 patients diagnosed with various kinds of non-ALS disorders (age at death: 64–96 years, mean: 81.0 years; 8 men and 2 women). None of the patients had a family history of ALS. Autopsy samples were obtained from patients of Gunma University Hospital and Geriatric Research Hospital, Japan, under established procedures after obtaining informed consent from the family of each patient. ALS patients were definitively diagnosed based on clinical and light microscopic findings. Samples were fixed with 4% paraformaldehyde in PBS (pH 7.4) and embedded in paraffin.

Five-micrometer-thick transverse paraffin sections of the lumbar spinal cords were prepared for HE staining and immunohistochemical analysis using a rabbit polyclonal anti-BTBD10 antibody (1:1000) generated in our laboratory. The rabbit polyclonal antibody was generated by immunization with a synthetic peptide, CPSGNSDLDP-DAQNPML, corresponding to the C-terminal 16-amino-acid peptide sequence of human BTBD10, and conjugated to keyhole limpet hemocyanin (Technical Keystone Craft, Takasaki, Japan).

The anti-BTBD10-antibody was used in immunoblot analysis of lysates from whole normal human brain (Novus Biologicals, Littleton, CO, USA) to investigate its speci-



**Fig. 2** BTBD10 (BTB/POZ domain-containing protein 10) immunoreactivity. Immunostaining for BTBD10 (A) and for BTBD10 preabsorbed with BTBD10 recombinant protein (B). BTBD10 immunostaining was obviously specific. Scale bar: 20  $\mu$ m.

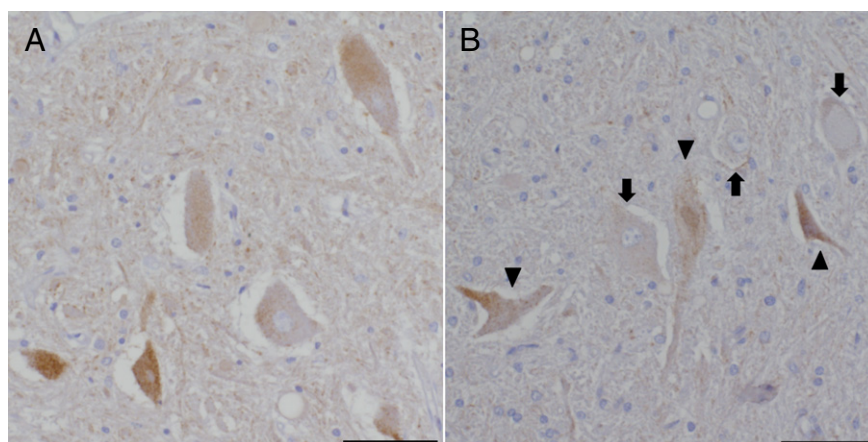
city. The immunoblotting revealed a single band at around 54 kDa (Fig. 1). Antibody specificity was also analyzed by adsorption tests using immunohistochemistry. A mirror section was immunostained after preadsorption of anti-BTBD10 antibody with BTBD10 recombinant protein. BTBD10-positive staining was not present as a result of the preadsorption (Fig. 2), indicating the fidelity of the immunoreactivity.

To enhance staining in the immunohistochemical analysis, the samples were autoclaved (121°C, 10 min). After endogenous peroxidase activity was quenched in 0.3%

H<sub>2</sub>O<sub>2</sub> (30 min), nonspecific binding sites on the sections were blocked with normal horse serum for 30 min at room temperature, then incubated with the primary antibody at 4°C overnight, washed in PBS for 30 min, incubated with the secondary antibody provided in a Histofine SAB-PO kit (Nichirei, Tokyo, Japan), and washed in PBS for 30 min. Finally, immunoreactivity was visualized using an avidin–biotin–peroxidase method. Sections were examined using an Olympus BX50 microscope.

After immunostaining the anterior horn cells of lumbar spinal cords from both sporadic ALS and non-ALS

**Fig. 3** Immunostaining with anti-BTBD10 (BTB/POZ domain-containing protein 10) antibody. In control cases (A), BTBD10-positive small granular cytoplasmic immunostaining was observed diffusely in the anterior horn cells. In amyotrophic lateral sclerosis (ALS) cases (B), the number of BTBD10-positive neurons was significantly lower in the anterior horns (arrow head). Neurons with reduced BTBD10 immunoreactivity are shown with arrows. The reduction in BTBD10 immunoreactivity was observed more frequently in large neurons than in small neurons. Bars, A, B: 30 µm.



**Table 1** The spinal cords in 13 sporadic ALS patients and 10 non-ALS patients were immunostained with antibody against BTBD10.

| Case | Sex | Age at death (years) | Diagnosis         | Duration of disease (months) | Respirator | Reduced BTBD10/All remaining neurons (%) |
|------|-----|----------------------|-------------------|------------------------------|------------|--|
| 1    | M   | 75                   | SALS              | 7                            | (-)        | 71/136 (52.2%)                           |
| 2    | F   | 69                   | SALS              | 15                           | (-)        | 65/135 (48.1%)                           |
| 3    | M   | 62                   | SALS              | 24                           | (-)        | 89/166 (53.6%)                           |
| 4    | F   | 56                   | SALS              | 25                           | (-)        | 77/148 (52.0%)                           |
| 5    | M   | 64                   | SALS              | 29                           | (-)        | 84/141 (59.5%)                           |
| 6    | F   | 68                   | SALS              | 30                           | (-)        | 86/171 (50.3%)                           |
| 7    | M   | 61                   | SALS              | 30                           | (-)        | 60/116 (51.7%)                           |
| 8    | F   | 60                   | SALS              | 33                           | 5 days     | 55/105 (52.4%)                           |
| 9    | F   | 70                   | SALS              | 36                           | (-)        | 80/153 (52.3%)                           |
| 10   | M   | 57                   | SALS              | 48                           | (-)        | 47/93 (50.5%)                            |
| 11   | F   | 64                   | SALS              | 84                           | (-)        | 67/122 (54.9%)                           |
| 12   | M   | 65                   | SALS              | 96                           | 31 months  | 54/97 (55.7%)                            |
| 13   | M   | 66                   | SALS              | 108                          | (-)        | 53/129 (42.1%)                           |
| 14   | M   | 90                   | AD                |                              |            | 9/189 (4.8%)                             |
| 15   | M   | 82                   | Pneumoniae        |                              |            | 11/221 (5.0%)                            |
| 16   | M   | 96                   | Colon carcinoma   |                              |            | 11/224 (4.9%)                            |
| 17   | F   | 81                   | AD                |                              |            | 14/274 (5.1%)                            |
| 18   | M   | 65                   | Pneumoniae        |                              |            | 11/223 (4.9%)                            |
| 19   | M   | 91                   | Pneumoniae        |                              |            | 9/206 (4.4%)                             |
| 20   | M   | 71                   | AMI               |                              |            | 11/227 (4.8%)                            |
| 21   | F   | 86                   | Ileus             |                              |            | 13/223 (5.8%)                            |
| 22   | M   | 75                   | RA                |                              |            | 8/201 (4.0%)                             |
| 23   | M   | 64                   | Gastric carcinoma |                              |            | 12/207 (5.8%)                            |

Reduction in BTBD10 (BTB/POZ domain-containing protein 10) immunoreactivity was observed more frequently in the anterior horn cells of patients with sporadic amyotrophic lateral sclerosis (SALS). There was no apparent relationship between the disease duration and the proportion of neurons with reduced BTBD10 immunoreactivity. AD, Alzheimer disease; AMI, acute myocardial infarction; F, female; M, male; RA, rheumatoid arthritis.

patients using the anti-BTBD10 antibody, the numbers of neurons with normal and reduced BTBD10 immunoreactivity were counted.

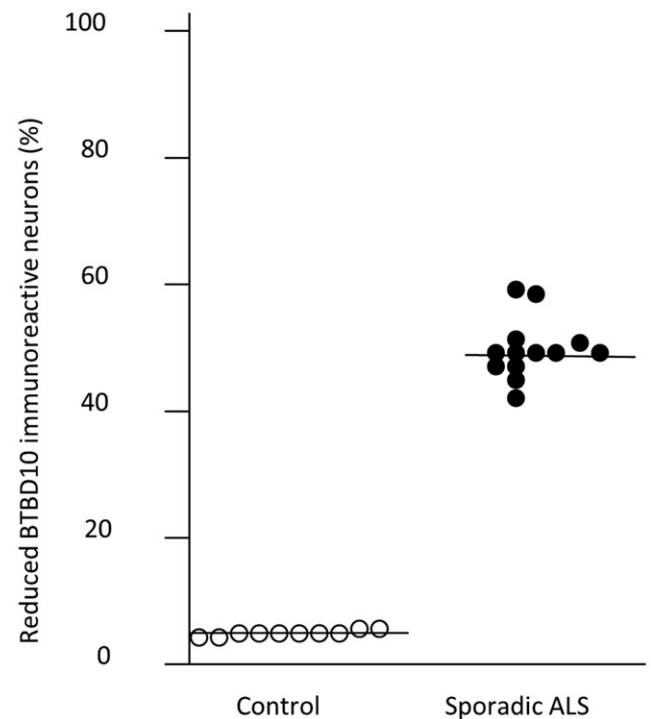
BTBD10 was found to be markedly expressed in neurons in the spinal anterior horns and only minimally expressed in astrocytes. BTBD10-positive motor neurons were defined as neurons with staining intensity stronger than that of the background neuropil of surrounding motor neurons (Fig. 3A). Most of the BTBD10-positive motor neurons were slightly positive. Other neurons with reduced staining were defined as neurons with reduced BTBD10 immunoreactivity (Fig. 3B). We investigated both large and small nucleated neurons in the anterior horn cells.

To investigate further the relationship between BTBD10 immunoreactivity and fragmentation of the GA or the pTDP-43 immunoreactivity, we immunostained 3- $\mu$ m-thick mirror sections of lumbar spinal cords from five patients with sporadic ALS using the anti-BTBD10 antibody (1:1000) and rabbit polyclonal anti-human trans-Golgi-network (TGN)-46 antibody (1:1000)<sup>15</sup> or rabbit polyclonal anti-pTDP-43 antibody (1:1000).<sup>16</sup> These three antibodies were generated in our laboratory. To detect immunoreactivity with the anti-TGN-46 antibody, the peroxidase-antibody complex was visualized using a VIP Substrate kit (Vector, Tokyo, Japan). To detect immunoreactivity with the anti-BTBD10 and anti-pTDP-43 antibodies, diaminobenzidine was used as the chromogen. Subsequently, micrographs of the sections were obtained.

## RESULTS

In sporadic ALS patients, approximately half (range, 42.1–59.5%) of the remaining anterior horn cells were identified as neurons with reduced BTBD10 immunoreactivity. Reduced BTBD10 immunoreactivity was more frequent in large neurons than in small neurons (Fig. 3). Bunina bodies and skein-like inclusions were BTBD10 negative. In contrast, in the non-ALS control cases, a reduction in BTBD10 immunoreactivity was identified in only approximately 5% (range, 4.4–5.8%) of the anterior horn cells. The level of BTBD10 was significantly decreased in the motor neurons of the spinal cords from sporadic ALS patients ( $P < 0.001$  by  $\chi^2$  test). However, we found no correlation between the ratio of neurons with reduced BTBD10 immunoreactivity to the number of all remaining neurons and the clinical course of the disease (age at onset and duration of illness) (Table 1; Fig. 4).

Next, we examined the relationship between reduced BTBD10 immunoreactivity and fragmentation of the GA. Immunohistochemical analysis of mirror sections of lumbar spinal cord tissue from patients with sporadic ALS showed that approximately 90% (range, 89.8–92.5%) of BTBD10-positive neurons had normal GA morphology



**Fig. 4** Percentages of neurons with reduced BTBD10 (BTB/POZ domain-containing protein 10) immunoreactivity. The median percentage of neurons with reduced BTBD10 immunoreactivity in all samples (control ( $n = 10$ ), sporadic amyotrophic lateral sclerosis (ALS) ( $n = 13$ )) was calculated. The average of the ratio of neurons with reduced BTBD10 immunoreactivity is indicated as a crossbar. The percentage of reduced BTBD10 immunoreactivity neurons was significantly higher in sporadic ALS ( $P < 0.001$  by  $\chi^2$  test).

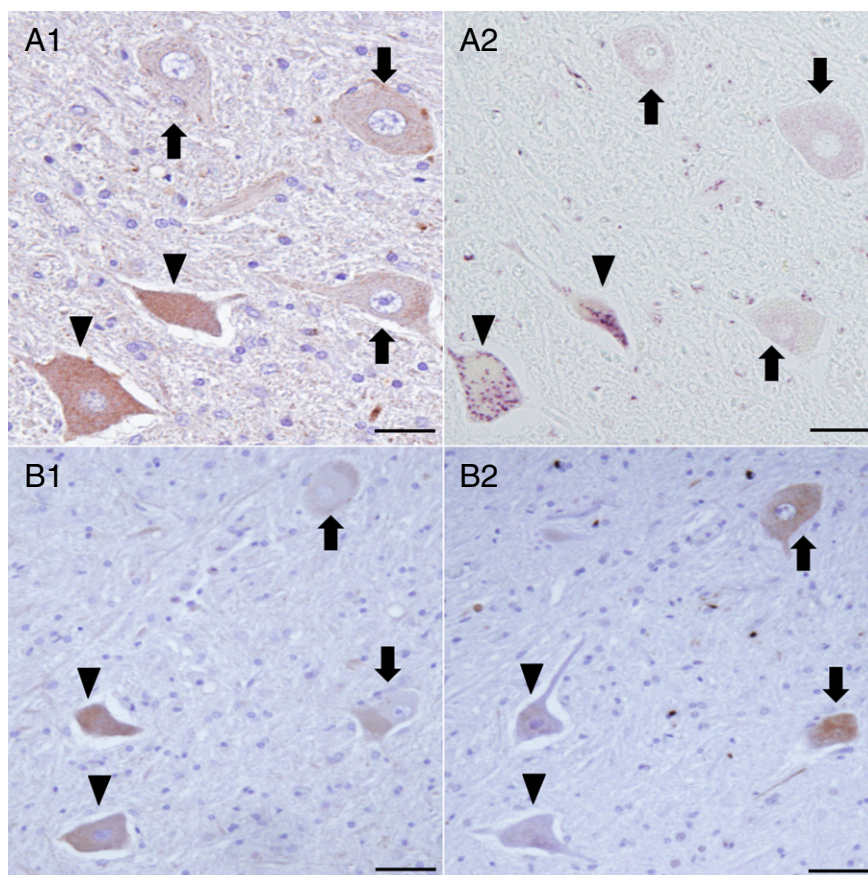
and most neurons with reduced BTBD10 immunoreactivity showed fragmentation of the GA (range, 92.9–94.3%) (Table 2; Figs 5A and 6A). There were few neurons with reduced BTBD10 immunoreactivity, but normal GA morphology (range, 5.7–7.1%) and normally stained BTBD10-positive neurons with GA fragmentation (range, 7.5–13.8%) (Fig. 6A). The percentage of normal GA morphology was significantly higher in BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity. The percentage of GA fragmentation was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons ( $P < 0.001$  by  $\chi^2$  test) (Fig. 6A).

Finally, we examined the relationship between reduced BTBD10 expression and pTDP-43 immunoreactivity in the anterior horn cells. Immunohistochemical analysis of mirror sections of lumbar cord tissue from patients with sporadic ALS showed that approximately 90% (range, 89.7–93.1%) of BTBD10-positive neurons had no pTDP-43-positive cytoplasmic aggregates. Almost all (range, 93.8–96.5%) neurons with reduced BTBD10 immunoreactivity had pTDP-43-positive cytoplasmic aggregates (Table 2;

**Table 2** Immunohistochemical analysis of mirror sections revealed that almost all neurons with reduced BTBD10 immunoreactivity had a fragmented GA and pTDP-43 immunoreactive aggregates, and almost all neurons with normal BTBD10 immunoreactivity had normal GA morphology and no abnormal pTDP-43-positive aggregates

| Case | Sex | Age at death (years) | Duration of disease (months) | Normal GA/normal BTBD10 (%) | GA fragmentations/reduced BTBD10 (%) | Negative pTDP-43/normal BTBD10 (%) | Positive pTDP-43/reduced BTBD10 (%) |
|------|-----|----------------------|------------------------------|-----------------------------|--------------------------------------|------------------------------------|-------------------------------------|
| 1    | M   | 79                   | 5                            | 44/49 (89.8%)               | 57/61 (93.4%)                        | 45/49 (91.8%)                      | 58/61 (95.1%)                       |
| 2    | M   | 75                   | 15                           | 55/62 (88.7%)               | 82/87 (94.3%)                        | 57/62 (91.9%)                      | 83/87 (95.4%)                       |
| 3    | M   | 64                   | 29                           | 50/58 (86.2%)               | 79/85 (92.9%)                        | 52/58 (89.7%)                      | 82/85 (96.5%)                       |
| 4    | M   | 61                   | 30                           | 49/53 (92.5%)               | 54/58 (93.1%)                        | 50/53 (93.1%)                      | 55/58 (94.8%)                       |
| 5    | F   | 64                   | 84                           | 49/54 (90.7%)               | 61/65 (93.8%)                        | 50/54 (92.6%)                      | 61/65 (93.8%)                       |

BTBD10, BTB/POZ domain-containing protein 10; F, female; GA, Golgi apparatus; M, male; pTDP-43, phosphorylated TAR DNA-binding protein of 43 kDa.

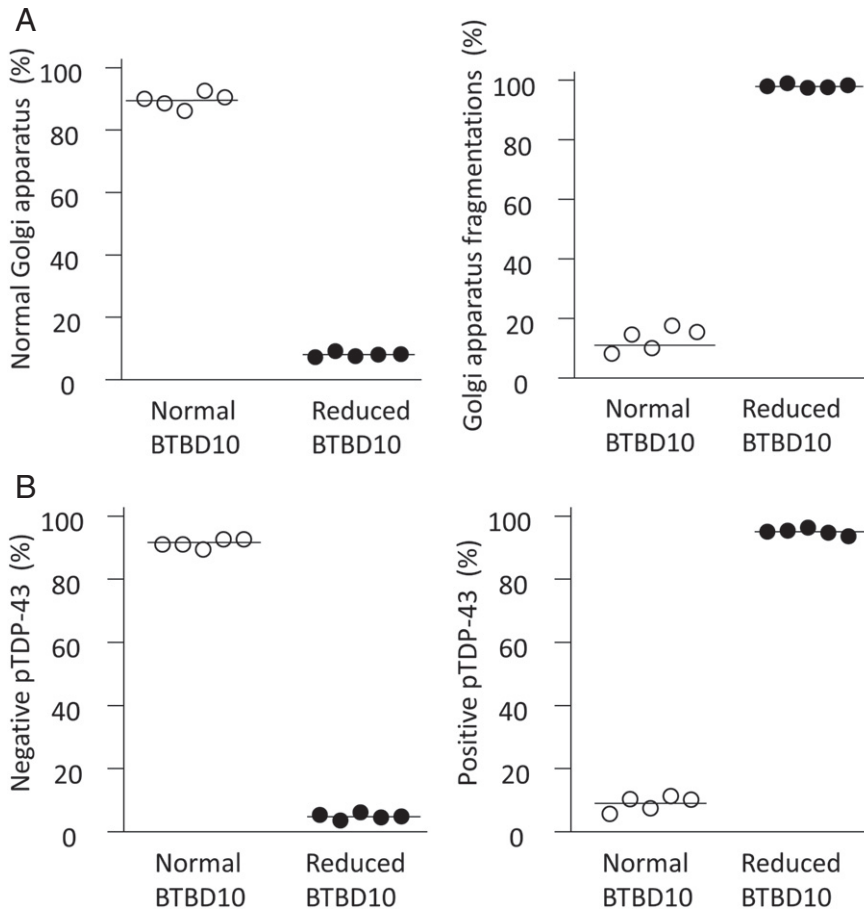


**Fig. 5** Mirror sections of the anterior horns in amyotrophic lateral sclerosis (ALS) cases. Immunostaining with anti-BTBD10 (BTB/POZ domain-containing protein 10) (A1, B1), anti-trans-Golgi-network (TGN)-46 antibodies (A2), and anti-phosphorylated TAR-DNA-binding protein 43 (pTDP-43) (B2). All arrows and arrowheads indicate neurons. In most neurons, those with reduced BTBD10 immunoreactivity (arrows in A1 and B1) showed fragmentation of the GA (A2, arrow) and pTDP-43-positive inclusions (B2, arrow), whereas neurons with normal BTBD10 immunoreactivity (arrow heads in A1 and B1) had normal Golgi apparatus (GA) morphology (A2, arrow head) and no pTDP-43-positive aggregates (B2, arrow head). Bars, A: 30 μm, B: 50 μm.

Figs 5B and 6B). We observed a few (range, 3.5–6.2%) neurons with reduced BTBD10 immunoreactivity, but without pTDP-43-positive cytoplasmic aggregates and a few (range, 6.9–10.3%) normal BTBD10-positive neurons with pTDP-43-positive cytoplasmic aggregates (Fig. 6B). The percentage of pTDP-43-negative neurons was significantly higher in the BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity. The percentage of pTDP-43-positive neurons was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons ( $P < 0.001$  by  $\chi^2$  test) (Fig. 6B).

## DISCUSSION

A recent study showed that the expression of BTBD10 was reduced in 44.6% of motor neurons in spinal cords from ALS patients, but only in 18.7% of motor neurons in spinal cords from control cases.<sup>8</sup> In the present study, we generated a new anti-BTBD10 antibody and confirmed that the expression of BTBD10 is markedly reduced in the anterior horn cells of spinal cords from patients with sporadic ALS compared with cords from control cases. These findings suggest that BTBD10 expression may decline during the process of neurodegeneration.



**Fig. 6** Comparison of BTBD10 (BTB/POZ domain-containing protein 10) and Golgi apparatus (GA)/phosphorylated TAR-DNA-binding protein 43 (pTDP-43) immunoreactivity. (A) The percentage of cells with normal GA morphology was significantly higher in BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity (left side). The percentage of cells with GA fragmentation was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons (right side) ( $P < 0.001$  by  $\chi^2$  test). Each crossbar indicates the average. (B) The percentage of pTDP-43-negative neurons was significantly higher in BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity (left side). The percentage of pTDP-43-positive neurons was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons (right side) ( $P < 0.001$  by  $\chi^2$  test). Each crossbar indicates the average.

TDP-43 was originally cloned as a human protein capable of binding to TAR DNA of the human immunodeficiency virus 1 long terminal repeat lesion. Physiologically, TDP-43 regulates a variety of RNA metabolisms.<sup>17</sup> Consistent with its function as an RNA-binding protein, TDP-43 associates with members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins such as hnRNP A2/B1, hnRNP A1, hnRNP C1/C2 and hnRNP A3.<sup>18–20</sup> The interaction of TDP-43 with hnRNPs is dependent on its C-terminal glycine-rich domain.<sup>18,21</sup> Dysregulation of TDP-43 accounts for neurodegeneration in most ALS cases.<sup>22</sup> We suggest that reduced BTBD10 expression may serve as a common mechanism underlying the pathogenesis of ALS based on the findings from the present study that the appearance of pTDP-43-positive aggregates correlated strongly with reduced BTBD10 immunoreactivity.

In our previous study, we demonstrated that the majority of neurons with pTDP-43-positive cytoplasmic aggregates had GA fragmentation, using TGN-46 immunostaining.<sup>23</sup> In the present study, we found that the majority of neurons with reduced BTBD10 expression simultaneously had both GA fragmentation and pTDP-43-positive cytoplasmic aggregates. The GA plays a key role in

the transportation, processing and targeting of numerous proteins destined for secretion, the plasma membrane and lysosomes.<sup>24,25</sup> In neurons, the GA is involved in the axoplasmic flow of numerous endogenous proteins and of exogenous macromolecules transported by orthograde, retrograde, and transsynaptic routes.<sup>26–28</sup> Therefore, GA fragmentation will have deleterious consequences for the proper function of axons and presynaptic terminals. Biologically, GA fragmentation is thought to be an early and probably irreversible lesion in neurodegeneration, caused by a variety of mechanisms, and is not necessarily secondary to apoptosis.<sup>29</sup> The findings in the present study suggest that GA fragmentation has a close relationship with reduced BTBD10 immunoreactivity. Dysfunction of stathmin, a regulator of microtubule polymerization, is thought to be linked to GA fragmentation.<sup>29</sup> It has been suggested that the phosphorylation of stathmin is an indicator of the activation of the PI3K/Akt signal.<sup>30</sup> Together, these results suggest that BTBD10 contributes indirectly to GA integrity by activating stathmin.

Finally, in the present study, we could not detect any relationship between the reduced BTBD10 immunoreactivity and the clinical course of the disease. Further studies are needed to determine the relationship between

BTBD10 reduction and clinical findings or course in ALS.

### ACKNOWLEDGMENTS

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