PCBP2 Is Downregulated in Degenerating Neurons and Rarely Observed in TDP-43-Positive Inclusions in Sporadic Amyotrophic Lateral Sclerosis

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PCBP2 is downregulated in degenerating neurons and rarely observed in TDP-43-positive inclusions in sporadic amyotrophic lateral sclerosis

Running title: PCBP2-positive inclusions in sporadic ALS

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ABSTRACT

Various heterogeneous nuclear ribonucleoproteins (hnRNPs) are deposited in pathological inclusions of amyotrophic lateral sclerosis (ALS) and related diseases such as frontotemporal lobar degeneration (FTLD). Recently, poly (rC)-binding protein 2 (PCBP2, hnRNP-E2), a member of the hnRNP family, was reported to be colocalized with transactivation-responsive DNA-binding protein 43 kDa (TDP-43)-immunopositive inclusions in cases of FTLD-TDP. Here, we used immunohistochemical methods to investigate PCBP1 and PCBP2 expression in the spinal cords of sporadic ALS patients, with special reference to TDP-43 positive inclusions. Thirty autopsy cases of sporadic ALS were examined by immunohistochemistry using antibodies against PCBP1, PCBP2, sequestosome 1 (p62), and TDP-43. In control subjects without neurological disorders, neurons predominantly expressed PCBP2, rather than PCBP1, in their cytoplasm and nuclei. Anterior horn cells of sporadic ALS patients often had various levels of PCBP2 expression, and motor neurons with skein-like inclusions often had reduced or lost cytoplasmic and nuclear PCBP2 staining. Notably, one case with FTLD-TDP subtype B pathology had marked

colocalization of TDP-43 and PCBP2 in the cytoplasmic inclusions and dystrophic neurites of the cerebral cortex, hippocampus, and spinal cord. In conclusion, PCBP2 was reduced in anterior horn cells of sporadic ALS, but its occurrence in TDP-43 inclusions was a rare phenomenon.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of both upper and lower motor neurons, which clinically leads to muscle weakness and eventual paralysis. Major pathological findings in ALS include degeneration of the precentral gyri, pyramidal tracts, spinal anterior horns, and lower cranial motor nuclei, in various combinations. In addition, the appearance of skein-like inclusions (SLIs) in spinal anterior horn cells is pathognomonic for sporadic ALS (1). The major constituent of these SLIs has been identified as transactive response DNA-binding protein 43 kDa (TDP-43) (2, 3), which in humans is encoded by the *TARDBP* gene (4). Diseases based on TDP-43 aggregation and accumulation are collectively referred to as TDP-43 proteinopathies, which range from ALS to frontotemporal lobar degeneration (FTLD) (2, 3, 5). TDP-43 belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) family, and a relationship between ALS and other hnRNPs has been reported for Fused in sarcoma (FUS) (6, 7), hnRNP A1 (8, 9), and TIA1 cytotoxic granule-associated RNA-binding protein (TIA1) (10).

Poly (rC)-binding proteins (PCBPs) are also members of the hnRNP family, and are characterized by their high-affinity and sequence-specific interactions with polycytosine. They are classified as PCBP1, PCBP2, PCBP3, PCBP4, and hnRNP K. In particular, PCBP1 and PCBP2 are also known as hnRNP-E1 and hnRNP-E2, respectively. All of these PCBPs have three K-homology (KH) domains, are able to shuttle between the nucleus and cytoplasm, and regulate gene expression at various levels including transcription, mRNA processing, mRNA stabilization, and translation (11, 12). PCBP1 is 83 % identical to PCBP2 at the DNA level and 90 % homologous at the amino acid level (13). However, several reports suggest that PCBP1 and PCBP2 may have different functions in the central nervous system. PCBP1 has been reported to induce apoptosis under conditions of severe oxidative stress (14), and forms intranuclear inclusions in affected neurons in Huntington's disease (15). In contrast, PCBP2 has been identified as a protein component of TDP-43-positive cytoplasmic inclusions in some types of FTLD-TDP (16, 17).

As yet, there have been no multi-case reports of PCBP1 and PCBP2 in sporadic ALS. We therefore studied alterations in PCBP2 expression in spinal motor

neurons of ALS cases, and investigated the possible association between PCBP2 and TDP-43 pathology.

MATERIALS AND METHODS

Case materials

We examined 30 autopsied cases that were clinicopathologically diagnosed with sporadic ALS, from Kyushu University Hospital and the National Omuta Hospital, Fukuoka, Japan. The two control cases, which showed no neurodegenerative changes, were a case of Duchenne muscular dystrophy (DMD) and a case of esophageal varix and liver cirrhosis. Characteristics of the cases are shown in Table 1. In addition to ALS pathology, FTLD-TDP pathology was observed in seven cases (cases 1–7 in Table 1). All seven of these cases were consistent with subtype B in the FTLD-TDP subset (18). All cases died between 2005 and 2019, and the autopsies were performed by pathologists of the Department of Neuropathology, Kyushu University. Written informed consent for autopsy, including the use of tissue for research purposes, was obtained. All analyses were performed in accordance with the Declaration of Helsinki.

This study was approved by the Ethics Committee of the Faculty of Medicine, Kyushu University (#2020-167).

Western blotting

Western blotting with anti-PCBP1 (rabbit polyclonal, ab74793, Abcam, Cambridge, UK) and anti-PCBP2 (mouse monoclonal (M07), Abnova, Taipei, Taiwan) antibodies was conducted to confirm their specificity and to assess the level of PCBP1 and PCBP2 in the brain tissue which from frontal cortex of the non-neurodegenerative case (control 1 in table 1). Cell extracts were prepared from HeLa S3 PCBP1- and PCBP2-knockout cell lines (14) using SDS sample buffer (60 mmol/L Tris-HCI (pH 6.8), 2 % SDS, and 10 % glycerol). Recombinant His-PCBP2 protein was expressed in *Escherichia coli* and purified using His affinity columns. The cell extracts and purified recombinant proteins were applied to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The immunoreactions were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Brain sample (frontal cortex of control 1 in Table 1) was frozen at the time of autopsy and stored at −80°C until further use. Control sample was

derived from a male of Duchenne muscular dystrophy. The sample was homogenized to a final concentration of 10 % in lysis buffer (1 % Nonidet P-40, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, 1 % sodium deoxycholate, 1 % SDS, 1 % Triton X-100, and 1 tablet cOmplete™ (Protease Inhibitor Cocktail Tablet, Roche Molecular Biochemicals, Rotkreuz, Switzerland)). The sample were electrophoresed by gels which were cast using a TGX™ Fast Cast™ Acrylamide kit 10 % (#1610173, Bio-Rad, Hercules, CA) and transferred onto polyvinylidene difluoride membrane. Anti-PCBP1 and anti-PCBP2 antibodies were used as the primary antibodies to detect PCBP1 and PCBP2, respectively. Peroxidase-conjugated anti-rabbit IgG (#1706515, 1:3000, Bio-Rad) and anti-mouse IgG (AP192P, 1:3000, Chemicon, Temecula, CA) were used for secondary antibodies. The immunoreaction was visualized using the same system.

Neuropathological examinations

The brains and spinal cords were fixed in 10 % buffered formalin and processed into paraffin-embedded sections. Specimens from the ALS cases included the middle frontal gyrus, precentral gyrus, superior and middle temporal gyri, inferior parietal lobule, anterior cingulate gyrus, amygdala, hippocampus with entorhinal and

transentorhinal cortices (at the level of the lateral geniculate body), calcarine cortex, basal ganglia, thalamus, cerebellum, midbrain, pons, medulla oblongata, and spinal cord (levels C4, C6, Th4, Th10, L3, and S2–3). Multiple 6 μm sections were routinely stained with hematoxylin and eosin (H&E) and Klüver–Barrera staining.

Immunohistochemistry

Immunohistochemistry was performed using primary antibodies against PCBP1 (rabbit polyclonal, 1:100; ab74793, Abcam), PCBP2 (mouse monoclonal (M07), 1:1000; Abnova), phosphorylated TARDBP (p-TDP-43; rabbit polyclonal, 1:5000; Cosmo Bio, Tokyo, Japan), TARDBP (TDP-43; rabbit polyclonal, 1:2000; Protein Tech Group Inc., Chicago, IL, USA), and sequestosome 1 (p62; rabbit polyclonal, 1:1000; MBL, Nagoya, Japan). Sections were deparaffinized in xylene and rehydrated in an ethanol gradient. Tissue was pretreated for antigen retrieval by autoclaving in 0.01 mol/L citrate buffer (pH 6.0) at 121°C for 10 min. Sections were then incubated with primary antibodies overnight at 4°C. After rinsing, immunoreaction products were detected using the polymer immunocomplex method (Envision system; DakoCytomation, Glostrup, Denmark). Immunoreactivity was visualized using 3,3'-

diaminobenzidine (Dojindo, Kumamoto, Japan) and specimens were lightly counterstained with hematoxylin.

Immunofluorescence

We performed double immunofluorescence staining with the following combinations of antibodies: anti-PCBP2 with anti-phosphorylated TARDBP, anti-PCBP2 with anti-TARDBP, anti-p62 with anti-PCBP2, and anti-p62 with anti-phosphorylated TARDBP. Alexa 488-labeled anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) and Alexa 546-labeled anti-mouse IgG (Invitrogen) were used as the secondary antibodies (1:50 dilution). Sections were deparaffinized and rehydrated, and antigen retrieval was performed as for the immunohistochemical experiments. Sections were then incubated with primary antibodies overnight at 4°C, rinsed, and incubated with appropriate combinations of secondary antibodies for 1 h at room temperature before being counterstained with 4',6-diamidino-2-phenylindole (Invitrogen). Specimens were observed using a ZEISS LSM 700 Laser Scanning Microscope (ZEISS, Oberkochen, Germany).

RESULTS

The specificities of the antibodies against PCBP1 and PCBP2

We first confirmed the specificities of the antibodies against PCBP1 and PCBP2 using western blotting, because of their high amino acid sequence homology. Each antibody showed high specificity for each protein, using cell homogenate samples from the knockout cell lines and recombinant proteins (Fig. 1).

The cellular distribution of PCBP1 and PCBP2 in the central nervous system

Next, we examined the cellular distribution of PCBP1 and PCBP2 in the central nervous system using immunohistochemistry. In the brains of the control cases, immunohistochemistry for PCBP1 and PCBP2 revealed differential neuronal expressions. Immunohistochemistry for PCBP1 revealed weak immunoreactivity in the cytoplasm of anterior horn cells of the spinal cord (Fig. 2A) and cerebral cortical neurons (Fig. 2C). Immunohistochemistry for PCBP2 in the spinal cord revealed strong cytoplasmic and nuclear staining in both anterior horn and posterior horn cells. The nuclear membrane and Nissl bodies were particularly accentuated (Fig. 2B). In layer 5

of motor cortical neurons at the precentral gyrus, the nuclei and perikarya were equally stained by PCBP2 immunostaining (Fig. 2D), and their intensities were stronger than those of PCBP1 immunostaining (Fig. 2C). These staining patterns were similarly observed in other cerebral neocortices.

To assess quantitatively whether the expression level of PCBP2 is dominant in cortical neurons, we performed western blot assay using the frontal cortex of the non-neurodegenerative case (control 1 in Table 1). The blot with anti-PCBP1 antibody showed a weak band near 37 kDa corresponding to PCBP1 (Fig. 2E). By contrast, the blot with anti-PCBP2 antibody showed distinct three bands around 40 kDa (Fig. 2F) whose pattern is similar to the sample of HeLa S3 (Fig. 1). Both these two lanes, anti-PCBP1 and anti-PCBP2 antibody, it was performed to stain whole protein with Ponceau S solution (P7170, Sigma-Aldrich, St. Lois, MO) before primary antibody response. It followed that these two lanes have almost same level of whole protein (Fig. 2E, F). Thus, PCBP2 was more abundantly expressed in the cortical neurons than PCBP1.

Reduced expression of PCBP2 in ALS cases

In 25 of the 30 ALS cases that were examined, SLIs were detected in the anterior horn cells of spinal cords using immunohistochemistry. Some cases without SLIs were at an advanced stage, with almost complete loss of the anterior horn cells. One case had ALS with FUS-positive basophilic inclusions. Anterior horn cells with SLIs often showed reduced expression of PCBP2 compared with that of anterior horn cells without SLIs (Fig. 3A, B). To semiquantitatively investigate this finding, 10 cases of ALS (cases 1, 2, 5, 8, 9, 11, 12, 19, 23, and 27 in Table 1) were selected because they had high numbers of SLIs. We calculated the ratio of PCBP2-negative anterior horn cells with SLIs to the total number of anterior horn cells with SLIs in these 10 ALS cases. We then compared this ratio with the ratio of PCBP2-negative anterior horn cells to the total number of anterior horn cells in the two control cases (control cases 1 and 2 in Table 1). There were 186 anterior horn cells with SLIs in the 10 ALS cases, of which 85 cells (45.7 %) showed a loss or markedly reduced expression of PCBP2. In the two control cases, 180 anterior horn cells were counted, and 37 of these (20.6 %) showed diminished PCBP2. We did not observe any notable differences in PCBP1 expression between ALS cases, FTLD-TDP cases, and control cases.

PCBP2-positive inclusions in ALS

Two cases of ALS (cases 1 and 8 in Table 1) had PCBP2-positive intracellular inclusions, and PCBP2 and p-TDP-43 were colocalized in SLIs in these cases. Immunohistochemistry for p-TDP-43 in case 1 revealed typical SLIs in spinal anterior horn cells (Fig. 4A), as well as round inclusions (Fig. 4B). Dystrophic neurites (Fig. 4C) and dot-like deposits (Fig. 4D) were also observed in the neuropil of the spinal anterior horn. Immunohistochemistry for PCBP2 demonstrated similar pathological structures, such as PCBP2-immunopositive SLIs (Fig. 4E), round inclusions (Fig. 4F), dystrophic neurites (Fig. 4G), and dot-like deposits (Fig. 4H) in the spinal anterior horn. In addition, the normal staining pattern of PCBP2 was markedly diminished (Fig. 4E, F) compared with control cases (Fig. 2B). Double immunofluorescence for PCBP2 and non-phosphorylated TDP-43 confirmed their almost complete colocalization in SLIs (Fig. 4I-K). Colocalization of p-TDP-43 and PCBP2 was also observed in dystrophic neurites (Fig. 4L–N). In anterior horn cells of

case 8, a few p-TDP-43-immunopositive SLIs were also colocalized with PCBP2 (Fig. 4O–Q).

Case 1 of ALS-FTLD with PCBP2-positive inclusions

A 71-year-old male (case 1 in Table 1) developed weakness of the distal muscles in his right upper limb. Muscle weakness and atrophy of the whole body occurred and he needed total assistance over the course of 1 year. Dysarthria and dysphagia gradually progressed, and the patient needed to be tube fed in the 6 months before his death as a result of complications of aspiration pneumonia. However, he was never treated with non-invasive or tracheostomy positive-pressure ventilation (NPPV, TPPV). Mild impairment of recent memory at the incipient stage of the disease was noted by neuropsychological evaluation (Hasegawa's Dementia Scale-Revised). Clinical symptoms of frontotemporal dementia-like changes in personality, social conduct, or semantic dementia were not evident. He died of respiratory failure as a result of ALS and aspiration pneumonia, aged 73 years.

The neuropathological examination of case 1 revealed FTLD-TDP subtype B pathology as well as ALS pathology (Fig. 5). His brain weighed 1300 g. The frontotemporal lobes were moderately atrophic (Fig. 5A), and spongiosis of the second layer of the frontal cortex was noted (Fig. 5D). Transverse sections of the cervical cord (C4) showed severe degeneration of the lateral corticospinal tracts (Fig. 5B) and a moderate loss of motor neurons (Fig. 5C). Immunohistochemistry for p-TDP-43 revealed SLIs in the lumbar cord (Fig. 5E), crescent-shaped inclusions in the hippocampal dentate gyrus (Fig. 5F), and neuronal cytoplasmic inclusions in the superficial (Fig. 5G) and deep (Fig. 5H) layers of the precentral gyrus. Several dystrophic neurites were also detected in the cortex (Fig. 5H). Thus, the autopsy findings were consistent with ALS-FTLD.

We further examined the cellular expression of PCBP2 in various regions of the central nervous system in case 1 (Fig. 6, 7). Immunohistochemistry with both anti-p-TDP-43 and anti-PCBP2 antibodies revealed spherical/crescent-shaped inclusions (Fig. 6A, D) or round neuronal cytoplasmic inclusions (Fig. 6A, C, D, F) in the cortex and in hippocampal dentate gyrus granule cells. Dystrophic neurites in the cortex were also

immunopositive for both p-TDP-43 and PCBP2 (Fig. 6B, E). Glial cells with p-TDP-43-positive inclusions were detected in the cortical deep layer and the subcortical white matter of the precentral gyrus (Fig. 6C inset), and PCBP2-positive glial inclusions were similarly identified (Fig. 6F inset). We observed neuronal cytoplasmic inclusions (Fig. 6G–I) and dystrophic neurites (Fig. 6J–L) in the precentral gyrus and the middle frontal gyrus, and there were also neuronal cytoplasmic inclusions in hippocampal dentate gyrus granule cells (Fig. 6M–O) using double immunofluorescence. Immunohistochemistry with anti-p62 antibody also demonstrated similar structures (Fig. 7A–E). Thus, we prepared mirror-image specimens and demonstrated the colocalization of p62, p-TDP-43, and PCBP2 in SLIs (Fig. 7F–M).

Absence of PCBP2-positive inclusions in the cerebral tissues of other sALS cases

Based on the results of case 1, the cerebral cortices of other sALS cases were examined to clarify the relationship between p-TDP-43-positive inclusions and PCBP2. Although many p-TDP-43-positive neuronal and glial cytoplasmic inclusions were observed in the precentral gyrus and frontal cortex of case 8, PCBP2-positive

aggregates were not detected. Likewise, other cases presenting p-TDP-43-positive aggregates in the frontal cortices (case 2–4, 6–8, 14, 15, and 17–28 in Table 1) did not revealed PCBP2-positive aggregates. In limbic system using sections of hippocampus with entorhinal and transentorhinal cortices at the level of the lateral geniculate body, there were 11 sALS cases which identified extra-motor TDP-43 pathology without antemortem clinically diagnosis of frontotemporal dementia (case 1–7, 15, 18, 21, and 23 in Table 1). In all cases except case 1, PCBP2-positive aggregates were not detected in the limbic lesions.

DISCUSSION

Various hnRNPs have been identified to be linked with ALS/FTLD (19). At least two common pathological events involving hnRNPs are observed in the affected neurons of ALS (11). One is the loss of the normal nuclear distribution of hnRNPs, and their translocation to the cytosol, where stress granules are formed. This finding supports the idea that altered RNA metabolism is an underlying pathomechanism for ALS/FTLD. For example, hnRNP A1 and A2/B1, which are highly expressed cellular

proteins (20), are sequestered in cytosolic stress granules together with other RNA-binding proteins that are causative for ALS/FTLD, such as FUS and TDP-43 (8, 21). Another common, pathognomonic finding of ALS/FTLD is the abnormal aggregation of hnRNPs. Mutations in the prion-like domains of hnRNP A1 and A2/B1 are causative for ALS20 and ALS/FTLD, respectively (9). In normal conditions, these hnRNPs have an intrinsic tendency to self-aggregate because of their prion-like domains, but this tendency is abnormally increased as a result of these disease-causing mutations (9). In the present study, we demonstrated that PCBP2 was also involved in the pathological processes of ALS. First, anterior horn cells with SLIs frequently showed a loss of cytoplasmic and nuclear PCBP2 staining. Second, we found two sporadic ALS cases in which SLIs in anterior horn cells had TDP-43 and PCBP2 colocalization.

The relationship between stress granules and the pathophysiology of ALS has attracted attention, and it has been reported that TDP-43 is recruited to stress granules in situations of oxidative stress (22, 23). Following oxidative stress, endogenous TDP-43 and PCBP2 colocalize within stress granules, and also colocalize with the stress granule

marker PABP1 (17). PCBP2 has been identified as a facilitator of internal ribosome entry site-mediated translation, and has an important role both in remodeling mRNAs in stress granules and in transferring specific mRNAs from stress granules to P-bodies for degradation (24, 25). Furthermore, a direct association between TDP-43 and PCBP2 has been detected using an enhanced cross-linking and immunoprecipitation protocol (eCLIP-seq); thus, PCBP2 is considered a target molecule for TDP-43 (26).

In our study, a single case (case 1) of sporadic ALS/FTLD showed extensive colocalization of TDP-43 and PCBP2 in the cerebrum and spinal cord. The TDP-43 pathology of this case corresponded to FTLD-TDP subtype B pathology, and the colocalization of TDP-43 and PCBP2 was readily detectable in cytoplasmic inclusions and dystrophic neurites in the cerebral cortices and hippocampal dentate gyrus granule cells. Kattuah et al. reported cortical cytoplasmic inclusions and dystrophic neurites with colocalized TDP-43 and PCBP2 in subtypes A and C of FTLD-TDP, but not in subtype B (17). Further study may therefore be necessary to clarify the clinicopathological relationship between FTLD-TDP subtypes and PCBP2-positive neuronal inclusions.

The unique molecular structures of PCBP1 and PCBP2, together with hnRNP K, contain KH domains that bind RNA. The KH domain is characterized by a 45-amino-acid repeat, and the core region has three-stranded antiparallel β-sheets together with three α-helices (βααββα). It has been shown using surface plasmon resonance that the KH domain has a high affinity for poly(C) repeats (27). So far, no mutations of PCBP2 have been identified in human neurological diseases. However, in the PCBP family, a heterozygous mutation in the hnRNP K gene has been reported to be causative for Okamoto syndrome (also known as Au–Kline syndrome) (28, 29), and to possibly be involved in ALS-FTLD (30). Although it remains uncertain whether PCBP2 can induce prion-like behavior, the identification of neuronal inclusions containing PCBP2 in the present study suggests that this may be a possibility.

Conclusions

Many RNA-binding proteins with prion-like domains have been strongly linked to stress granule assembly and neurodegenerative diseases (31-33). Likewise, PCBP2 may be involved in the pathways by which TDP-43 positive cytoplasmic

inclusions are formed and anterior horn cells are degenerated in ALS and FTLD-TDP subtype B.

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REFERENCES

1. Mizusawa H, Nakamura H, Wakayama I, et al. Skein-like inclusions in the anterior horn cells in motor neuron disease. *J Neurol Sci* 1991;105:14-21

2. Arai T, Hasegawa M, Akiyama H, et al. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun* 2006;351:602-611

3. Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006;314:130-133

4. Ignatius SH, Wu F, Harrich D, et al. Cloning and Characterization of a Novel Cellular Protein, Tdp-43, That Binds to Human-Immunodeficiency-Virus Type-1 Tar DNA-Sequence Motifs. *Journal of Virology* 1995;69:3584-3596

5. Kwong LK, Neumann M, Sampathu DM, et al. TDP-43 proteinopathy: the neuropathology underlying major forms of sporadic and familial frontotemporal lobar degeneration and motor neuron disease. *Acta Neuropathol* 2007;114:63-70

6. Kwiatkowski TJ, Jr., Bosco DA, Leclerc AL, et al. Mutations in the FUS/TLS

gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 2009;323:1205-1208

7. Vance C, Rogelj B, Hortobagyi T, et al. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 2009;323:1208-1211

8. Honda H, Hamasaki H, Wakamiya T, et al. Loss of hnRNPA1 in ALS spinal cord motor neurons with TDP-43-positive inclusions. *Neuropathology* 2015;35:37-43

9. Kim HJ, Kim NC, Wang YD, et al. Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* 2013;495:467-473

10. Mackenzie IR, Nicholson AM, Sarkar M, et al. TIA1 Mutations in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia Promote Phase Separation and Alter Stress Granule Dynamics. *Neuron* 2017;95:808-816 e809

11. Geuens T, Bouhy D, Timmerman V. The hnRNP family: insights into their role in health and disease. *Hum Genet* 2016;135:851-867

12. Makeyev AV, Liebhaber SA. The poly(C)-binding proteins: a multiplicity of functions and a search for mechanisms. *RNA* 2002;8:265-278

13. Leffers H, Dejgaard K, Celis JE. Characterisation of Two Major Cellular Poly(rC)-Binding Human Proteins, Each Containing Three K-homologous (KH) Domains. *European Journal of Biochemistry* 1995;230:447-453

14. Ishii T, Hayakawa H, Igawa T, et al. Specific binding of PCBP1 to heavily oxidized RNA to induce cell death. *Proc Natl Acad Sci U S A* 2018;115:6715-6720

15. Mori S, Honda H, Ishii T, et al. Expanded polyglutamine impairs normal nuclear distribution of fused in sarcoma and poly (rC)-binding protein 1 in Huntington's disease. *Neuropathology* 2019;39:358-367

16. Davidson YS, Robinson AC, Flood L, et al. Heterogeneous ribonuclear protein E2 (hnRNP E2) is associated with TDP-43-immunoreactive neurites in Semantic Dementia but not with other TDP-43 pathological subtypes of Frontotemporal Lobar Degeneration. *Acta Neuropathol Commun* 2017;5:54

17. Kattuah W, Rogelj B, King A, et al. Heterogeneous Nuclear Ribonucleoprotein E2 (hnRNP E2) Is a Component of TDP-43 Aggregates Specifically in the A and C Pathological Subtypes of Frontotemporal Lobar Degeneration. *Front Neurosci* 2019;13:551

18. Mackenzie IR, Neumann M, Baborie A, et al. A harmonized classification system for FTLD-TDP pathology. *Acta Neuropathol* 2011;122:111-113

19. Zhao M, Kim JR, van Bruggen R, et al. RNA-Binding Proteins in Amyotrophic Lateral Sclerosis. *Mol Cells* 2018;41:818-829

20. Dreyfuss G, Kim VN, Kataoka N. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol* 2002;3:195-205

21. Molliex A, Temirov J, Lee J, et al. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* 2015;163:123-133

22. Ayala YM, Zago P, D'Ambrogio A, et al. Structural determinants of the cellular localization and shuttling of TDP-43. *J Cell Sci* 2008;121:3778-3785

23. Colombrita C, Zennaro E, Fallini C, et al. TDP-43 is recruited to stress granules in conditions of oxidative insult. *J Neurochem* 2009;111:1051-1061

24. Fujimura K, Kano F, Murata M. Identification of PCBP2, a facilitator of IRES-mediated translation, as a novel constituent of stress granules and processing bodies. *RNA* 2008;14:425-431

25. Fujimura K, Katahira J, Kano F, et al. Selective localization of PCBP2 to cytoplasmic processing bodies. *Biochim Biophys Acta* 2009;1793:878-887

26. Tam OH, Rozhkov NV, Shaw R, et al. Postmortem Cortex Samples Identify Distinct Molecular Subtypes of ALS: Retrotransposon Activation, Oxidative Stress, and Activated Glia. *Cell Rep* 2019;29:1164-1177 e1165

27. Sidiqi M, Wilce JA, Vivian JP, et al. Structure and RNA binding of the third KH domain of poly(C)-binding protein 1. *Nucleic Acids Res* 2005;33:1213-1221

28. Au PYB, You J, Caluseriu O, et al. GeneMatcher aids in the identification of a new malformation syndrome with intellectual disability, unique facial dysmorphisms, and skeletal and connective tissue abnormalities caused by de novo variants in HNRNPK. *Hum Mutat* 2015;36:1009-1014

29. Okamoto N. Okamoto syndrome has features overlapping with Au-Kline syndrome and is caused by HNRNPK mutation. *Am J Med Genet A* 2019;179:822-826

30. Moujalled D, Grubman A, Acevedo K, et al. TDP-43 mutations causing amyotrophic lateral sclerosis are associated with altered expression of RNA-binding protein hnRNP K and affect the Nrf2 antioxidant pathway. *Hum Mol Genet*

2017;26:1732-1746

31. Gitler AD, Shorter J. RNA-binding proteins with prion-like domains in ALS and FTLD-U. *Prion* 2011;5:179-187

32. King OD, Gitler AD, Shorter J. The tip of the iceberg: RNA-binding proteins with prion-like domains in neurodegenerative disease. *Brain Res* 2012;1462:61-80

33. Wolozin B. Regulated protein aggregation: stress granules and neurodegeneration. *Mol Neurodegener* 2012;7:56

FIGURE LEGENDS

Figure 1

Western blot assays probed with anti-PCBP1 and anti-PCBP2 antibodies. The samples were extracted from HeLa S3 PCBP1- and PCBP2- knockout cell lines, shown as PCBP1(-) and PCBP2(-), respectively. Recombinant His-PCBP1 protein (PCBP1-His_6) and recombinant His-PCBP2 protein (PCBP2-His_6) were purified using His affinity columns. The cell extracts (each containing 7.5 μg protein) and purified proteins (each containing 3.75 ng protein) were applied to SDS-PAGE and used to examine the specificities of anti-PCBP1 (left) and anti-PCBP2 (right) antibodies, respectively. Immunoblot with anti-PCBP1 antibody shows a single main band at 37 kDa (white arrowhead), and immunoblot with anti-PCBP2 antibody shows a main band at 40 kDa (black arrowhead) with several lower-molecular weight bands.

Figure 2

Cellular distribution of PCBP1 (A, C) and PCBP2 (B, D) in the two control cases (A, B: control 2 in Table 1. C, D: control 1 in Table 1). (A) With PCBP1

immunohistochemistry in the anterior horn of the spinal cord, the nucleus and cytoplasm of neurons were weakly stained, and slight neuropil staining was diffusely observed. Blood vessels and glial cells was also positively stained. (B) With PCBP2 immunohistochemistry in the anterior horn of the spinal cord, the nucleus, rough endoplasmic reticulum, and cytoplasm of the motor neurons were more selectively stained than with PCBP1 immunohistochemistry. Lipofuscin in the anterior horn cell (arrow) did not stain. (C) With PCBP1 immunohistochemistry in layer 5 of the motor cortex (precentral gyrus), weak expression was observed both in the pyramidal neurons and neuropil. PCBP1 was expressed strongly in certain glia (arrows). (D) With PCBP2 immunohistochemistry in the cortex of the precentral gyrus, neurons were more selectively stained than with PCBP1 immunohistochemistry. Scale bar: 50 μm.

(E, F) Western blot analysis of the total homogenate of frontal cortex (control 1 in Table 1) using anti-PCBP1 and anti-PCBP2 antibodies, respectively. Immunoblot with anti-PCBP1 antibody showed several weak bands around 40 kDa (white arrowhead), and immunoblot with anti-PCBP2 showed a major band (black arrowhead). Total proteins of each lane was stained with Ponceau S solution to check the evenness of the sample

loading. The expression of PCBP2 protein was more abundant than that of PCBP1 protein in the frontal cortex.

Figure 3

Immunohistochemistry was performed using anti-p-TDP-43 (A) and anti-PCBP2 (B) antibodies in the spinal cord of case 11. There were several anterior horn cells with skein-like inclusions that had reduced PCBP2 expression (asterisk). Sections are mirror images (two consecutive sections) and one has been turned upside down. Scale bar: 100 μm.

Figure 4

Immunohistochemistry for p-TDP-43 (A–D) and PCBP2 (E–H) in spinal anterior horn cells of case 1. Skein-like inclusions (A, E), round inclusions (B, F), dystrophic neurites (C, G), and dot-like deposits (D, H) were observed. Double immunofluorescence for PCBP2 and TDP-43/p-TDP-43 in the spinal anterior horn of case 1 (I–N) and case 8

(O–Q). Skein-like inclusions (I–K, O–Q) and dystrophic neurites (L–N) were observed. Scale bars: 20 μm.

Figure 5

Macroscopic specimen and histopathology of case 1 is shown. The brain weight was 1300 g, and frontal-lobe-dominated atrophy was observed (A). In the cervical spinal cord, Klüver–Barrera (KB) staining revealed atrophy of the anterior horn and degeneration of the lateral corticospinal tract (B). Atrophy and reduction of anterior horn cells were obvious (C). In the cortex of the precentral gyri, vacuolar degeneration and gliosis were observed with hematoxylin and eosin staining (D). Immunohistochemistry for p-TDP-43 revealed skein-like inclusions in anterior horn cells of the spinal cord (E). In the brain, spherical/crescent-shaped or round neuronal cytoplasmic inclusions were also observed in hippocampal dentate gyrus granule cells (F) and cortical neurons in the superficial (G) and deep (H) layers. Scale bars: C, D = 20 μm. E–H = 50 μm.

Figure 6

Immunohistochemistry for p-TDP-43 (A–C) and PCBP2 (D–F) in hippocampal dentate gyrus granule cells (A, D), the entorhinal cortex (B, E), the middle frontal gyrus (C, F), and the precentral gyrus (insets of C and F) of case 1. Double immunofluorescence for PCBP2 and p-TDP-43 (G–O) in the precentral gyrus (G–I), middle temporal gyri (J–L), and hippocampal dentate gyrus granules cells (M–O). Scale bars: 20 μm.

Figure 7

Immunohistochemistry for p62 in the spinal cord (A, B), middle temporal gyri (C), entorhinal cortex (D), and hippocampal dentate gyrus granule cells (E). Double immunofluorescence for p-TDP-43 and p62 (F–I), and PCBP2 and p62 (J–M), in spinal anterior horn cells with skein-like inclusions from case 1 (two consecutive sections). Scale bars: 20 μm.