Poly-A Binding Protein-1 Localization to a Subset of TDP-43 Inclusions in Amyotrophic Lateral Sclerosis Occurs More Frequently in Patients Harboring an Expansion in C9orf72

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Abstract
Amyotrophic lateral sclerosis (ALS) is an adult-onset motor neuron disease in which the loss of spinal cord motor neurons leads to paralysis and death within a few years of clinical disease onset. In almost all cases of ALS, transactive response DNA binding protein of 43 kDa (TDP-43) forms cytoplasmic neuronal inclusions. A second causative gene for a subset of ALS is fused in sarcoma, an RNA binding protein that also forms cytoplasmic inclusions in spinal cord motor neurons. Poly-A binding protein-1 (PABP-1) is a marker of stress granules (i.e. accumulations of proteins and RNA indicative of translational arrest in cells under stress). We report on the colocalization of PABP-1 to both TDP-43 and fused-in-sarcoma inclusions in 4 patient cohorts: ALS without a mutation, ALS with an intermediate polyglutamine repeat expansion in ATXN2, ALS with a GGGGCC hexanucleotide repeat expansion in C9orf72, and ALS with basophilic inclusion body disease. Notably, PABP-1 colocalization to TDP-43 was twice as frequent in ALS with C9orf72 expansions compared to ALS with no mutation. This study highlights PABP-1 as a protein that is important to the pathology of ALS and indicates that the proteomic profile of TDP-43 inclusions in ALS may differ depending on the causative genetic mutation.

Key Words: Amyotrophic lateral sclerosis, ATXN2, C9orf72, FUS, Inclusion, Motor neuron, PABP-1, Stress granule, TDP-43.

INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is an adult-onset motor neuron disease in which the loss of spinal cord motor neurons leads to a relatively rapid onset of paralysis and death. To date, the only effective treatment is benzothiazole riluzole, which only modestly extends patient survival (1). A common pathologic feature in almost all cases of ALS is the formation of neuronal cytoplasmic inclusions (NCIs) and dystrophic neurites containing the normally nuclear transactive response DNA binding protein of 43 kDa (TDP-43) (2). A second RNA binding protein that forms intraneuronal inclusions and is causative of a subset of ALS is the fused in sarcoma (FUS) protein (3–11). Detailed analysis of proteins that help form and maintain pathologic inclusions aids in understanding the underlying cellular mechanisms that lead to degeneration of motor neurons.

The pathologic accumulation of TDP-43 and FUS into NCIs is common in several neurodegenerative disorders. In frontotemporal degeneration (FTD), which shows clinical and pathologic overlap with ALS (12, 13), TDP-43 forms NCIs in a number of regions within the brain (2, 14, 15). Another example of pathologic inclusions found in ALS and FTD are basophilic inclusions. Basophilic inclusions are negative for TDP-43 but are positive for FUS and a number of other RNA binding proteins (3–5, 9, 16–19). Both TDP-43 and FUS are proteins involved in a range of RNA biogenesis processes, including the transport of RNA transcripts into cytoplasmic stress granules (20, 21). Stress granules are cytosolic structures that store RNA in a translationally repressed manner upon exposure to stress (22). It has been proposed that the localization of TDP-43 and FUS to cytoplasmic stress granules may lead to the formation of TDP-43 and FUS NCIs in ALS (19, 22, 23). Several reports have indicated that some, but not all, stress granule markers colocalize to TDP-43 NCIs and to basophilic inclusions in spinal cord motor neurons in ALS (18, 19, 24).

We reported that stress granule–associated pathways can modulate TDP-43 toxicity in Drosophila, yeast, and mammalian cells, and that poly-A binding protein-1 (PABP-1) forms NCIs in ALS motor neurons; like ataxin 2 (a gene associated with ALS/FTD and a stress granule protein), PABP-1 can modulate TDP-43 toxicity in Drosophila (25, 26). Poly-A binding protein-1 pathology has been noted in TDP-43 NCIs in the motor neurons of ALS patients of undocumented genetic background, in basophilic inclusions in ALS motor neurons, and NCIs in a mouse model of ALS (18, 27, 28). Given that we have shown that PABP-1 function is a regulator of TDP-43 toxicity in vivo model systems, we now translate these studies into the human setting and thoroughly examine the pathology of PABP-1 in the spinal cord of human ALS. We demonstrate that PABP-1 pathology is a common feature of multiple RNA binding
protein–associated inclusions in various genetic subtypes of ALS patients and, notably, is twice as prevalent in patients harboring a GGGGCC repeat expansion in C9orf72.

**MATERIALS AND METHODS**

**Clinical Data**

Patients diagnosed with ALS were selected based on the presence of phosphorylated TDP-43 (pTDP-43) pathology in the motor neurons of the spinal cord. Basophilic inclusion body disease (BIBD) cases were selected based on the presence of FUS pathology in spinal cord motor neurons. Table 1 provides summaries for each patient studied at the Center for Neurodegenerative Disease Research Brain Bank at the University of Pennsylvania.

**Immunohistochemistry**

Tissue was examined by routine neuropathologic diagnostic methods, as described previously (2, 29–31). Briefly, spinal cord samples were fixed in 10% neutral buffered formalin (ALS cases with no known mutation and ALS cases with mutation in C9orf72) or 70% ethanol with 150 mmol/L NaCl (ALS cases with mutation in ATXN2 and 2 BIBD cases with ALS), and 7-μm-thick sections were cut. Testing of cases where both ethanol- and formalin-fixed tissues were available showed robust staining of both PABP-1 and pTDP-43 inclusions and did not reveal qualitative differences in immunoreactivity. For detection of 3,3-diaminobenzidine, immunohistochemistry was performed on serial sections using standard avidin-biotin complex ABC detection methods (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) with citrate microwave antigen retrieval (Vector Laboratories). The antibodies used were rat anti-pTDP-43 monoclonal antibody (S409/410, 1:500), rabbit anti-PABP-1 antibody (1:800 for 3,3-diaminobenzidine detection and 1:100 for immunofluorescence; Cell Signaling Technology, Danvers, MA), and mouse anti-FUS antibody (1:1000; Proteintech Group Inc, Chicago, IL). Sections were counterstained either with hematoxylin for 3,3-diaminobenzidine detection or with DAPI for immunofluorescence. Inclusions positive for pTDP-43, FUS, and/or PABP-1 were calculated for each case. To determine the frequency of colocalization between pTDP-43/FUS and PABP-1 in each genetic background, we quantified the inclusions across all cases.

**Consent**

All patients preconsented for autopsy, and consent for autopsy was reobtained from the next of kin at the time of death. The University of Pennsylvania Institutional Review Board confirmed that the Center for Neurodegenerative Disease Research Neurodegenerative Disease Autopsy Brain Bank protocols are exempt from full human subjects research review.

**TABLE 1. Cases**

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<th>Diagnosis</th>
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<th>Age at Death, years</th>
<th>Region Analyzed</th>
<th>Mutation Status</th>
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<td>M</td>
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<td>78</td>
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</table>

(—) No known mutation in TARDBP, UBQLN2, ATXN2, and C9orf72. (•) Data unknown. ATXN2 refers to an intermediate polyQ expansion in ataxin 2 (pathologic repeat length is indicated in brackets). C9orf72 refers to a GGGGCC hexanucleotide repeat expansion.

ALS-D, ALS with dementia; F, female; M, male.
RESULTS

Study Subjects: Clinical Characteristics and Diagnosis

We examined spinal cord tissue from a total of 25 subjects (Table 1). Ten of the patients had no known mutations in the coding regions of TARDBP, UBQLN2, ATXN2, and C9orf72, and all had TDP-43 pathology in the motor neurons of the spinal cord (32, 33). The median age at onset of ALS without an identified mutation was 66.3 years and the median disease duration was 4.9 years; 5 were male and 5 were female. Four patients had an intermediate polyglutamine (polyQ) expansion.

FIGURE 1. Pathology of TDP-43 and that of PABP-1 occur in the same motor neurons. Mirror sections from the spinal cord of ALS patients were stained for either pTDP-43 or PABP-1. **(A)** Pathology of TDP-43 in ALS (Case 3) motor neurons (arrowheads); 1 motor neuron contained a round-like inclusion (arrow). **(B)** The mirror section was stained for PABP-1, and the same motor neurons were identified (arrowheads). The same motor neuron that contained a round-like pTDP-43 inclusion contained a round-like PABP-1 inclusion (arrow). **(C)** Skein-like accumulations of TDP-43 were identified in the motor neurons of the spinal cord of a patient with ALS (Case 5) (arrows). **(D)** The same motor neurons in mirror sections were immunoreactive to PABP-1 (arrows). **(E)** Motor neurons of the spinal cord of a control patient without ALS (arrows) showed an even cytoplasmic localization pattern for PABP-1 and a general lack of PABP-1 in the nucleus (arrowheads). Scale bar = 30 μm.
TABLE 2. Quantification of PABP-1 and pTDP-43 Inclusions in Mirror Sections From the Spinal Cord of ALS Patients

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Motor Neurons With pTDP-43</th>
<th>Motor Neurons With pTDP-43 and PABP-1</th>
<th>Motor Neurons With pTDP-43 and PABP-1 Inclusions, %</th>
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<tbody>
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<tr>
<td>Mean</td>
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(27–33 CAG repeats) in ATXN2, with a median age at onset of 63.5 years and a median disease duration of 1 year; 2 were male and 2 were female. Nine patients had a C9orf72 repeat expansion; the median age at onset was 62.9 years and the median disease duration was 2.9 years; 7 were male and 2 were female. Two patients had BIBD with ALS; the median age at onset was 70 years and the median disease duration was 5 years; 1 was male and 1 was female.

**PABP-1 Forms Pathologic Accumulations in ALS Motor Neurons With TDP-43 Pathology**

To provide detailed insights into PABP-1 pathology in the motor neurons of ALS patients, we determined whether PABP-1 pathology overlapped with pathologic TDP-43 inclusions. Serial mirror sections from the spinal cord of 5 ALS patients were stained with either an antibody that detects only the pathologic form of TDP-43 (phosphorylated TDP-43, pTDP-43) or an antibody to PABP-1. Pathology of TDP-43 was assessed as preinclusion, round-like or Skein-like (Figs. 1A, C). Motor neurons with pTDP-43 accumulations were scored for the presence of PABP-1 accumulations on the mirror section. This analysis showed that PABP-1 occurred in motor neurons that contained pTDP-43 inclusions (Figs. 1A–D), with 20% of pTDP-43-containing motor neurons also showing PABP-1 pathology (Table 2).

**PABP-1 Colocalizes to TDP-43 Inclusions in ALS Motor Neurons**

To determine whether PABP-1 pathology was present within the pTDP-43 inclusions, we stained the spinal cord tissue of 10 ALS patients without an associated mutation (ALS–no mut) for PABP-1 and pTDP-43 using double label immunofluorescence. This approach demonstrated that PABP-1 did not colocalize with preinclusions of pTDP-43 (Figs. 2A–C), but that PABP-1 colocalized with pTDP-43 Skein-like accumulations (Figs. 2D–F) and round-like accumulations (Figs. 2G–I). Not all of the cases examined had pTDP-43 pathology in the sections used for immunofluorescence; those cases were excluded from further analysis (Table 3). Of the ALS–no mut cases in which TDP-43 pathology was observed, PABP-1 was identified in 36% of pTDP-43 inclusions (Fig. 3), a value similar to that of the smaller subset of patients analyzed by serial section immunostaining (Table 2).

We found no evidence of PABP-1 pathology that was independent of pTDP-43 inclusions (data not shown), indicating that PABP-1 is a feature of mature inclusions. In addition, PABP-1 cannot bind to the cleaved form of TDP-43 and, as a result, does not localize to pTDP-43 inclusions in the motor cortex, where pathologic TDP-43 is mainly cleaved (27). We confirmed the absence of PABP-1 pathology in the motor cortex in 6 cases with pTDP-43 inclusions (data not shown). The absence of PABP-1 pathology in the motor cortex suggests that the localization of PABP-1 to pTDP-43 inclusions in the motor neurons of the spinal cord is selective and is not the result of a nonspecific RNA binding capacity of PABP-1 to pathogenic inclusions in the CNS.

**PABP-1 Colocalization to pTDP-43 Occurs in ALS Patients Harboring an Intermediate PolyQ Repeat Expansion in ATXN2**

Intermediate polyQ expansions of 27 to 33 repeats in ATXN2 are a risk factor for ALS (ATXN2-ALS) (26, 34–40);
recent data indicate familial association of expansions with disease (41, 42). In addition, evidence suggests that modulation of TDP-43 toxicity by ataxin 2 is mediated through PABP (25, 26). Therefore, to assess whether PABP-1 pathology was also a feature of ATXN2-ALS, we examined PABP-1 colocalization to TDP-43 inclusions in such patients (Tables 1, 3). Only pTDP-43 Skein-like accumulations were observed in tissue from the 5 ALS patient samples examined (Table 3). This is consistent with previous findings suggesting that Skein-like inclusions are the predominant pathology in ATXN2-ALS spinal cord motor neurons (43). From the 4 cases, 11 Skein-like accumulations were observed in total (Table 3), with PABP-1 colocalizing with 47% of TDP-43 Skein-like inclusions (Figs. 3A–F, 4).

PABP-1 Colocalization to TDP-43 Inclusions Is More Prevalent in Patients With a GGGGCC Hexanucleotide Repeat Expansion in C9orf72

The most common genetic cause of ALS and FTD to date is a GGGGCC hexanucleotide (G4C2) repeat expansion in the C9orf72 gene (C9-ALS) (44, 45). The GGGGCC hexanucleotide repeat expansion confers specific clinical features on C9-ALS (i.e. earlier age of death and bulbar onset); however, no differences in TDP-43 pathology in the motor neurons have been observed between C9-ALS patients and ALS–no mut patients (32, 46). To determine whether PABP-1 pathology was a feature of C9-ALS, we analyzed spinal cord tissue from 9 cases harboring the C9orf72 repeat expansion for PABP-1 and pTDP-43 inclusions (Table 1).

PABP-1 Colocalizes to Pathologic FUS Inclusions in Motor Neurons

Fused in sarcoma is an RNA binding protein that, like TDP-43, forms NCIs in a number of neurodegenerative diseases (3–11). One such disease in which FUS forms NCIs in spinal cord motor neurons is BIBD, a progressive neurodegenerative disease with variable clinical phenotypes ranging from ALS to FTD (3, 11). Similar to TDP-43, FUS localizes to stress granules (27, 47). We examined 2 cases of ALS with BIBD for potential colocalization of FUS and PABP-1 to motor neuron NCIs (Table 1). We found evidence of 2 types of FUS inclusions: round basophilic inclusions in Case 25 (Fig. 5A) and large NCIs in Case 24 (Fig. 5B). In Case 25, PABP-1 colocalized with FUS to basophilic inclusions (Figs. 5A–C). Three large NCIs immunoreactive to FUS were also observed, but only 1 contained PABP-1. In Case 24, only large NCIs were observed; 2 of 14 costained with PABP-1 (Figs. 5F–H). These data suggest that PABP-1 is a protein that is involved in the pathology of both TDP-43 and FUS.

**DISCUSSION**

Poly-A binding protein-1 is a marker of stress granules—cellular accumulations that contain proteins and RNAs. Under stress conditions, cells reprogram their transcriptome to generate proteins that can aid in situations of stress. Because proteins associated with ALS are RNA binding proteins that can localize to stress granules, it has been suggested that the inclusions in ALS are stress granule remnants. Previously, we reported that the stress granule marker PABP-1 is important for mediating the toxicity of TDP-43 in model systems, and we identified PABP-1 inclusions in the ALS spinal cord (25). To further extend the functional relationship between TDP-43 and PABP-1, we examined the relationship between PABP-1 inclusions and pTDP-43 in the spinal cord of ALS patients. Our studies indicate that PABP-1 can be found in TDP-43 inclusions in the tissue of ALS patients with no known mutations (ALS–no mut), in those with an intermediate polyQ repeat expansion in ATXN2 (ATXN2-ALS), patients harboring a GGGGCC hexanucleotide repeat expansion in C9orf72 (C9-ALS), and in patients with FUS pathology. Thus, PABP-1 is a protein that pathologically associates with inclusions in ALS of multiple genetic associations.

Interestingly, PABP-1 does not localize to TDP-43 preinclusions; rather, PABP-1 colocalizes with mature TDP-43 inclusions in both Skein-like and round-like inclusions of ALS–no mut patients. Poly-A binding protein-1 also colocalized with TDP-43 inclusions in ATXN2-ALS, in which only Skein-like inclusions were observed. Poly-A binding protein-1, however, colocalized to both round-like and Skein-like inclusions in C9-ALS spinal cord motor neurons. Thus, PABP-1 is a common pathogenic protein that colocalizes to NCIs in a number of different subtypes of ALS. Quantification of PABP-1 colocalization with TDP-43 inclusions indicates that PABP-1 is present in TDP-43 inclusions more frequently in the spinal cord of C9-ALS. C9-ALS patients have an earlier age at onset and a more rapid disease progression, but no difference in TDP-43 pathology in spinal cord motor neurons has been observed (32, 44). Our data suggest that there is a difference in NCI pathology in the spinal cord motor neurons of C9-ALS patients; intriguingly, this difference does not reside with pathogenic TDP-43 but may reside with the pathologic proteins that colocalize to the TDP-43 inclusions.

Modulation of stress granule components modulates TDP-43 toxicity in Drosophila (25). Among these proteins, ataxin 2 and fly PABP show striking interactions with TDP-43; reduction of PABP mitigates TDP-43 toxicity in the nervous system, whereas expression of human ataxin 2 in the nervous system enhances TDP-43 toxicity. The interaction between ataxin 2 and TDP-43 is dependent on the PABP binding domain of ataxin 2 (25). In human spinal cord motor neurons, PABP-1 may act to promote formation of TDP-43 inclusions, may stabilize the TDP-43 inclusions, or may be involved in the maturation of TDP-43 inclusions. Decreasing the levels of PABP-1 may reduce the toxic effect of TDP-43 NCIs on spinal cord motor neurons by slowing the rate of formation, stabilization, or maturation of pathogenic NCIs. Alternatively, reduced PABP-1 levels may prevent PABP-1–associated proteins, such as ataxin 2, from colocalizing to TDP-43 inclusions, thereby reducing the overall toxicity that the NCIs exert on the spinal cord motor neurons (25, 26).

Taken together, these studies highlight that combining functional studies in model systems with neuropathologic data can give important insights into pathologic mechanisms that may be perturbed in patients. In addition, the mechanistic overlap between TDP-43 and another causative gene for ALS (FUS) and the finding reported here that PABP-1 localizes to FUS inclusions in the motor neurons of the spinal cord tissue
of 2 cases of BIBD with ALS underscore the broad relevance of interaction data from model systems to neuropathologic studies. Our findings highlight the pathologic association of PABP-1 in ALS with cases with a range of genetic backgrounds and in situations of FUS pathology.

ACKNOWLEDGMENTS

We thank all the patients and their families who were involved in this study, and we thank John Robinson and Theresa Schuck for advice with immunohistochemistry questions.

REFERENCES


FUS
PABP-1
merge/DAPI

FIGURE 5. Poly-A binding protein-1 colocalizes with pathologic FUS in the spinal cord motor neurons of patients with FUS pathology. (A–C) Poly-A binding protein-1 (PABP-1) colocalized with round-like FUS inclusions in a motor neuron from the spinal cord tissue of a patient with BIBD (Case 25). (D–F) Large round FUS filamentous inclusions were observed in the spinal cord motor neurons of a patient with BIBD (Case 24) (D). The large round FUS inclusions were typically negative for PABP-1 (arrows), although PABP-1 occasionally colocalized with FUS in the large round inclusions in a patient with BIBD (Case 24). (G–I) Two of 14 FUS-positive large round inclusions were also positive for PABP-1. Scale bar = 20 μm.
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