FUS-Immunoreactive Inclusions Are a Common Feature in Sporadic and Non-SOD1 Familial Amyotrophic Lateral Sclerosis

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Objective: Amyotrophic lateral sclerosis (ALS) is a fatal disorder of motor neuron degeneration. Most cases of ALS are sporadic (SALS), but about 5 to 10% of ALS cases are familial (FALS). Recent studies have shown that mutations in FUS are causal in approximately 4 to 5% of FALS and some apparent SALS cases. The pathogenic mechanism of the mutant FUS-mediated ALS and potential roles of FUS in non-FUS ALS remain to be investigated.

Methods: Immunostaining was performed on postmortem spinal cords from 78 ALS cases, including SALS (n = 52), ALS with dementia (ALS/dementia, n = 10), and FALS (n = 16). In addition, postmortem brains or spinal cords from 22 cases with or without frontotemporal lobar degeneration were also studied. In total, 100 cases were studied.

Results: FUS-immunoreactive inclusions were observed in spinal anterior horn neurons in all SALS and FALS cases, except for those with SOD1 mutations. The FUS-containing inclusions were also immunoreactive with antibodies to TDP43, p62, and ubiquitin. A fraction of tested FUS antibodies recognized FUS inclusions, and specific antigen retrieval protocol appeared to be important for detection of the skein-like FUS inclusions.

Interpretation: Although mutations in FUS account for only a small fraction of FALS and SALS, our data suggest that FUS protein may be a common component of the cellular inclusions in non-SOD1 ALS and some other neurodegenerative conditions, implying a shared pathogenic pathway underlying SALS, non-SOD1 FALS, ALS/dementia, and related disorders. Our data also indicate that SOD1-linked ALS may have a pathogenic pathway distinct from SALS and other types of FALS.

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SALS cases.9–14 Altogether, mutations in specific genes have been identified in about 30% of FALS cases. The causes for the remaining FALS cases have yet to be identified.

In contrast to FALS, the etiology and the pathogenic mechanisms underlying SALS, the most common form of ALS, remain largely unknown. Understanding the causes and pathogenic mechanisms of SALS remains a major challenge.

Protein aggregation has been recognized as a pathological hallmark in several neurodegenerative disorders, such as extracellular amyloid-beta plaques and intracellular tau neurofibrillary tangles in Alzheimer disease, and alpha-synuclein containing Lewy bodies in Parkinson disease.15 In ALS, protein aggregates are most common in spinal motor neurons, and the ubiquitin-immunoreactive and skein-like aggregates are thought to be pathognomonic of ALS.16,17 SOD1-immunoreactive aggregates are a prominent pathology in SOD1-mediated ALS, but not in SALS.18 On the other hand, TDP43-immunoreactive aggregates have been identified in SALS, but not in SOD1-ALS.19,20

Both TDP43 and FUS are DNA/RNA-binding proteins. They are predominantly present in cell nuclei in physiological condition. However, in neurons with cytoplasmic TDP43 aggregates, nuclear TDP43 is diminished.19,20 It is currently unknown if FUS is involved in the pathogenesis of SALS and other forms of FALS without FUS mutations.

In this study, we analyzed postmortem tissues using immunostaining to explore post-translational involvement of FUS in different types of ALS and other neurodegenerative disorders, as well as neurological disease-free controls.

Patients and Methods

Cases

Spinal cord sections from a total of 78 clinically and pathologically diagnosed ALS cases were analyzed using immunostaining in this study. The analyzed ALS cases included SALS (n = 52), ALS with dementia (ALS/dementia, n = 10), and FALS (n = 16). We also analyzed frontal lobe or hippocampus sections from patients with frontotemporal lobar degeneration (FTLD) with (n = 4) or without progranulin mutations (n = 5), atypical FTLD with neuronal inclusions composed of an unidentified ubiquitinated protein (aFTLD-U or FTLD-FUS) (n = 2), and controls without history of apparent neurological disorders (n = 5). Spinal cord controls without motor neuron diseases and other neurological disorders (n = 6) were also included in this study. In total, 100 cases were analyzed.

Genetic Analysis

Genomic DNA was extracted from whole blood, transformed lymphoblastoid cell lines, or tissues by standard methods (Qiagen, Valencia, CA). Exonic DNA fragments of the TDP43, FUS, and SOD1 genes were directly sequenced for 24 cases of SALS, 6 cases of ALS/dementia, and 16 cases of FALS, using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

Immunohistochemistry and Confocal Microscopy

Immunohistochemistry and confocal microscopy were performed using previously described methods.21 In brief, 6-mm sections were cut from formalin-fixed, paraffin-embedded spinal cord and brain regions containing frontal lobe or hippocampus. The sections were deparaffinized and rehydrated by passing the slides in serial solutions: 3× for 10 minutes each in xylene, 3× for 5 minutes each in 100% ethanol, 3× for 3 minutes each in 95% ethanol, once for 5 minutes each in 75% ethanol, 50% ethanol, deionized water, and phosphate-buffered saline (PBS). The antigens in the sections were retrieved using a decloaking chamber with a 1X Antigen Decloaker solution (Biocare Medical, Walnut Creek, CA) at 125°C for 20 minutes. The sections were cooled to room temperature for 30 minutes and rinsed with deionized water for 5 minutes. For immunohistochemistry, the endogenous peroxidase activities were blocked with 2% hydrogen peroxide. Nonspecific background was blocked with 1% bovine serum albumin in PBS for 20 minutes at room temperature. An affinity-purified rabbit antihuman FUS polyclonal antibody (11570-1-AP, Proteintech Group, Chicago, IL) at a concentration of 0.5 to 3 μg/ml was applied to the slides and incubated at room temperature for 1 hour. After rinsing the slides 3× for 5 minutes each, a biotinylated goat antirabbit immunoglobulin G (IgG) was applied to the slides and incubated at room temperature for 30 minutes. The excess secondary antibody was removed by rinsing the slides 3×, 5 minutes for each time. The signals were detected with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) using 3-amin-9-ethylcarbazole as a chromogen. The slides were counterstained with hematoxylin and sealed with Aqua Poly/Mount (Polyscience, Warrington, PA). The slides were examined and photographed under a light microscope. In addition to the anti-FUS antibody 11570-1-AP, we also tested 8 other anti-FUS antibodies, including A300-292A, A300-293A, A300-294A, and A300-302A (Bethyl Laboratories, Montgomery, TX), ab23439 (Abcam, Cambridge, MA), sc-25540 and sc-47711 (Santa Cruz Biotechnology, Santa Cruz, CA), and HPA008784 (Sigma-Aldrich, Saint Louis, MO). For confocal microscopy, FUS monoclonal or polyclonal antibodies were used in combination with either mouse anti-TDP43 monoclonal antibody (60019-2-Ig, 6 μg/ml, Protein Tech Group, Chicago, IL), rabbit anti-TDP43 polyclonal antibody (10782-2-AP, 5 μg/ml, Protein Tech Group), mouse anti-ubiquitin monoclonal antibody (10R-U101B, 3 μg/ml, Fitzgerald Industries International, Concord, MA), or mouse anti-p62 monoclonal antibody (H00008878...
Results

**FUS-Immunoreactive Aggregates/Inclusions in SALS, ALS/Dementia, and Non-SOD1 FALS**

Sequencing analysis of the FUS, TDP43, and SOD1 genes in 46 ALS samples revealed 3 cases with SOD1\(^{A4V}\) mutation, and 1 case each with SOD1\(^{G85R}\), FUS\(^{R521L}\), or TDP43\(^{G298S}\) mutation among the 16 FALS cases. No mutations in the SOD1, FUS, and TDP43 genes were found in 24 SALS and 6 ALS/dementia cases (unpublished data). Nine antibodies against FUS were initially tested on spinal cord sections of 10 SALS cases (Supplementary Table). Among these antibodies, 2 affinity-purified FUS antibodies against human FUS (11570-1-AP and HPA008784) revealed FUS-immunoreactive inclusions (Fig 1). Because the antibody 11570-1-AP yielded more robust signals (see Fig 1 and Supplementary Table), it was then used for further analysis throughout this study. Immunohistochemical analysis of the spinal cord sections revealed that FUS-immunoreactive inclusions were present in some of the surviving spinal anterior horn neurons from all 52 SALS cases, 12 SOD1-negative FALS cases, and 10 ALS/dementia cases (Fig 2 and Supplementary Fig 1), but not in the 4 FALS cases with SOD1 mutations. Previous studies have demonstrated that SOD1/ubiquitin-positive inclusions in spinal motor neurons are a pathological hallmark in ALS patients with SOD1 mutations and transgenic mice overexpressing mutant SOD1.\(^{18,22}\) Supporting the notion that the FUS-positive inclusions are absent in SOD1-linked ALS, we found that the ubiquitin-positive inclusions in spinal neurons were FUS-negative in patients with SOD1\(^{G85R}\) or SOD1\(^{A4V}\) mutation and in the transgenic mice overexpressing SOD1\(^{G93A}\) and SOD1\(^{L126Z}\) (Supplementary Fig 2).\(^{21,23}\) We observed FUS-immunoreactive inclusions in a case with FUS\(^{R521L}\) mutation. Interestingly, we also observed FUS-immunoreactive inclusions in a patient with TDP43\(^{G298S}\) mutation. Most of the inclusions were skein-like. Some compact inclusions were also observed. The FUS inclusions appeared to be present predominantly in large anterior horn motor neurons and their neuritic processes (see Figs 1 and 2 and Supplementary Fig 1). Significantly increased FUS staining was also observed in some glial cells. In spinal cord sections with preserved dorsal root ganglia of 1 SALS patient, FUS inclusions were observed in the spinal anterior horn motor neurons.
neurons, but not in the ganglionic primary sensory neurons. In the motor neurons, the FUS inclusions were located primarily in the cytoplasm and neurites. The shape and distribution pattern of the FUS inclusions were similar to those of TDP43 inclusions observed in non-SOD1 ALS patients. The skein-like, cytoplasmic, and neuritic FUS-positive inclusions appeared characteristic in these non-SOD1 ALS cases. Even in some neurons with abundant lipofuscin, these FUS inclusions were apparent (see Supplementary Fig 1U, V). But these inclusions were not observed in 6 spinal cord samples from control cases without ALS (see Supplementary Fig 1W–B).

**Colocalization of TDP43, p62, and Ubiquitin with FUS in Spinal Neuron Inclusions in Non-SOD1 ALS Cases**

Because the FUS inclusions were similar in morphology and distribution pattern to the TDP43 inclusions observed in non-SOD1 ALS patients, we tested if FUS and TDP43 were colocalized. Confocal microscopy demonstrated that the FUS-positive inclusions were also immunoreactive with the same TDP43 antibody used to detect TDP43 inclusions in ALS (Fig 3A–Q).

Ubiquitin- and p62-immunoreactive inclusions in anterior horn motor neurons are hallmarks of ALS. To test if the FUS- and TDP43-containing inclusions were also immunoreactive to p62 and ubiquitin, we analyzed inclusions using confocal microscopy. We found that the FUS inclusions were also immunoreactive to p62 (see Fig 3D–F) and ubiquitin (see Fig 3G–I) antibodies. Consistent with the data seen in our immunohistochemical studies, these inclusions were present in cytosol and neurites. Using immunohistochemistry, we observed FUS-positive inclusions in spinal neurons of a patient with TDP43G298S mutation (see Fig 2E). Confocal microscopy demonstrated that TDP43-positive inclusions in this patient were also immunoreactive to the anti-FUS antibody (see Fig 3J–L).

As the characteristic skein-like FUS inclusions detected with the FUS antibody 11570-1-AP were also immunoreactive to antibodies against TDP43, ubiquitin, and p62, we further tested by confocal microscopy whether the TDP43- and ubiquitin-positive inclusions could be detected with other commercially available FUS...
antibodies. Consistent with immunohistochemistry, HPA008784 also recognized the skein-like and TDP43-positive inclusions (Fig 4A–C). In addition, the TDP43-positive inclusions were also positive with FUS monoclonal antibody sc-47711 (see Fig 4D–F). However, the 11570-1-AP antibody yielded apparently stronger FUS immunofluorescence (IF) intensity than the other 2 (see Figs 3 and 4 and Supplementary Fig 3). In approximately two-thirds of the cells with TDP43-positive inclusions, the FUS IF signals yielded by HPA008784 and sc-47711 were colocalized with strong TDP43 signals, but they were less robust when TDP43 signals were weak (see Supplementary Fig 3D–I). The other FUS antibodies, however, did not show apparent costaining (see Supplementary Fig 3J–R and Supplementary Table). We also observed nuclear staining of FUS in some cells where FUS and TDP43 inclusions were present, although nuclear staining of the TDP43 were not apparent (see Supplementary Fig 3D–F). Notably, the antibodies HPA008784 and sc-47711, which recognize the inclusions, are raised using 2 separate polypeptides that are 61 amino acids apart (Supplementary Fig 4).

**FIGURE 4:** Confocal analysis of the FUS pathology with different FUS antibodies. The representative FUS pathology is shown with FUS antibodies HPA008784 (A-C) and sc-47711 (D-F) in spinal neurons of sporadic amyotrophic lateral sclerosis cases. The ubiquitin-positive inclusions in atypical frontotemporal lobar degeneration with neuronal inclusions composed of an unidentified ubiquitinated protein were immunoreactive to FUS antibody (G–I), but not to TDP43 antibody (J–L).

**FUS pathology is independent of TDP43 pathology in aFTLD-U**

Most cases of frontotemporal dementia (FTD) are associated with inclusions containing TDP43. Recently, a subgroup of approximately 10% FTD cases has been described, which presents an unusual clinical phenotype and pathology characterized by frontotemporal lobar degeneration with neuronal inclusions composed of an unidentified ubiquitinated protein (atypical FTD-U, aFTLD-U). The inclusion in aFTLD-U are positive for ubiquitin and FUS, but are negative for TDP43 (aFTLD-U or FTLD-FUS). To test if FUS-positive inclusions are the result of cross-reactivity of tested FUS antibodies to TDP43, we analyzed hippocampus sections of 2 patients with aFTLD-U. Consistent with previous data, we found that pronounced oval or crescentic ubiquitin-positive inclusions in the dentate granule cells of the hippocampus were strongly immunoreactive with the FUS antibody 11570-1-AP (see Fig 4G–I). However, these FUS-positive inclusions were negative for TDP43 (see Fig 4J–L). Western blot using antibody 11570-1-AP showed a single band of the expected size that did not overlap with the TDP43 bands (Fig 5).

The robust immunoreactivity of the well-defined in-
Inclusions observed with 11570-1-AP in the FTLD-FUS led us to further evaluate the sensitivity of the other FUS antibodies to the inclusions in the FTLD-FUS cases. Consistent with the data obtained from spinal cord sections, the HPA008784 yielded strong signals, and the sc-47711 generated moderate signals (Fig 6). In addition, A300-302A also yielded moderate signals, and the ab23439 revealed weak signals. The other 4 antibodies (sc-25540, HPA008784, sc-47711, ab23439, and A300-293A) showed varying levels of signal intensity, indicating the need for careful selection of antibodies for FUS protopathological analyses.
A300-292, A300-293, and A300-294) did not show apparent signals on the ubiquitin-positive inclusions (see Fig 6M–O, and Supplementary Table).

**Antigen Retrieval-Related Detection of FUS Pathology**

FUS does not share homology with TDP43 at the protein level. We observed FUS-positive inclusions in SALS and non-SOD1 ALS using 3 of the 9 antibodies tested in this study. Although the epitopes of 11570-1-AP overlap or partially overlap with those of antibodies HPA008784 and sc-47711, the antibodies HPA008784 and sc-47711 have been raised using 2 separate polypeptides without any overlap and are 61 amino acids apart in different species of hosts (see Supplementary Fig 4). Moreover, sc-47711 is a monoclonal antibody. These FUS antibodies also detected FUS inclusions in FTLD-FUS, where TDP-43 inclusions were absent. Immunoblot using antibody 11570-1-AP, which is raised using the longest polypeptide of the FUS, showed a single band that did not overlap with the TDP43 bands. These data suggest that the presence of the inclusions immunoreactive to FUS antibodies is unlikely due to cross-reactivity of the FUS antibodies to TDP-43; rather, FUS is a component of the inclusions.

The FUS pathology in spinal cord has been previously evaluated using immunostaining, and FUS-positive inclusions have not been identified in SALS and non-FUS ALS cases. In our previous studies of SOD1 inclusions in spinal cord of SOD1-linked FALS patients and SOD1 transgenic mice, we observed that antigen retrieval methodology played an essential role in detection of SOD1 inclusions in lower motor neurons, especially in some human cases (unpublished data). We compared 3 antigen retrieval protocols: boiling and microwave, which are most commonly used, and high-pressure decloaking chamber. We found that antigen retrieval using the high-pressure decloaking chamber was the most efficient in detection of SOD1 inclusions, and have therefore adopted this protocol as the routine method for antigen retrieval. Because other studies failed to show FUS pathology in SALS using microwave protocol for antigen retrieval, we assumed that in addition to the sensitivity of the antibodies, antigen retrieval methodology may be an important factor for influencing the detection of FUS-positive inclusions. To test this possibility, we compared boiling and microwave protocols to the high-pressure decloaking chamber protocol using spinal cord sections from SALS cases. We observed that the FUS-positive inclusions were only observed when the high-pressure decloaking chamber protocol was used, and the ubiquitin- and p62-positive inclusions were consistently negative for FUS when boiling or microwave protocol was used (Fig 7A–L). However, the ubiquitin-, p62-, and TDP43-positive inclusions were consistently observed with all 3 antigen retrieval protocols (see Fig 7A–P).

**FUS Pathology in the Brain of FTLD-TDP43 with or without Progranulin Mutations, and ALS/Dementia**

We further tested frontal lobe sections from 4 FTLD cases with different progranulin mutations and another 5 FTLD cases without progranulin mutations. We observed that ubiquitin- and TDP43-immunoreactive inclusions were also positive for FUS, although the inclusions were...
rare in these cases (Supplementary Fig 5A–F). In sections of the neocortex and hippocampus from 3 ALS/dementia patients, rare ubiquitin-positive inclusions were also positive for FUS (see Supplementary Fig 5G–L), but the FUS-immunoreactivity of the ubiquitin-positive inclusions in granule cells of hippocampal dentate gyrus appeared to be less intense compared to those in FTLD-FUS cases (see Supplementary Fig 5J–L). We also examined brain sections from frontal and hippocampal regions from 5 control cases without dementia, ranging in age from 56 to 75 years. We did not observe FUS-positive inclusions in these cases.

**Discussion**

In this study, we observed FUS-positive inclusions in anterior horn neurons and their neurites in spinal cord of 52 SALS cases, 10 ALS/dementia cases, 1 case each with TDP43 or FUS mutation, and other 10 FALS cases with unknown genetic defects, but not in 4 cases with SOD1 mutations or SOD1-ALS transgenic mice. These data indicate that the FUS inclusions are involved not only in mutant FUS-linked FALS, but also in sporadic ALS and non-SOD1 FALS, representing a new finding with potentially significant implications.8,27 These data suggest that the pathogenic pathway of mutant SOD1-mediated ALS is largely independent from other forms of ALS, including mutant FUS-linked ALS. We then explored possible explanations for the apparent discrepancy between our data and previous reports, in which FUS-positive inclusions were not observed in SALS or FALS without FUS mutation.8,25,26 We found that the high pressure decloaking chamber technique might be more effective than boiling and microwave methods for antigen retrieval of FUS in the inclusions. Although the discrepancy in the immunostaining remains to be further studied, our data suggest that the FUS in the inclusions of spinal motor neurons may have a conformation that requires optimized conditions such as efficient antigen retrieval or that FUS may be a less abundant component in the inclusions compared to TDP43.

Three of the 9 antibodies to FUS evaluated in the present study can be used for detection of FUS inclusions in spinal cord sections by immunostaining. The 11570-1-AP yielded the most robust signals. The HPA008784 also revealed FUS signals, but less robust than the 11570-1-AP. These 2 antibodies were generated using much longer polypeptides as immunogens (349 and 128 amino acids, respectively). The other polyclonal antibodies generated using much shorter polypeptides failed to detect FUS inclusions in ALS with immunostaining. A monoclonal antibody sc-47711 could also be used to detect FUS inclusions by immunofluorescence, but not by immunohistochemistry. This antibody was generated using the C-terminal half of FUS, but the epitope remains to be mapped. In ALS cases, both FUS and TDP43 were present in the inclusions. However, the presence of FUS in TDP43-negative inclusions in the aFTLD-U cases suggests that neuronal FUS pathology is independent of TDP43 pathology, at least in aFTLD-U. Although we did not observe apparent FUS-positive inclusions in non-SOD1 ALS cases with 6 other antibodies in this study, optimization of the immunostaining method may further increase the sensitivity for detection of the FUS pathology with other FUS antibodies.

A previous report demonstrated TDP43/ubiquitin inclusions in all evaluated cases of SALS, ALS/dementia, and SOD1-negative FALS.19 Our data support this observation. Confocal analysis demonstrated that the FUS-immunoreactive inclusions were also immunoreactive with antibodies to TDP43, ubiquitin, and p62. Our study indicated that the FUS/TDP43/ubiquitin/p62-positive inclusions are a common feature in SALS, ALS/dementia, FUS or TDP43 mutation-linked FALS, and other SOD1-negative FALS. These data suggest that SALS, ALS/dementia, FUS or TDP43 mutation-linked FALS, and other SOD1-negative FALS may share a common downstream pathway in the pathogenesis of motor neuron degeneration, although the initial cause of disease in each specific case may differ. Obviously, SOD1-linked ALS appears to have a pathogenic pathway that is independent, at least in part, from other forms of ALS.

The presence of FUS pathology in sporadic and most familial forms of ALS parallels that of TDP43 in ALS, β-amyloid in Alzheimer disease and α-synuclein in Parkinson disease. Like TDP43, β-amyloid, and α-synuclein, FUS is involved in the formation of inclusions in sporadic disease, although genetic mutations only account for a small fraction of the familial form of these disorders. Moreover, FUS pathology has recently been identified in aFTLD-U,25 neuronal intermediate filament inclusion disease,26 and basophilic inclusion body disease.28 We showed FUS pathology in FTLD with or without progranulin mutations, as well as ALS/dementia. These data suggest that post-translational modification of FUS is involved in a wider range of neurodegenerative disorders in addition to motor neuron degeneration in ALS.

The TDP43 and FUS are multifunctional proteins with DNA/RNA binding properties. They have been implicated in transcription, splicing, and transport of RNA, protein translation, microRNA biogenesis, apoptosis, and...
cell division. The pathogenetic mechanisms by which FUS mutations cause motor neuron degeneration remain to be elucidated. It is not clear whether these FUS/TDP43/ubiquitin/p62-positive inclusions per se present as toxic components to motor neurons, or if the formation of inclusions sequesters and thereby deprives neurons of other essential proteins, or RNA, or whether some other mechanism exists. Transgenic and knockout animal models may help address these issues. A previous study demonstrated that FUS-deficiency (FUS−/−) leads to perinatal death in mice, whereas heterozygous mice (FUS+−/) appear to have normal phenotype, favoring but not proving the hypothesis that mutations in FUS cause the disease through a gain of function mechanism. Colocalization of FUS and TDP43 in neuronal inclusions raises the possibility that interactions of FUS and TDP43 with other cellular components may be a common theme in motor neuron degeneration in most, if not all, SOD1-negative forms of ALS. Examination of the common pathway involving these post-translationally modified proteins may offer a novel avenue for developing therapies for most forms of ALS and related disorders. We believe that demonstration of FUS, along with TDP43 in SALS, non-SOD1 ALS, and ALS/dementia, is a major step forward in formulating a common pathogenetic pathway for these disorders.

Acknowledgment
This study was supported by the National Institutes of Health (grant NS050641), Les Turner ALS Foundation, Vena E. Schaff ALS Research Fund, Harold Post Research Professorship, Herbert and Florence C. Wenske Foundation, David C. Asselin MD Memorial Fund, and Les Turner ALS Foundation/Herbert and Florence C. Wenske Professorship to T.S. K.A. is a post-doctoral fellow of Blazeman Foundation for ALS.

We thank the following for retrieval of the autopsy tissues: M. Myers, R. Macke, P. C. Allen, C. M. Hulette, J. S. Lewis, and C. R. Miller. We thank J. Caliendo for proofreading the manuscript.

Potential Conflicts of Interest
Nothing to report.

References


