ORIGINAL CONTRIBUTION

Differential Involvement of Optineurin in Amyotrophic Lateral Sclerosis With or Without *SOD1* Mutations

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Background: Mutations in optineurin have recently been linked to amyotrophic lateral sclerosis (ALS).

Objective: To determine whether optineurin-positive skeinlike inclusions are a common pathologic feature in ALS, including *SOD1*-linked ALS.

Design: Clinical case series.

Setting: Academic referral center.

Subjects: We analyzed spinal cord sections from 46 clinically and pathologically diagnosed ALS cases and ALS transgenic mouse models overexpressing ALS-linked *SOD1* mutations G93A or L126Z.

Results: We observed optineurin-immunoreactive skeinlike inclusions in all the sporadic ALS and familial ALS cases without *SOD1* mutation, but not in cases with *SOD1* mutations or in transgenic mice overexpressing the ALS-linked *SOD1* mutations G93A or L126Z.

Conclusion: The data from this study provide evidence that optineurin is involved in the pathogenesis of sporadic ALS and non-*SOD1* familial ALS, thus supporting the hypothesis that these forms of ALS share a pathway that is distinct from that of *SOD1*-linked ALS.

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AMYOTROPHIC LATERAL SCLEROSIS (ALS) is a fatal paralytic disorder caused by degeneration of motor neurons in the brain and spinal cord. Most cases appear as sporadic ALS (SALS) of unknown etiology. Approximately 5% to 10% of patients with ALS have a family history of the disease, but familial ALS (FALS) is genetically heterogeneous. Genetic mutations in the Cu/Zn superoxide dismutase gene, *SOD1*, are the most frequent cause of ALS, accounting for approximately 20% of FALS and 1% of SALS cases.[1,2] An additional 8% to 10% of FALS is accounted for by mutations in the *TDP43* and *FUS* genes.[3-6] Mutations in several other genes, including *DCTN1*, *ALSIN*, *SETX*, *VAPB*, *ANG*, *FIG4*, *PONs*, *DAO*, and *VCP*, have been linked[7-16] to fewer cases of ALS or ALS-like syndromes.

Because FALS and SALS are often clinically indistinguishable, it has been speculated that they may share the same or similar pathogenic pathways. Indeed, recent studies[17,18] of TDP43 and FUS have provided important lines of evidence supporting the hypothesis that FALS and SALS share some elements of molecular pathologic characteristics. However, they have one major difference: inclusions in the lower motor neurons are positive with TDP43 and FUS immunohistochemistry in *SOD1*-negative FALS and SALS, but they are negative in *SOD1*-linked ALS.

Recently, mutations in the optineurin (OPTN) gene, *OPTN*, have been linked to a small number of ALS cases.[19] Eight patients with *OPTN* mutations had ALS disease onset at ages ranging from 30 to 60 years, mostly with relatively slow progression and long duration. In a patient with *OPTN* E478G mutation and in patients with SALS, round hyaline inclusions in the anterior horn cells were OPTN-immunoreactive. The ubiquitin- and TDP43-positive and skeinlike inclusions were also immunoreactive for OPTN in patients with SALS. Moreover, tests for SOD1-positive Lewy body–like hyaline inclusions from cases linked to *SOD1* were also immunopositive for OPTN. These novel observations raise the possibility that OPTN staining appears to be a more general marker for inclusions in various types of ALS. Therefore, OPTN might be involved in the pathogenesis of the widest spectrum of ALS to date, including *SOD1*-linked ALS[19]

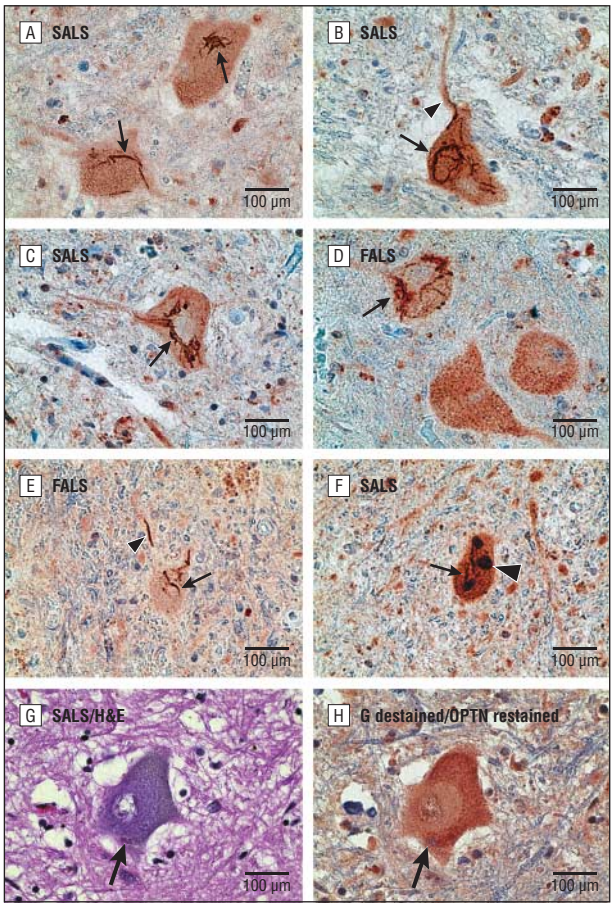

Figure 1. Optineurin (OPTN)-immunoreactive inclusions in amyotrophic lateral sclerosis (ALS). Representative OPTN-immunoreactive inclusions were detected by antibody C-term in sporadic ALS (SALS) (A-C and F) and non-*SOD1* familial ALS (FALS) (D and E). The typical skeinlike inclusions in soma and neurites are shown by small arrows and arrowheads, respectively. Some inclusions appeared to be compact (large arrowhead in panel F). Representative Bunina bodies (large arrow in panel G) stained with hematoxylin and eosin (H&E) were destained, then restained with OPTN antibody and were negative for OPTN (H).

rather than just SALS, although *SOD1*-linked ALS is thought to have a pathogenic mechanism distinct from other forms of ALS.[17,18]

Immunostaining of spinal cord autopsy samples reveals immunoreactive intracellular structures that might be related to the pathogenic processes. Many factors may influence immunostaining, such as sensitivity and specificity of the antibodies, tissue preservation and processing, and detection protocols.[17] Nevertheless, certain structures detected by immunostains are unique and diagnostic for some diseases. In ALS, the skeinlike inclusions in the spinal anterior horn cells are usually considered to be characteristic of ALS; they have not been reported in other neurodegenerative disorders. The protein composition of skeinlike inclusions may vary depending on the cause of ALS; they are not easily seen with hematoxylin-eosin staining, but can be readily detected by immunohistochemistry with antibodies against ubiquitin and p62. It has been shown[17,18] that SOD1-positive skeinlike inclusions are present in ALS cases with *SOD1* mutations, but not in other types of ALS. On the other hand, TDP43- and FUS-positive skeinlike inclusions are present in SALS and most FALS cases, but not in those with *SOD1* mutations. Because TDP43 and FUS appear to be mutually exclusive from SOD1 in skeinlike inclusions, it remains unclear whether OPTN-positive skeinlike inclusions are a common pathologic feature in ALS, especially *SOD1*-linked ALS.

In this study, we analyzed a large series of postmortem tissues with immunostaining to further explore the possible involvement of OPTN in different types of ALS.

METHODS

CASES

We analyzed spinal cord sections from 46 clinically and pathologically diagnosed ALS cases, including SALS (n=32) and FALS (n=14). Among the 14 FALS cases, 6 were linked to mutations in *SOD1* (A4V [4 cases]; and G85R [2 cases]). Spinal cord control sections without ALS (n=6) were also included in this study. In total, 52 cases were analyzed. In addition, spinal cord sections from well-characterized ALS transgenic mouse models overexpressing ALS-linked *SOD1* mutations G93A or L126Z were included.[20,21] In general, 2 to 3 spinal cord sections were analyzed for each case. More sections (up to 8) were analyzed in 8 cases with extensive motor neuron loss.

WESTERN BLOT, IMMUNOHISTOCHEMISTRY, AND CONFOCAL MICROSCOPY

Western blot, immunohistochemistry, and confocal microscopy were performed using previously described methods.[17] The epitope retrieval was carried out using a high-pressure chamber.[17] Two affinity-purified polyclonal antibodies against OPTN were tested: (1) OPTN C-term polyclonal antibody (amino acids 571-591, 0.2 µg/mL, catalog No. 100000; Cayman Chemical, Ann Arbor, Michigan) and (2) OPTN INT polyclonal antibody (amino acids 115-130, 1.0 µg/mL, catalog No. 100002; Cayman Chemical). These are the same antibodies that were used by Maruyama and coworkers[19] in their novel investigation. The other antibodies, including those against ubiquitin, p62, TDP43, FUS, and SOD1, were the same as previously described.[17,20] For testing the immunoreactivity of the small eosinophilic Bunina bodies, we first identified Bunina bodies in motor neurons in the ALS spinal cord sections stained with hematoxylin-eosin (**Figure 1** G). After photography, we removed the coverslips from the slides in xylene and destained the sections in alcohol. The sections were then restained with immunohistochemistry, using the OPTN antibody. The immunoreactivity of the Bunina bodies was examined microscopically, using the previous hematoxylin-eosin photographs as reference.

RESULTS

The 2 OPTN polyclonal antibodies that we tested (C-term and INT) were the same ones used in a previously reported study.[19] Both antibodies yielded immunoreactive signals in the spinal cord sections of 5 SALS cases. Because the signal generated with antibody C-term was more robust and Western blot with antibody C-term revealed a single band of expected size (eFigure; http://www.archneurol.com), we used that antibody throughout this study. Immunohistochemical staining of the spinal cord sections revealed that OPTN-immunoreactive skeinlike inclusions were present in some of the spared spi-

nal anterior horn neurons from all 32 SALS cases (Figure 1A-C), supporting the hypothesis that OPTN is involved in the pathogenesis of SALS.[19] The OPTN-immunoreactive skeinlike inclusions were also observed in a subset of the remaining spinal anterior horn neurons in all 8 FALS cases without mutations in SOD1 (Figure 1D and E). These inclusions were located in the cell bodies and neurites. The OPTN-positive inclusions were typically skeinlike on morphologic examination (Figure 1A-E), but some appeared to be relatively more compact as spherical inclusions (Figure 1F). In contrast, we did not observe OPTN-positive inclusions in any of the non-ALS controls or in the 6 cases with mutations in *SOD1* (A4V or G85R), although multiple sections were analyzed.

Two major types of inclusions can be observed in surviving spinal motor neurons in all types of ALS cases: skeinlike/spherical inclusions and Bunina bodies. The skeinlike/spherical inclusions are ubiquitin-positive, and Bunina bodies are small and eosinophilic but ubiquitin-negative. In a study[22] of 102 ALS cases, the skeinlike/spherical inclusions were found in all cases and Bunina bodies were found in 88 cases (86%). In our experience, the skeinlike/spherical inclusions may be seen in approximately 5% to 30% of the motor neurons in affected regions of the spinal cord, but Bunina bodies appear much less frequently, in approximately 1% of motor neurons in the same region. To date, Bunina bodies have been shown[23] to be immunoreactive for only 2 proteins: cystatin C[24] and transferrin. To determine whether Bunina bodies contain OPTN, we tested spinal cord sections from 3 ALS cases. The small eosinophilic Bunina bodies were first identified in some anterior horn large neurons in the sections stained with hematoxylin-eosin (Figure 1G). After photography, the sections were destained and then restained with immunohistochemistry, using the OPTN C-term antibody. We found no OPTN in the Bunina bodies (Figure 1H).

The skeinlike inclusions in spinal motor neurons in FALS cases with *SOD1* mutations are immunoreactive for ubiquitin. To minimize the possibility that our failure to detect OPTN-positive inclusions in our *SOD1* linked cases could be due to the absence of inclusion-bearing neurons in the slides that we analyzed, we performed 2-color confocal immunofluorescence microscopy, using antibodies to ubiquitin and OPTN. We observed that skeinlike inclusions were immunoreactive with antibodies against ubiquitin and OPTN in cases with SALS (**Figure 2**A-C). We also observed ubiquitin-positive skeinlike inclusions in all the *SOD1*-linked FALS cases, but these inclusions were consistently negative for OPTN (Figure 2D-I). We further analyzed 2 well-characterized ALS transgenic murine models overexpressing the ALS-linked *SOD1* mutations G93A or L126Z.[20,21] We found that ubiquitin-positive inclusions were also negative for OPTN in these transgenic mice (Figure 2J-O).

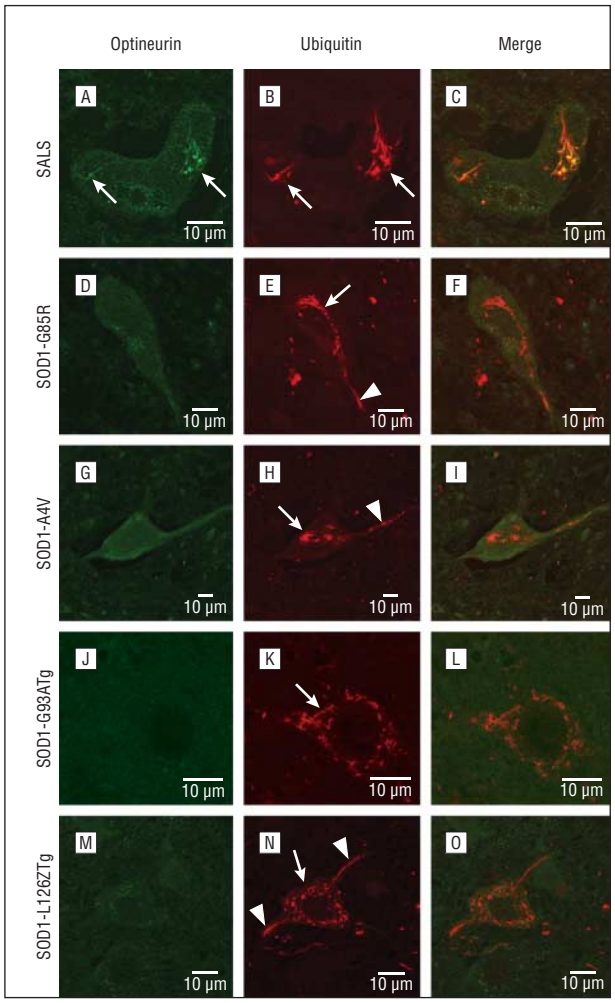

Figure 2. Immunoreactivity of optineurin in ubiquitin-positive inclusions in sporadic amyotrophic lateral sclerosis (SALS) cases, *SOD1*-linked ALS, and SOD1 transgenic (Tg) mice. Confocal microscopy was performed using antibodies to optineurin (green) and ubiquitin (red) on the spinal cord sections from ALS cases and Tg mice. Representative images show that the ubiquitin-positive inclusions are optineurin-positive in SALS cases (A-C), but negative in SOD1-G85R (D-F) and SOD1-A4V (G-I), as well as in ALS Tg mice overexpressing SOD1-G93A (J-L) or SOD1-L126Z (M-O). Representative optineurin-positive skeinlike inclusions in an SALS case are indicated by arrows in panel A. The ubiquitin-positive inclusions in soma and neurites are indicated by arrows and arrowheads, respectively, in panels B, E, H, K, and N.

COMMENTS

In this study, we analyzed a large series of postmortem spinal cord samples from cases of both SALS and FALS and 2 *SOD1*-linked ALS mouse models, using antibodies to OPTN and ubiquitin. The results indicate that OPTN is localized to characteristic skeinlike inclusions of anterior horn neurons and their neurites in spinal cords of SALS and some FALS cases, but not in the cases linked to *SOD1* or in the ALS transgenic mouse models overexpressing ALS-linked mutant *SOD1*. These data indicate that OPTN is also involved in SALS and some types of FALS, although *OPTN* mutations account for only a small fraction of ALS. Optineurin, however, does not appear to be involved in *SOD1*-linked ALS.

Motor neuron degeneration is a shared downstream event in all types of ALS. However, upstream pathways are likely to be different, depending on the cause of the disease. It has been shown[17,18] that TDP43 and FUS, 2 proteins that are mutated in some ALS cases, are commonly involved in non-*SOD1* ALS, but not in *SOD1*-linked ALS. The results of the present study demon-

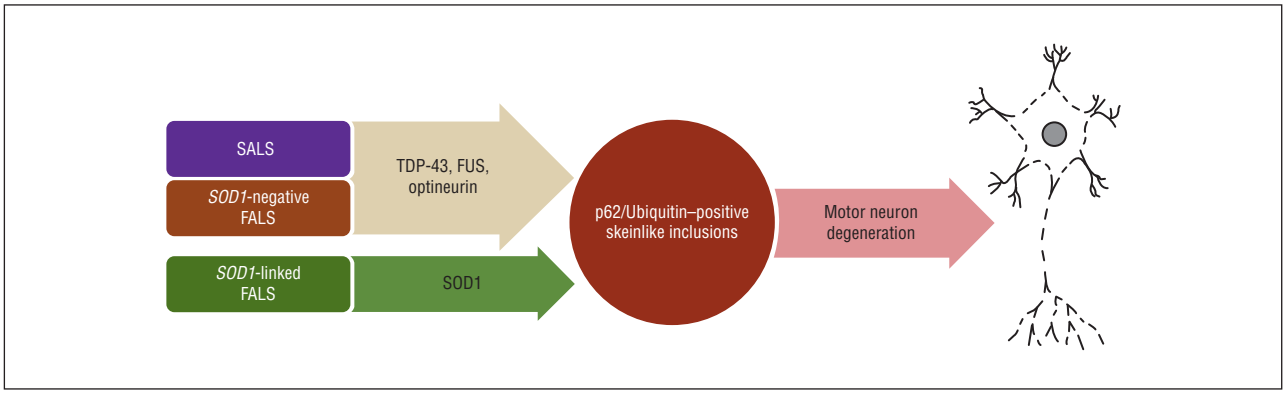

Figure 3. Schematic model depicting 2 parallel pathways involved in the pathogenesis of amyotrophic lateral sclerosis (ALS). TDP-43, FUS, and optineurin are components of pathologic inclusions in sporadic ALS (SALS) and *SOD1*-negative familial ALS (FALS). SOD1-positive inclusions are exclusively present in *SOD1*-linked FALS. Ubiquitin and p62 may represent a common pathway involved in the formation of skeinlike inclusions, eventually leading to motor neuron degeneration.

strate that OPTN, another protein mutated in a small fraction of ALS cases, is also involved in non-*SOD1* ALS. The findings suggest that OPTN, similarly to TDP43 and FUS, may play a role in the pathogenesis of most cases of ALS, whereas *SOD1*-linked ALS has a distinct pathogenic pathway (**Figure 3**).

We used immunostaining to examine the presence of OPTN in the ALS-specific skeinlike inclusions in different types of ALS. Immunostaining involves the binding of an antibody to a cellular or tissue epitope of interest and visualization of the bound product by a detection system. The sensitivity and specificity of immunostaining can be affected by several factors, such as tissue preservation and processing, epitope retrieval procedure, the amount of epitopes to be detected in the tissue, and the quality of the antibody and detection system. In our previous study,[17] we noticed that only 3 of 9 FUS antibodies could be successfully used for detection of FUS inclusions in non-*SOD1* ALS cases; moreover, the intensity of signals yielded by these 3 antibodies might vary depending on the antibody used. These observations suggest the importance of antibody sensitivity for immunostaining. Another issue related to antibody sensitivity may involve the relative amount of specific epitopes in the inclusions. If sufficient amounts of epitopes are present, they may be readily detected with most relevant antibodies, as shown by SOD1-positive inclusions detected by different SOD1 antibodies in *SOD1*-linked ALS.[20] Based on these considerations, although we are not able to exclude the possibility that OPTN is present in the SOD1-immunoreactive skeinlike inclusions in *SOD1*-linked ALS, it is plausible that OPTN is involved to a far less degree (or perhaps not involved) in *SOD1*-linked ALS than in non-*SOD1* ALS. Therefore, *SOD1*-linked ALS may represent a unique type of ALS in which the skeinlike inclusions are predominantly composed of SOD1. However, the skeinlike inclusions in non-*SOD1* ALS may be composed of many other ALS-risk constituents, including TDP43, FUS, OPTN, and unidentified proteins. In fact, we noticed that immunoreactivity of the skeinlike inclusions in non-*SOD1* ALS cases was strongest with the TDP43 antibody (10782-2-AP; ProteinTech Group, Inc, Chicago, Illinois), followed by FUS antibody (11570-1-AP; ProteinTech Group, Inc) and OPTN C-term antibody (catalog No. 100000, Cayman Chemical). However, the relevance of the immunoreactivity strength to the relative roles of these proteins in the pathogenic process in non-*SOD1* ALS is unclear.

The exact physiologic function of OPTN and its role in ALS remain to be elucidated. It has been shown that OPTN can interact with adenovirus E3-14.7K protein,[25] huntingtin,[26] transcription factor IIIA,[27] Rab8,[28] myosin VI,[29] and ubiquitinated receptor-interacting protein.[30] Through these interactions, OPTN may play important roles in various cellular processes, such as apoptosis, inflammation, membrane and vesicle trafficking, and transcriptional activation. Optineurin competes with nuclear factor κB essential modulator for binding to the receptor-interacting protein and inhibits nuclear factor κB activation.[30] Notably, the ALS-linked OPTN mutations result in an altered pattern of intracellular distribution and/or a loss of ability to inhibit nuclear factor κB activity.[19]

Divergent mechanisms have been proposed[19] for *OPTN*-linked ALS, depending on the types of mutation. For the homozygous premature termination codon mutations, such as exon 5 deletion and Q398X, loss of OPTN functions are apparently involved; for heterozygous E478G, the abnormal accumulation of the mutant OPTN may be deleterious.[19] The characteristic skeinlike inclusions identified in non-*SOD1* ALS cases have suggested pathogenic roles of these inclusions in non-*SOD1* ALS. Two likely mechanisms may exist: (1) the inclusions are deleterious and (2) the inclusions, which may or may not be "protective" to the cell, trap some cellular proteins that are essential for normal cellular function, leading to a loss-of-function effect. Identification of the loss-of-function mutations in *OPTN*-linked ALS, together with the observation that OPTN is present in the skeinlike inclusions in most non-*OPTN* ALS, supports the notion that OPTN is one of such essential proteins for extended motor neuron survival. Co-localization of the 3 known ALS-linked proteins (TDP43, FUS, and OPTN) in the skeinlike inclusions in non-*SOD1* ALS suggests a pathogenic role of the aberrant interaction of specific proteins in the pathogenesis of the disease. Further studies of TDP43, FUS, and OPTN should provide new insight toward an understanding of the pathogenic mechanisms responsible not only for the subtypes of FALS but also for SALS,

thereby providing a rational pathogenic basis for targeted therapeutic intervention.

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Online-Only Material: The eFigure is available at http://www.archneurol.com.

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