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Fast Progression in Amyotrophic Lateral Sclerosis Is Associated With Greater TDP-43 Burden in Spinal Cord

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Abstract

Upper and lower motor neuron pathologies are critical to the autopsy diagnosis of amyotrophic lateral sclerosis (ALS). Further investigation is needed to determine how the relative burden of these pathologies affects the disease course. We performed a blinded, retrospective study of 38 ALS patients, examining the association between pathologic measures in motor cortex, hypoglossal nucleus, and lumbar cord with clinical data, including progression rate and disease duration, site of symptom onset, and upper and lower motor neuron signs. The most critical finding in our study was that TAR DNA-binding protein 43 kDa (TDP-43) pathologic burden in lumbar cord and hypoglossal nucleus was significantly associated with a faster progression rate with reduced survival ($p < 0.02$). There was no correlation between TDP-43 burden and the severity of cell loss, and no significant clinical associations were identified for motor cortex TDP-43 burden or severity of cell loss in motor cortex. *C9orf72* expansion was associated with shorter disease duration ($p < 0.001$) but was not significantly associated with pathologic measures in these regions. The association between lower motor neuron TDP-43 burden and fast progression with reduced survival in ALS provides further support for the study of TDP-43 as a disease biomarker.

Key Words: Amyotrophic lateral sclerosis, Lower motor neuron, Motor cortex, Progression rate, Spinal cord, TAR DNA-binding protein 43 kDa (TDP-43), Upper motor neuron.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is characterized by upper motor neuron (UMN) and lower motor neuron (LMN) signs, progressive weakness, and muscle atrophy (1). Extramotor symptoms such as cognitive dysfunction are also common (1−3), which may reflect an underlying multisystem TDP-43 proteinopathy (4, 5). ALS is characterized pathologically by the accumulation of ubiquitinated proteins, including TAR DNA-binding protein 43 kDa (TDP-43) (6), and this is thought to be detrimental to normal cellular function. TDP-43 pathology has been noted in upper and lower motor neuron pools, non-motor neurons, and glia of ALS patients (2, 7, 8), as well as within skeletal muscle (9) and axons (10). Elevated levels of TDP-43 have also been detected in the plasma of ALS patients compared with controls (11), suggesting that widespread dysregulation of RNA-binding proteins, including TDP-43, is a fundamental feature of the disease. The pathologic heterogeneity of TDP-43 across patients has been well characterized (12, 13) and a greater whole brain TDP-43 burden is associated with both cognitive impairment and *C9orf72* expansion (14, 15).

TDP-43 is an informative protein that may be used to better understand how cellular pathology contributes to patient symptoms in ALS, particularly since misfolded TDP-43 may be an early marker of cellular dysfunction that appears before evident neuron loss. However, it remains unclear how TDP-43 relates to rate of disease progression and disease duration, as well as to the site of symptom onset and relative balance of UMN and LMN signs. TDP-43 burden and cell loss in UMN and LMN pools may also be useful in better understanding current models of anterograde and retrograde injury in ALS (16−18). For instance, to examine whether the burden of TDP-43 and cell loss in UMN and LMN pools strongly associates with patterns of disease onset, or other clinical features. Regarding TDP-43 burden and disease duration, few clinico-pathologic studies of ALS have looked directly at this question. Several studies have suggested a surprising trend of greater whole brain TDP-43 burden with shorter disease duration in ALS (12, 19). This differs from neurodegenerative diseases where the burden of misfolded protein is typically greatest in patients with the longest disease duration (20, 21).

Examining whole brain TDP-43 burden in ALS, we previously reported that a "TDP-43 severe" group had the shortest duration and fastest progression mean values (14). That study did not focus on the motor system specifically, nor the association between motor system pathologies and clinical findings.

The aim of this study was to examine the clinical associations of pathology in UMN and LMN pools in a blinded, retrospective clinicopathologic analysis of 38 autopsied ALS patients (21 men, 17 women, median age of onset 61 years, and median duration 36 months). We assessed neuronal loss in motor cortex (UMNs) and lumbar cord and hypoglossal nucleus (LMNs), as well as the inclusion density of TDP-43 pathology in the same. The addition of TDP-43 to the study design was critical because TDP-43 burden and neuron loss do not always correlate (22) and TDP-43 may be a more sensitive marker of the disease process and early cell injury (23). Independently collected clinical data were then examined to determine whether patterns of cell loss and/or TDP-43 pathology associated with salient clinical variables, such as onset site, the balance of UMN and LMN signs, and the rate of disease progression and disease duration.

MATERIALS AND METHODS

Case Identification

The patients included in the study all came to autopsy at Houston Methodist Hospital. The patients were evaluated in the clinic of 2 of the study authors specializing in neuromuscular disease. Final pathologic assessments were performed in all cases by Houston Methodist neuropathologists, confirming the clinical diagnosis of ALS. Informed written consent was previously obtained from patients or their next of kin with research permissions provided. The study was performed with the approval of the Institutional Review Board at Houston Methodist Hospital (IRB Pro00010377).

Hematoxylin and eosin (H&E)-stained material for each of the 38 ALS autopsies was reviewed by a neuropathologist (M.D.C.) to determine 3 regions of interest (ROIs) were present per case: (i) primary motor cortex, characterized by thick agranular frontal cortex with large pyramidal cells in lamina V, including Betz cells; (ii) lower motor neurons of hypoglossal nucleus; and (iii) lower motor neurons of lumbar spinal cord. Cases were excluded if the ROIs could not be sufficiently defined for both UMN and LMN pools, the orientation was insufficient for further pathologic evaluation, or there were confounding factors, such as diffuse hypoxic/ischemic injury. The list of suitable cases was then provided to 2 study authors (S.J.C., S.H.A.), who were blinded to pathologic data and details of autopsy diagnoses. These 2 authors reviewed and collated the salient clinical information per patient prior to further analyses.

Clinical Assessment

A complete clinical chart review was performed by the lead author, supervised by a neurologist and neuromuscular disease specialist (S.H.A.). Clinical variables recorded included: (i) age of onset and disease duration (months), (ii) site of disease onset (lower extremity [LE]; upper extremity [UE];

bulbar; or diffuse), (iii) UMN versus LMN symptom predominance at onset, based on first documented physical exam tone, strength, and reflexes, (iv) Appel ALS (AALS) score monthly rate of change (24), (v) *C9orf72* expansion status (also see below under Pathologic Assessment), and (vi) cognitive impairment. For each patient, site of disease onset, UMN versus LMN predominance, and AALS progression rate were reviewed and agreed upon by these 2 authors.

The AALS monthly rate of change was calculated using the initial and most advanced AALS scores. If only an initial score was available (e.g. in a rapidly progressive patient), AALS rate of change was calculated using reported time of symptom onset (month 0, considered to be a normal AALS score of 30) and the initial documented AALS score. Rates were stratified into 4 progression categories (slow, $1 = <2$ points per month; intermediate, $2 = 2-3.49$ points per month; fast, $3 = 3.5-5.99$ points per month; very fast, $4 = \geq 6$ points per month), or were not available (6 patients). Cognitive impairment was assessed by neuropsychological evaluation, MoCA, MMSE, or clinical descriptions. Missing data included rate of progression (6 patients), cognitive impairment status (4 patients), and disease duration (one patient).

Histological and Immunohistochemical Procedures

The autopsy procedure at our institution for ALS patients has previously been described in detail (4, 14). As described above, only cases with UMN pools in frontal (motor) cortex and LMNs in hypoglossal nucleus and lumbar cord were studied (3 blocks per patient). The staining protocol for tissue examination included H&E and full-length TDP-43 immunostain (Proteintech, 10782-2-AP, rabbit polyclonal antibody, 1:200; Rosemont, IL). Immunostaining was performed on brain tissue fixed in 20% formalin following brain removal, typically within 8–24 hours of the patient's death per research protocol. Formalin-fixed paraffin embedded tissue was sectioned at 4 μm, mounted on charged slides and dried at 60°C. Immunostaining was performed on a BenchMark ULTRA platform (Ventana Medical Systems, Inc., Tucson, AZ) with appropriate positive and negative controls. Full-length (N-terminal) TDP-43 immunostain was used because this is very sensitive to pathologic inclusions in ALS (14), as it facilitates the identification of "pre-inclusion" pathology wherein granular cytoplasmic aggregates are detected in neurons coupled with loss of nuclear staining (25). (As described below, phospho-TDP staining was also performed in spinal cord samples to examine the correlation of inclusion pathology by both approaches.) *C9orf72* expansion status was determined or confirmed by assessing dipeptide repeat proteins in the hippocampal tissue of each case using antibodies to poly-glycine-alanine (poly-GA) (mouse monoclonal, 1:200, EMD Millipore, MABN889) and poly-glycine-proline (poly-GP) (rabbit polyclonal, 1:1000, EMD Millipore, ABN455), as previously described (26).

Neuropathological Assessment: H&E and TDP-43 Pathologies

Each slide was coded with a unique pathology identifier such that the corresponding patient identification was not known to the lead author, a neuropathologist and the primary rater for H&E and TDP-43 pathologies. For each of the 3 study regions of interest (ROI), a qualitative assessment of neuron loss was made from H&E preparations and a semi-quantitative assessment of TDP-43 inclusion density was made, resulting in 6 pathologic parameters per case (2 per ROI). Pathologic rating of each sample was performed blinded to all clinical and autopsy report data. Every slide and neuropathologic assessment by the lead author was subsequently reviewed with a second neuropathologist (M.D.C.) at a multi-headed microscope. Prior to statistical analyses, and blinded to clinical data, final pathologic assessments provided to the study biostatistician (L.E.P.) were agreed upon by 2 neuropathologists (S.J.C., M.D.C.). This resulted in rare modifications to initial scoring (∼6.5% of all pathology measures), typically by a single degree in the semiquantitative scales described below. Modification of a rating of no pathologic alteration in motor cortex (score 0) to mild pathology (score 1) was the most common alteration, and modifications were less common to LMN assessments or TDP-43 inclusion density measures.

For H&E pathologies, both UMN (motor cortex) and LMN (hypoglossal nucleus, lumbar cord) ROIs were each rated on a four-point scale from no pathologic alteration (score 0) to advanced/severe pathology (score 3). For motor cortex, ratings included: no pathologic alteration (score 0); mild pathology (score 1), characterized by mild neuron loss, mild cortical atrophy, and vascular congestion; moderate pathology (score 2), characterized by moderate neuronal loss, moderate cortical atrophy and mild to moderate vacuolization of superficial cortical laminae; and severe pathology (score 3), characterized by severe, pan-cortical neuron loss, atrophy and collapse of cortex, and a spongiform appearance of cortex (Fig. 1, top and middle rows). For LMN pools, the same scoring system was used with an emphasis on residual α-motor neuron density. For LMN pools, ratings included: No pathologic alteration (score 0); mild pathology (score 1), characterized by mild LMN loss, gliosis, and early parenchymal vacuolization and vascular congestion; moderate pathology (score 2), consisting of moderate LMN loss and moderate parenchymal vacuolization, gliosis, and vascular congestion; and severe (score 3) pathology, reserved for near-total LMN loss, gliosis and severe vacuolization and rarefaction (infarct-like) (Fig. 1, bottom row).

The density of TDP-43 inclusion pathology was assessed in study ROIs using a semiquantitative approach. For each ROI, the focus of greatest pathologic involvement was identified, and TDP-43 inclusion density was recorded as the number of total pathologic inclusions (neuronal, glial, and neuritic) in 3 successive fields at 400× magnification. For samples with extremely dense TDP-43 inclusion pathology (>30 to innumerable), the TDP-43 density value was recorded as 40 for statistical analysis (14).

To examine the relationship of N-terminal TDP-43 and phospho-TDP-43 (pTDP-43) inclusion pathology, additional staining was carried out in 35 study spinal cords. Sections were stained for phospho-TDP43 (Ser409/410) (22309-1-AP, 1:500, Proteintech), using a previously described immunostaining approach (9). Representative fields of pTDP-43 pathology in the ventral horn of spinal cord were captured at 200× magnification in cellSens software 1.13 (Olympus America, Inc., Center Valley, PA), using a DP71 camera mounted on a Olympus BX-43 Microscope. Image acquisition parameters were held constant and photomicrographs were loaded into ImageJ (27) for quantitation using a thresholding approach (28). Color thresholding (RGB mode) was used to generate a binary image from which DAB-positive, pTDP-43 inclusions were automatically counted using the analyze particles tool under the Analyze menu.

Neuropathological Assessment: UMN Versus LMN Predominant Patterns

The morphologic (H&E) and TDP-43 inclusion density measures were subsequently utilized to determine the predominant site of ALS-related pathology for each variable and patient sample. For H&E pathologies, categories included: LMN predominant cell loss (lumbar or hypoglossal pathology scores > cortex) and cortical predominant cell loss (cortex > lumbar or hypoglossal scores). Cases without a predominant site were categorized as: diffuse-severe cell loss (scores of ≥2 in UMN and LMN ROIs) and diffuse-mild cell loss (scores of ≤1 in UMN and LMN ROIs). TDP-43 inclusion density was similarly categorized as cortical predominant TDP, LMN predominant TDP, and diffuse TDP. Cases with diffuse-severe TDP pathology had a 0% difference between UMN and LMN TDP-43 values and maximal inclusion density at both sites. Cases with diffuse-mild TDP pathology had a 0% difference (4 cases) or 18% difference (one case) between UMN and LMN sites and no ROI with a density value exceeding 10.

Statistical Methods

Analysis of variance (ANOVA) and t-tests were employed to test for equality of means between various pathologic and clinical groups. Welch ANOVA and Welch t-tests were used for tests involving unequal variances. Prior to clinico-pathologic associations, it was determined that duration of disease and AALS progression rate were very strongly correlated (p < 0.01). Therefore, duration was split into 4 quartiles, which were used as the final duration/progression rate metric for statistical testing. Pearson and Spearman rank correlation were used to examine significant correlations between pathologic (H&E, N-terminal TDP-43) and clinical data and the directionality of the correlations. Chi-squared contingency table analysis was also used for tests of independence for categorical variables. All statistical tests were two-sided and a p < 0.05 was considered significant.

RESULTS

Clinical Characteristics

A summary of demographic and clinical data is provided in Table 1. Briefly, the 38 patients studied included 21
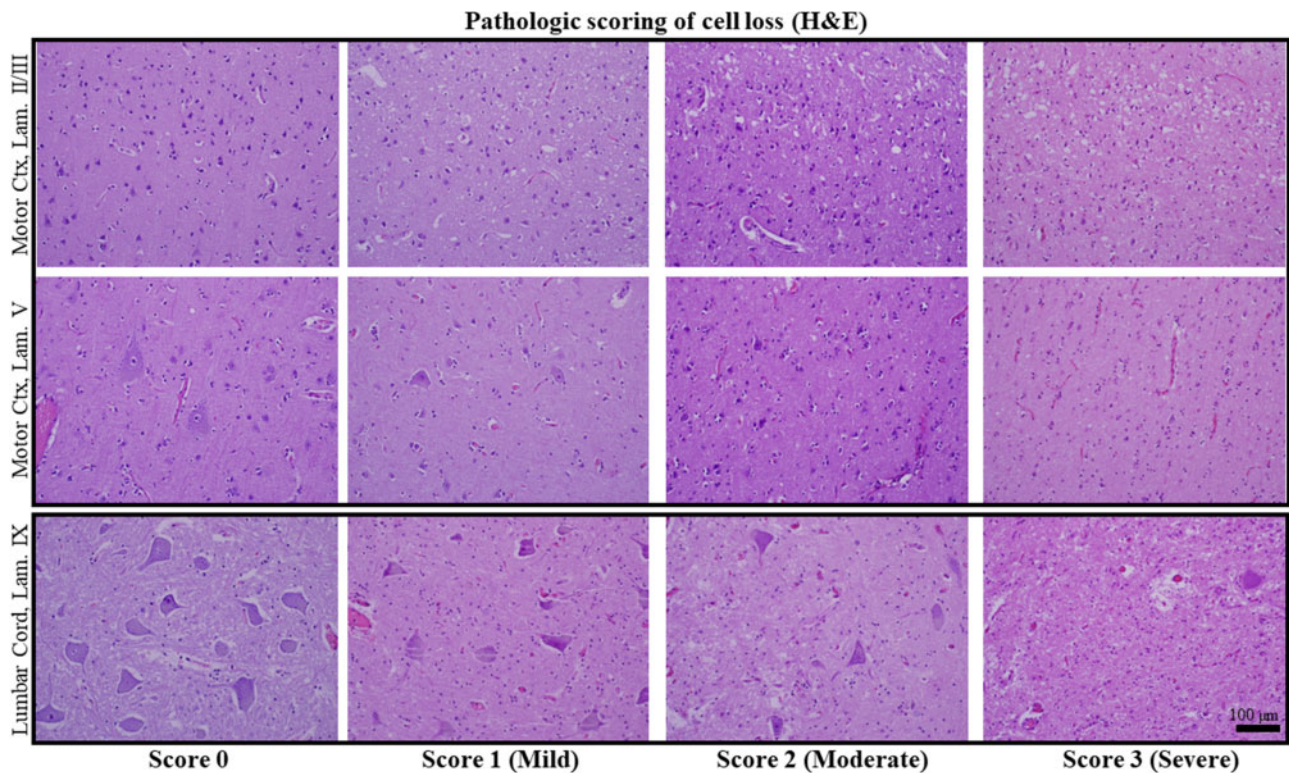
FIGURE 1. Pathologic scoring of cell loss in H&E-stained autopsy material. Scores ranged from no evident cell loss (score 0; left-most column) to severe cell loss (score 3; right-most column) (see "Materials and Methods" for detail). The top 2 rows show lamina II and III (top row) and lamina V (middle row) in 4 ALS patients with scores $0-3$. In the left-most panel of the middle row (score 0), large pyramidal cells (Betz cells) in lamina V are evident and in the score 3 example this layer is depleted of neurons. The bottom row shows residual α-motor neurons in the lateral portion of Lamina IX of the lumbar cord, ranging from score 0 (a non-ALS example; no ALS case in this study was score 0) to scores $1-3$ (study samples shown). All images taken at $\times200$ magnification and the scale bar in the bottom right panel applies to all images.

men and 17 women, hereafter referred to as ALS01 to ALS38, with a median age of 61 years at disease onset (interquartile range [IQR], 7 years; ranging from 41 to 79 years) and a median disease duration of 36 months (IQR, 34; ranging from 11 to 117 months). Six patients were designated as familial ALS (fALS) (16.2%), 31 patients as sporadic (sALS) (83.8%), and for one patient the fALS/sALS designation was not known. Nine patients had *C9orf72* expansion (24.3%) as determined by premortem molecular testing and/or dipeptide repeat pathology (poly-GA, poly-GP) in autopsy samples. Thus, the molecular basis in 5 of 6 fALS patients was accounted for by *C9orf72* expansion.

The earliest site of symptoms at disease onset was lower extremity in 18 patients (47.4%), upper extremity in 10 patients (26.3%), bulbar in 7 patients (18.4%), and was multifocal in 3 (7.9%). Of the 30 patients, where UMN and LMN predominance at onset could be accurately determined, 6 patients (20%) had UMN-predominant symptoms, 11 patients (36.7%) demonstrated LMN-predominant symptoms, and 13 patients (43.3%) demonstrated equal UMN and LMN signs and symptoms. None of the patients were diagnosed as having the primary lateral sclerosis or primary muscular atrophy variants of ALS.

Duration was known in all but one patient, and speed of progression could be determined in 32 patients. Three patients (9.4%) were "slow progressors" (range of monthly rate of change in AALS score, $0.96-1.67$ and disease duration range, $82-117$ months) and 5 patients (15.6%) were "rapid progressors" (range of monthly rate of change in AALS score, $6.7-9.4$; disease duration range, $12-25$ months). As described above, duration (months) and progression rate were very strongly correlated, such that quartiles of duration were used for statistical analyses. Mild cognitive impairment was present in 17 of the 34 patients (50%) where cognitive status was documented, and 35% of these cognitively impaired patients had *C9orf72* expansion.

Neuropathologic Characteristics

Neuropathologic data are summarized in the bottom half of Table 1. Of 38 patients studied, LMN and UMN ROIs with H&E and TDP-43 data were available in 37 (one patient had partial ROI data). An LMN predominant cell loss pattern (H&E stain) was by far the most prevalent pattern, being seen in 28 patients (75.7%). This was followed by a diffuse pattern in 8 patients (21.6%) (7 diffuse-severe cell loss, 1 diffuse-mild cell loss). Only a single patient had cortical predominant cell loss (2.7%). Notably, this occurred in a patient with *C9orf72*

TABLE 1. Clinical and Neuropathology Data in 38 Study Patients

Variable	N (%)	Median	Other
Age at onset		61 years	$41 - 79$ years (IQR 7)
Men/women	21 (55)/17 (45)		
Duration (months)*		36 months	$11 - 117$ months (IQR 34)
Site of symptom onset			
Lower extremity (LE)	18 (47.4)	–	–
Upper extremity (UE)	10 (26.3)	–	–
Bulbar	7 (18.4)	–	–
Multifocal (UE, LE, and/or Bulbar)	3 (7.9)	–	–
UMN vs LMN signs and symptoms[†]			
UMN prominence	6 (20.0)	–	–
LMN prominence	11 (36.7)	–	–
Equal UMN and LMN prominence	13 (43.3)	–	–
Familial ALS	6 (16.2)	62 years[‡]	–
C9orf72 expansion[#]	5	–	–
Sporadic ALS	31 (83.8)	67 years	–
C9orf72 expansion	4	–	–
AALS score progression rate			
Slow (<2 points per month)	3 (9.4)	1.33 pts/month	83 months duration*
Intermediate ($2 - 3.49$ points per month)	10 (31.3)	2.37 pts/month	54 months duration
Fast ($3.5 - 6$ points per month)	14 (43.8)	4.56 pts/month	32 months duration
Very fast (>6 points per month)	5 (15.6)	9.00 pts/month	20 months duration
Cognitive status[$]			
Impaired	17 (50%)	–	6 C9ORF72 patients
Intact	17 (50%)	–	3 C9ORF72 patients
Pathologic pattern (H&E)			
Cortical predominant cell loss	1 (2.7)	–	–
LMN predominant cell loss	28 (75.7)	–	–
Diffuse, severe cell loss	7 (18.9)	–	–
Diffuse, mild cell loss	1 (2.7)	–	–
TDP-43 inclusion density			
Cortical TDP predominant	15 (40.5)	–	67% LMN predom path[+]
LMN TDP predominant	12 (32.5)	–	92% LMN predom path
Diffuse, severe TDP	6 (16.2)	–	–
Diffuse, mild TDP	4 (10.8)	–	–

*AALS Progression Rate and Duration (months) were significantly correlated (p < 0.01). Median duration shown by AALS score progression rate. Duration was used in statistical analyses.

[†]UMN versus LMN predominance at onset (clinically) could not be determined in eight patients.

[‡]Median age at death.

[#]Patients with *C9orf72* expansion (N = 9) accounted for 24.3% of the study cohort.

[$]Cognitive status was not known in four patients.

[+]Independent of TDP predominance, the majority in both groups had LMN predominant cell loss by H&E and no correlations between inclusion density and cell loss were present in any ROI.

expansion, rapidly progressive disease, and diffuse-severe TDP pathology.

In contrast, the predominant region of TDP-43 pathology was more evenly distributed. Twelve patients (32.5%) had LMN predominant TDP pathology, 10 patients had a diffuse TDP pattern (27.0%) (4 diffuse-mild TDP, 6 diffuse-severe TDP), and 15 patients (40.5%) had cortical predominant TDP. The majority of LMN predominant TDP (92%) and cortical predominant TDP (67%) patients had LMN predominant cell loss on H&E. As such, there was no significant association between the pattern of TDP pathology and cell loss ($\chi^2 = 2.9$, p = 0.09). Neither were there correlations between TDP-43 inclusion density and H&E-based ratings of cell loss in lumbar (Spearman rho = -0.08), hypoglossal (Spearman rho = -0.17), and motor cortex (Spearman rho = 0.19) ROIs. N-terminal TDP-43 inclusion density and phospho-TDP-43 inclusion counts in ImageJ were strongly correlated across ALS spinal cords (rho = 0.47, p = 0.0041) (Spearman's rank correlation as implemented in R [29]), supporting the use of the N-terminal TDP-43 measures for subsequent clinicopathologic associations.

As described in the "Materials and Methods", TDP-43 inclusion density was recorded as the number of total pathologic inclusions (neuronal, glial, and neuritic) in 3 successive

TABLE 2. Groupwise Comparisons of Average Pathologic Scores

Variable	Disease Duration (Quartiles)					*C9orf72* Expansion		
	Q1 (n = 10)	Q2 (n = 9)	Q3 (n = 9)	Q4 (n = 10)	p value	No (n = 28)	Yes (n = 9)	p value
Months Survival (Average)	11 − 24 (18.6)	25 − 36 (28.4)	37 − 58 (44.0)	60 − 117 (81.6)	<0.0005	50.8	25.4	<0.0005
Progression Rate*	Very Fast	Fast	Intermediate	Slow	−	−	−	−
Age at Onset	66.0	59.1	56.3	62.2	0.036	60.5	62.1	0.60
H&E Pathologic Rating								
Lumbar Cord	2.1	2.1	2.6	2.4	0.3981	2.4	2.0	0.1804
Hypoglossal Nucleus	1.9	2.1	2.1	2.5	0.3115	2.0	2.3	0.3555
Motor Cortex	1.4	1.2	1.4	1.6	0.7936	1.4	1.4	0.9583
TDP-43 Density								
Lumbar Cord	18.4	15.3	17.9	6.9	0.0196	13.3	19.5	0.2412
Hypoglossal Nucleus	23.0	13.6	14.1	5.7	0.0118	14.3	15.0	0.8907
Motor Cortex	16.4	18.9	21.2	18.1	0.9165	19.8	16.6	0.5984

*Approximate progression rate of each duration quartile. Progression rate and duration were significantly correlated (p < 0.01) (see "Results").

fields at 400× magnification. Variably dense glial cytoplasmic inclusion (GCI) pathology was noted in both motor cortex and spinal cord across patients, but GCI pathology was not enumerated separately.

Clinicopathologic Associations

Clinicopathologic associations are summarized in Table 2. The most notable pathologic association with duration was TDP-43 inclusion density in both LMN ROIs, which was not seen for the motor cortex ROI. TDP-43 inclusion density was greatest in patients with short disease duration (n = 10; average duration 18.6 months, SD = 4.8) and lowest in slowly progressive, longer disease duration patients (n = 10; average duration 81.6 months, SD = 20.6), for both the lumbar cord (p = 0.0196) and the hypoglossal nucleus (p = 0.0118) (Fig. 2).

Spearman rank correlation also revealed significant, negative correlations between duration (in months) and TDP-43 inclusion density in lumbar cord (rho = -0.34, p < 0.05) and hypoglossal nucleus (rho = -0.50, p < 0.01) (i.e. greater TDP-43 inclusion density with shorter duration). Likewise, Spearman rank correlation indicated a significant, negative correlation between phospho-TDP-43 inclusion counts in ALS spinal cord and disease duration (months) (rho = -0.36, p < 0.05). TDP-43 pathology in several short- and long-lived patient examples are shown in Figure 3.

Spearman correlation indicated a strong, positive correlation of TDP-43 inclusion density between lumbar and hypoglossal ROIs (rho = 0.65, p < 0.01) but no correlation between the TDP-43 density in either LMN ROI with motor cortex. Duration quartiles did not differ by age at onset, cognitive impairment status, H&E-based measures of cell loss, or cortical TDP-43 inclusion density.

A cortical predominant TDP pattern was noted in longer-lived patients, and 13 of the 15 cases (87%) with this pattern were in the longer-lived third and fourth quartiles of duration (median survival 59 months) (χ^2 = 13.3, p < 0.001) (Fig. 2). However, the cortical predominant TDP pattern did not equate to an UMN predominant clinical presentation, or a cortical predominant cell loss pattern (a rare pattern by H&E). For example, of 13 study patients with survival exceeding ≥50 months, cortical predominant TDP was seen in 9 patients (69.2%). However, features of LMN-predominant disease were seen in 6 of these patients, or there were equal UMN and LMN features (5 patients) (UMN/LMN predominance at onset was unknown for 2). None of these cases had cortical predominant cell loss by H&E, with cell loss being rated as diffuse-severe (2 patients), diffuse-mild (1 patient), or LMN predominant (10 patients).

Patients with *C9orf72* expansion had a significantly shorter duration of disease (25.4 months) than those patients without *C9orf72* expansion (50.8 months) (p < 0.001). However, there was no significant difference in the severity of cell loss or TDP-43 inclusion density between patients with and without *C9orf72* expansion. Pathologic associations of UMN versus LMN symptom predominance at onset were less clear, in part because only 6 cases had UMN-predominant symptoms at onset and 4 of these patients (67%) had *C9orf72* expansion.

Stratified by site of clinical onset—lower extremity, upper extremity, bulbar, and diffuse groups—there was no significant difference in age at onset, duration of disease, *C9orf72* status, or TDP-43 inclusion density. A trend was identified, however, between severity of neuron loss in the lumbar cord ROI and lower extremity onset (p = 0.0577). Likewise, 10 of the 15 patients (67%) with severe neuron loss in lumbar cord (score 3) presented with lower extremity onset clinically, compared with 32% of cases with mild (score 1) or moderate (score 2) lumbar cord pathology (χ^2 = 4.4, p = 0.037).

DISCUSSION

Here, we examined the relationship between salient clinical features of ALS with UMN and LMN neuropathologies. A major strength of the research design is that the neuropathologic analysis was performed blinded to independently collected clinical data, including disease duration, progression
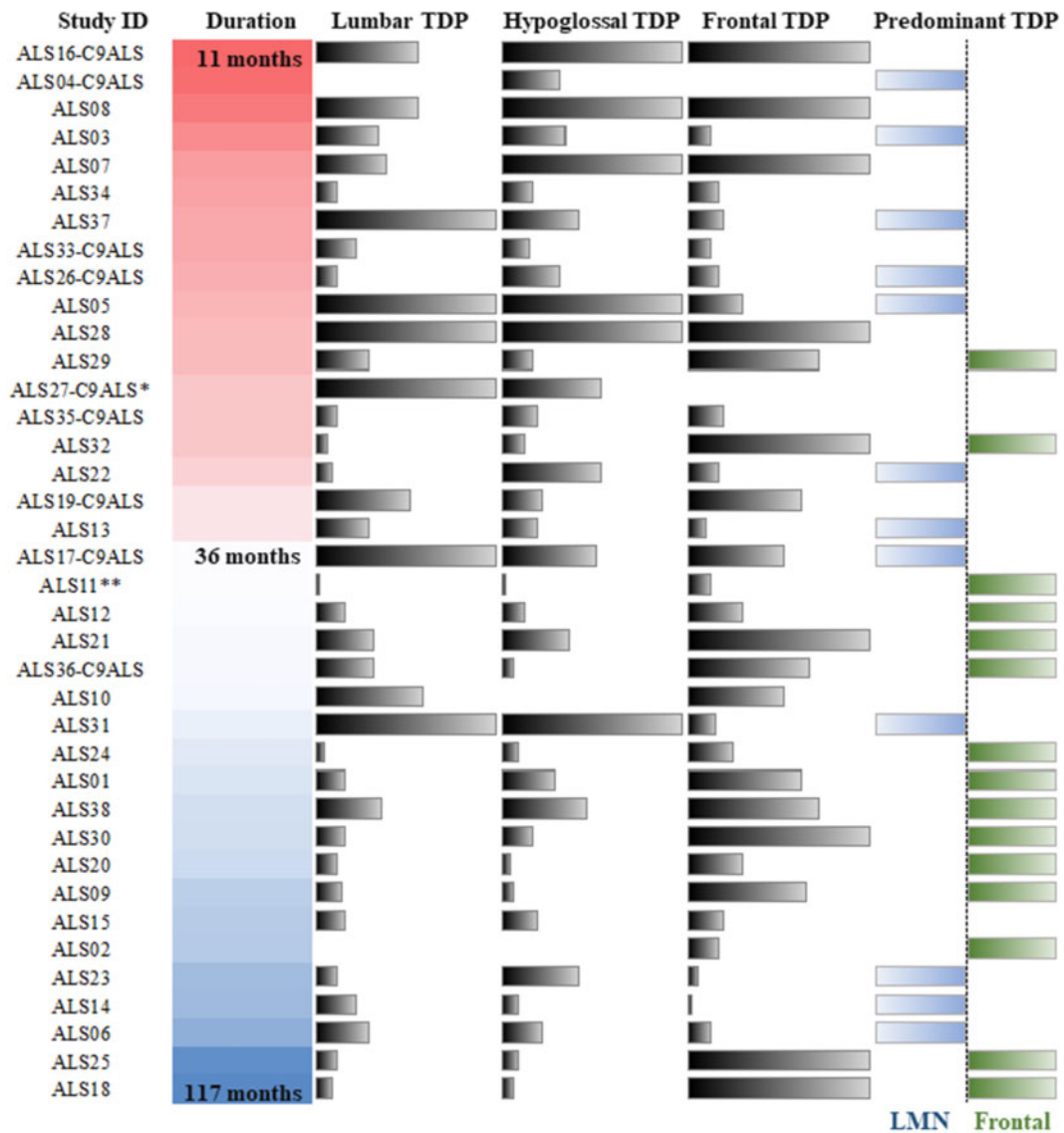
FIGURE 2. Relationship between disease duration and predominant site of TDP-43 pathology. Disease duration, shown from 11 months (sample ALS16) to 117 months (sample ALS18) and TDP-43 burden and predominance. For Lumbar, Hypoglossal, and Frontal TDP bars, the value shown indicates the TDP-43 density for that patient and ROI. The "Predominant TDP" column indicates cases with LMN-predominant TDP (leftward bars), cortical-predominant TDP (rightward bars), or diffuse TDP pathology with no predominant site (no bar shown). *Notes*: Patients with *C9orf72* expansion are indicated by "C9ALS" appended after the sample ID. *This sample lacked frontal TDP data. **Duration was not known for this sample, so the median duration of all samples is shown.

rate and onset site. We found that cell loss in the LMNs of lumbar spinal cord was nearly uniform across patients and that this loss exceeded that of UMN pools in nearly all cases. Moreover, we found that greater TDP-43 pathology in LMN pools was significantly associated with faster disease progression and reduced overall survival in ALS (for spinal cord this was identified using both N-terminal and phospho-TDP-43-based measures). Notably, spinal cord LMN loss did not correlate with either TDP-43 pathologic burden or disease duration. As such, the statistical association between greater TDP-43 burden and faster progression/reduced survival did not appear to be driven by clearance of pathologic protein in long-lived

ALS patients with fewer residual LMNs. Further, among the 8 patients with a diffuse pattern of UMN and LMN cell loss, 83% had "fast" or "very fast" progression rate with a median survival of 30 months. This suggests that a subset of patients have more extensive and severe motor neuron loss at disease onset, and that this is not necessarily associated with *C9orf72* status. In contrast to the associations identified for LMN pools, primary motor cortex (M1) cell loss was absent (n = 4) or mild (n = 20) in the majority of cases (63.2%) and an UMN-predominant pattern of cell loss was seen in only a single patient (2.6%). Likewise, TDP-43 burden in M1 had no association with disease duration or predominance of UMN or LMN
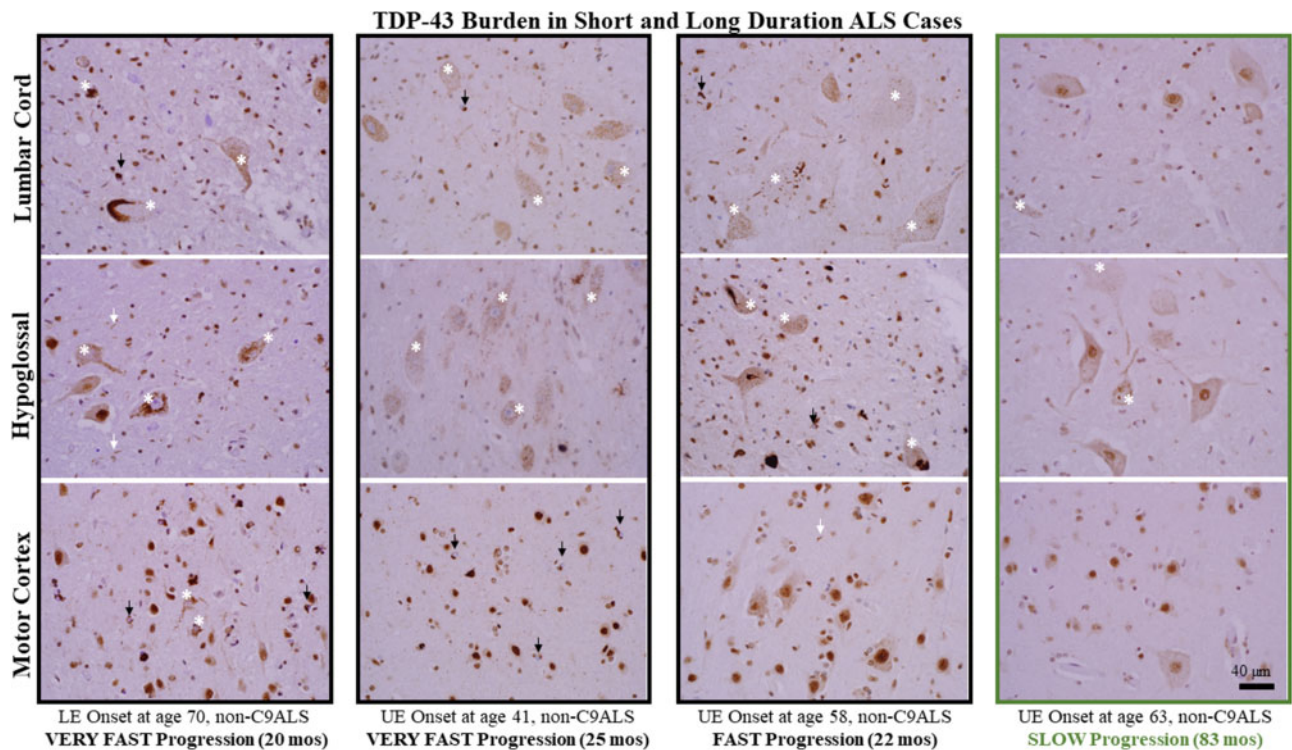
FIGURE 3. TDP-43 burden in short and long duration ALS cases. TDP-43 burden in 3 short-lived, rapidly progressive patients (first 3 columns) and one long-lived, slowly progressive patient (right-most column). For each set of patient images, N-terminal TDP-43 staining is shown for lumbar cord (top row), hypoglossal nucleus (middle row) and motor cortex (bottom row). The images are representative of study samples and demonstrate greater TDP-43 inclusion pathology in the LMN pools of more rapidly progressive patients. The images also show the heterogeneity of neuronal pathology (white asterisks), including granular "pre-inclusions" with loss of nuclear staining (see text for detail), short neurites (white arrows), and oligodendroglial cytoplasmic inclusions (black arrows). Note that in several images the granular, pre-inclusion pathology with loss of nuclear staining is predominant (e.g. LMN pools of the patient shown in the second column with onset at age 41). All images taken at ×400 magnification and the scale bar in the bottom right panel applies to all images.

findings at onset. Finally, variably dense GCI pathology was seen across the motor system regions-of-interest studied here. We have previously found GCI density in white matter is greatest in cases with the most extensive and severe TDP-43 pathology in whole brain (a "TDP43-severe" subgroup) (14). Future studies are needed to determine the clinical and pathologic associations, if any, of GCI density in UMN and LMN regions-of-interest and associated white matter tracts.

The burden of TDP-43 pathology was greater in the LMN pools of rapidly progressive ALS patients studied here. There was no association between the severity of LMN loss and TDP-43 pathology. This suggests a complex relationship between misfolded TDP-43 and cell death and dysfunction in LMN pools, potentially mediated by a pro-inflammatory environment (30), the astrocytic response to neuronal injury (31), or other processes, such as mitochondrial dysfunction (32). The paradoxical relationship of greater TDP-43 pathology with shorter disease duration contrasts with neurodegenerative diseases, such as Alzheimer disease (20) and multiple system atrophy (21), where greater pathologic burden is typically seen in the longest-lived patients. Misfolded TDP-43 and SOD1, like phospho-tau and α-synuclein, may demonstrate seeding activity that causes conformational changes in wild-type protein (33). A time-dependent, spatial spread model of ALS has been proposed (17), potentially involving prion-like mechanisms (16). It is therefore reasonable to hypothesize that TDP-43 burden in the brain would be greatest in the longest-lived ALS patients. Yet autopsy studies of long-lived ALS patients have shown TDP-43 pathology may actually be very mild and limited in anatomic distribution (34). Likewise, a recent study of military veterans with ALS and long duration (>10 years) demonstrated that the long-lived patients had less TDP-43 pathology than short-lived patients (35). A prior study by our group also found a trend between reduced patient survival and greater whole brain TDP-43 burden (14). One possible explanation for these results is heterogeneity of TDP-43 strains, as described in FTLD-TDP (36), with more virulent forms leading to a rapid clinical course and a more fulminant TDP-43 proteinopathy. Another possibility is that brain and spinal cord TDP-43 burden also reflects intrinsic cellular insults that may be more widespread in rapidly progressive patients. The finding that greater TDP-43 burden in spinal cord—and potentially in other brain areas—correlates with faster progression and reduced survival in ALS supports the proposed use of TDP-43 as a biomarker in the disease (23). In addition, the majority of patient samples studied here had a

LMN-predominant pattern of cell loss, as previously reported in several older studies (37–40), all performed prior to the discovery of TDP-43. With the limitations of an autopsy-based study, this does provide some support for a retrograde model of ALS progression, wherein the initial site of injury involves LMN pools. Earlier studies found ALS patients with severe LMN loss and near normal UMN density with only rare cases having severe UMN pathology and a mildly affected cord (39)—the same result was seen here. Neither were associations seen between UMN and LMN cell loss, also consistent with earlier studies (37, 39). As described above, we also found that TDP-43 burden in LMNs significantly associated with progression speed and overall survival. Exactly how TDP-43 contributes to LMN and axonal injury in ALS is not known, but this may involve mechanisms of cytoskeletal disruption (41), sequestration of mRNA-binding proteins necessary for normal axonal function (42), or mitochondrial dysfunction with increased production of reactive oxygen species (32).

Motor cortex cell loss and TDP-43 pathology did not associate with clinical parameters, such as onset site, duration, progression rate, or predominance of UMN symptoms. Surprisingly, only 9 patients had maximal TDP-43 inclusion density in motor cortex, and only one of these cases had an UMN-predominant clinical presentation. Further, among 5 cortical predominant TDP patients with severe UMN TDP-43 and minimal LMN TDP-43 pathology, none had *C9orf72* expansion, none had an UMN-predominant clinical presentation, 3 had LMN-predominant signs at presentation, and 4 had cognitive impairment. Motor cortex TDP-43 burden may therefore reflect other disease features not sufficiently captured in this study, or associate with whole brain TDP-43 burden and impaired cognition (12, 14). Along these lines, 64% of cognitively impaired, non-C9ALS patients had moderate or severe motor cortex TDP-43 burden in this study. One limitation to our assessment of motor cortex pathology is that lateral tract degeneration, using LFB-stained or similar materials, was not considered, and this may have limited our assessment of more mild forms of UMN pathology.

C9orf72 expansion is associated with greater global TDP-43 burden (12, 14, 15), a more rapid clinical course, and cognitive impairment (43, 44). Based on these associations, we hypothesized that greater TDP-43 burden would be identified in the motor system of C9ALS patients, contributing to cell toxicity in UMNs and LMNs and reduced survival. Consistent with earlier studies, we found reduced survival in C9ALS patients. However, we did not identify any differences in motor system TDP-43 burden or in the severity of motor neuron loss. C9ALS patients with rapid progression, cognitive impairment and very mild TDP-43 burden have been described, demonstrating that other pathologies may mediate progression in this form of ALS (e.g. RNA foci, poly-GA and poly-GR aggregates). Additional studies are needed to examine the burden of C9ALS-related pathologies, including RNA foci, DPRs, TDP-43, and microglial activation, in the motor system of C9ALS patients to determine which of these pathologies correlates with progression speed. Our findings would suggest this is unlikely to be TDP-43 burden alone.

In conclusion, we found that TDP-43 pathologic burden in LMN pools, but not motor cortex, is associated with faster disease progression with reduced survival in ALS. We also found that LMN cell loss exceeds motor cortex cell loss in most ALS patients and identified a trend between severity of lumbar cord LMN pathology and lower extremity onset. Future studies are needed to examine whether clearance of misfolded TDP-43 in LMN pools and blocking of dysfunctional cellular responses activated by misfolded TDP-43 would help to slow the progression of disease.

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DATA AVAILABILITY

Data and/or supporting microscopic images from this study are available from the corresponding author upon reasonable request.

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