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Prolyl Isomerase Pin1 Expression in the Spinal Motor Neurons of Patients With Sporadic Amyotrophic Lateral Sclerosis

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Background and Purpose Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease. Selective deficiency of edited adenosine deaminase acting on RNA 2 (ADAR2), a key molecule in the acquisition of Ca^{2+} resistance in motor neurons, has been reported in sporadic ALS (sALS) spinal motor neurons. Since ADAR2 activity is positively regulated by prolyl isomerase Protein never in mitosis gene A interacting-1 (Pin1), a known phosphorylation-dependent peptidyl-prolyl cis/trans isomerase, we investigated Pin1 expression in spinal motor neurons in sALS.

Methods Specimens of the spinal cord were obtained from the lumbar region in eight sALS patients and age-matched five controls after postmortem examinations. The specimens were double stained with anti-Pin1 and anti-TAR DNA-binding protein of 43 kDa (TDP-43) antibodies, and examined under a fluorescence microscope.

Results This study analyzed 254 and 422 spinal motor neurons from 8 sALS patients and 5 control subjects, respectively. The frequency of motor neurons with high cytoplasmic Pin1 expression from the spinal cord did not differ significantly between sALS specimens without cytoplasmic TDP-43 inclusions and control specimens. However, in sALS specimens, neurons for which the Pin1 immunoluminescence intensity in the cytoplasm was at least twice that in the background were more common in specimens with cytoplasmic TDP-43 inclusions ($p<0.05$ in χ^2 test).

Conclusions In sALS, neurons with higher expression levels of Pin1 levels had more TDP-43 inclusions. Despite the feedback mechanism between Pin1 and ADAR2 being unclear, since Pin1 positively regulates ADAR2, our results suggest that higher Pin1 expression levels in motor neurons with TDP-43 pathology from sALS patients represent a compensatory mechanism.

Keywords ADAR2; amyotrophic lateral sclerosis; prolyl isomerase Pin1; spinal motor neuron; TDP-43.

INTRODUCTION

Elucidating the pathophysiology of neurodegenerative diseases is essential to developing appropriate treatments. However, the pathophysiology of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease, remains unclear, which restricts treatment options. Upper and lower motor neurons are affected in patients with ALS, and the degeneration of spinal lower motor neurons can cause respiratory failure and death. Cytoplasmic TAR DNA-binding protein of 43 kDa (TDP-43) inclusions have been described in the spinal motor neurons of sporadic ALS (sALS) patients,[1,2] and these inclusions are considered the main pathological feature of sALS. In addition, adenosine deaminase acting on RNA 2 (ADAR2) deficiency is specifically observed in the spinal motor neurons of sALS patients but not in either healthy normal or disease control patients.[3] ADAR2 regulates Ca^{2+} influx in motor neurons through

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α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors via adenosine-to-inosine (A-to-I) conversion at the glutamine/arginine (Q/R) site of glutamate-gated ion-channel-receptor subunit 2 (GluA2) mRNA.[4] Moreover, Q/R-site-unedited GluA2 mRNA is expressed in the motor neurons of sALS patients.[5-7] An analysis of ADAR2 conditional knockout mice (ADAR2$^{flox/flox}$/VAChT-Cre or AR2 mice) demonstrated that the failure of A-to-I conversion at this site causes the degeneration of spinal motor neurons and progressive limb weakness.[7] Motor neurons with decreased ADAR2 immunoactivity show TDP-43 pathology,[8] and ADAR2-deficient motor neurons exhibit abnormal TDP-43 localization in AR2 mice.[9] Together these findings indicate that ADAR2 reduction is a cause of neuronal death and TDP-43 pathology in the motor neurons of patients with sALS, and hence is an important pathophysiological feature in sALS. Protein never in mitosis gene A interacting-1 (Pin1), a phosphorylation-dependent peptidyl-prolyl cis/trans isomerase, is required for ADAR2 editing, nuclear localization, and stabilization.[10] Based on the above observations, we investigated the expression of Pin1—which positively regulates ADAR2 activity—in the spinal motor neurons of sALS patients and controls.

METHODS

We examined specimens from the lumbar region of the spinal cord obtained via postmortem examinations of patients with sALS and controls. Eight patients with sALS (6 males, 2 females; age 66.9±5.7 years; median age 68 years) and 5 controls (4 males, 1 female; age 73.0±4.6 years; median age 73 years) participated in this study. The participants' characteristics are listed in Table 1. Written informed consent was obtained from their families for performing autopsies and using their tissue specimens in research. The protocol of this research was approved by the Institutional Human Ethics Committee of Tokyo Medical University (No. 3374).

The obtained specimens were fixed in 10% neutral buffered formalin for approximately 7 days, embedded in paraffin, and then cut into 4-μm-thick serial sections that were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. To retrieve the antigen, the sections were heated in citrate buffer (pH 6.0) at 121°C for 10 min and then washed with phosphate-buffered saline (PBS). Normal serum (3:200 dilution of normal horse serum in PK-4002, Vector Laboratories, Burlingame, CA, USA) was applied at room temperature for 20 min to the sections, which were then incubated with a primary antibody overnight at 4°C. The following primary antibodies were used: mouse monoclonal anti-Pin1 (1:100 dilution of sc-46660, Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit polyclonal anti-TDP-43 (1:100 dilution of 10782-2-AP, Proteintech Group, Rosemont, IL, USA). The primary and secondary antibodies were diluted with an immunoreaction enhancer solution (Can Get Signal, NKB-601, TOYOBO, Osaka, Japan). The sections were subsequently washed with PBS and incubated with a secondary antibody at room temperature for 60 min. The following species-appropriate secondary antibodies were used: 1:1,000 dilution of Alexa Fluor 488 (A-11001, Thermo Fisher Scientific, Waltham, MA, USA) for Pin1, and Alexa Fluor 594 (A-11072, Thermo Fisher Scientific) for anti-TDP-43. The sections were also stained with 4', 6-diamidino-2-phenylindole (1:2,000 dilution of D1306, Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature for nuclear staining before being mounted onto slides with an antifade reagent (Fluoromount/Plus K048, Diagnostic Biosystems, Pleasanton, CA, USA).

Table 1. Characteristics of the participants who provided specimens for this study, all from the lumbar spinal cord

Case no.	Age at death (yr)	Sex	Diagnosis
A1	69	M	sALS
A2	69	M	sALS
A3	66	M	sALS
A4	59	M	sALS
A5	70	F	sALS
A6	76	F	sALS
A7	59	M	sALS
A8	67	M	sALS
C1	70	F	LGMD
C2	73	M	Theophylline intoxication
C3	77	M	LGMD
C4	78	M	Meningitis
C5	67	M	MyD

F, female; LGMD, limb-girdle muscular dystrophy; M, male; MyD, myotonic dystrophy; sALS, sporadic amyotrophic lateral sclerosis.

We examined the sections under a fluorescence microscope (BZ-X800, Keyence Corporation, Osaka, Japan) and analyzed the data using the analyzer provided with the microscope. Example images are shown in Fig. 1.

We counted the number of motor neurons in the anterior horn and measured the intensity of Pin1 immunoluminescence in each neuron. A hybrid cell counting technique provided by the BZ-X800 analyzer was used to detect the immunoluminescence intensities of Pin1 in the cytoplasm and background (Fig. 2). This hybrid cell counting technique allows quantification of immunoluminescence intensity on each pixel and provides the average intensity of an enclosed area. To examine Pin1 expression in the cytoplasm of spinal motor neurons, we extracted the cytoplasm areas and evaluated those cells with cytoplasms that were 100 μm^2 or larger. We also examined Pin1 expression in a selected background area that was 2,000 μm^2

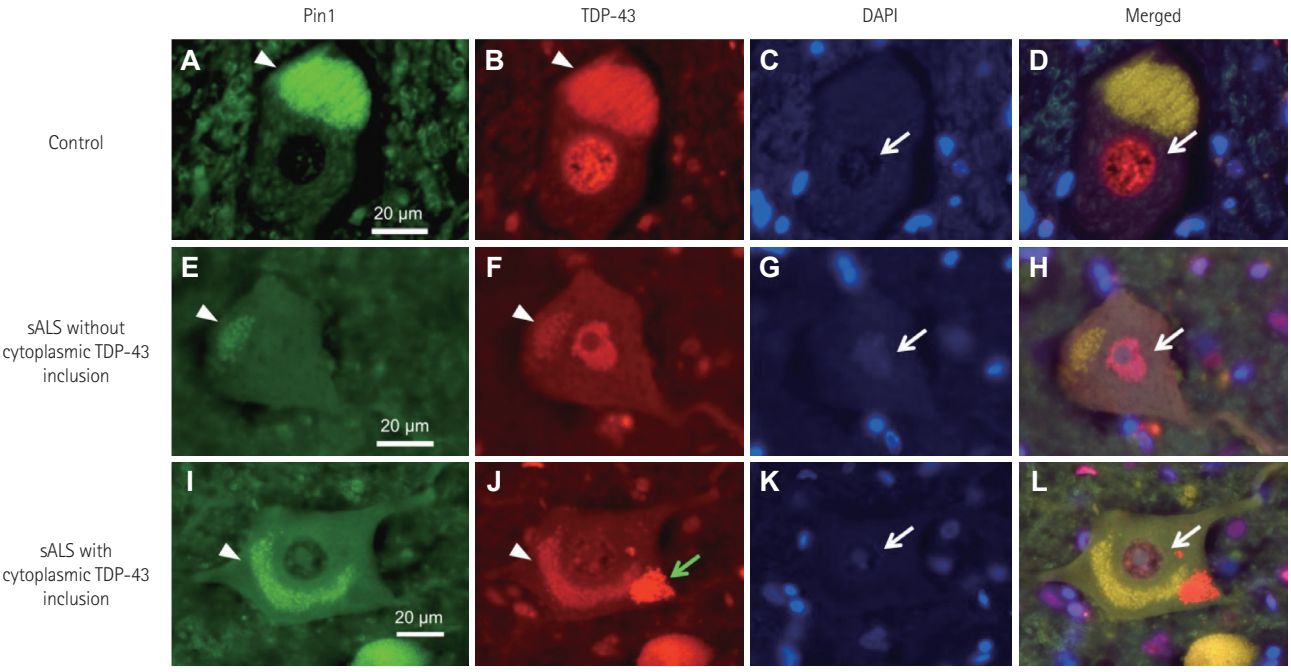

Fig. 1. Pin1 and TDP-43 expression levels in spinal motor neurons of controls and sALS patients. Spinal motor neurons from controls (A–D), in addition to those without (E–H) and with (I–L) cytoplasmic TDP-43 inclusions from sALS patients are shown. The neuron in panel I shows high Pin1 expression in the cytoplasm and is categorized as H-type. Conversely, the neurons shown in panels A and E were categorized as L-type (low Pin1 expression) (Green arrow, cytoplasmic TDP-43 inclusions; White arrows, nuclei; Arrowheads, lipofuscin). DAPI, 4', 6-diamidino-2-phenylindole; H-type, high-Pin1-expression neuron type; Pin1, protein never in mitosis gene A interacting-1; sALS, sporadic amyotrophic lateral sclerosis; TDP-43, TAR DNA-binding protein of 43 kDa.

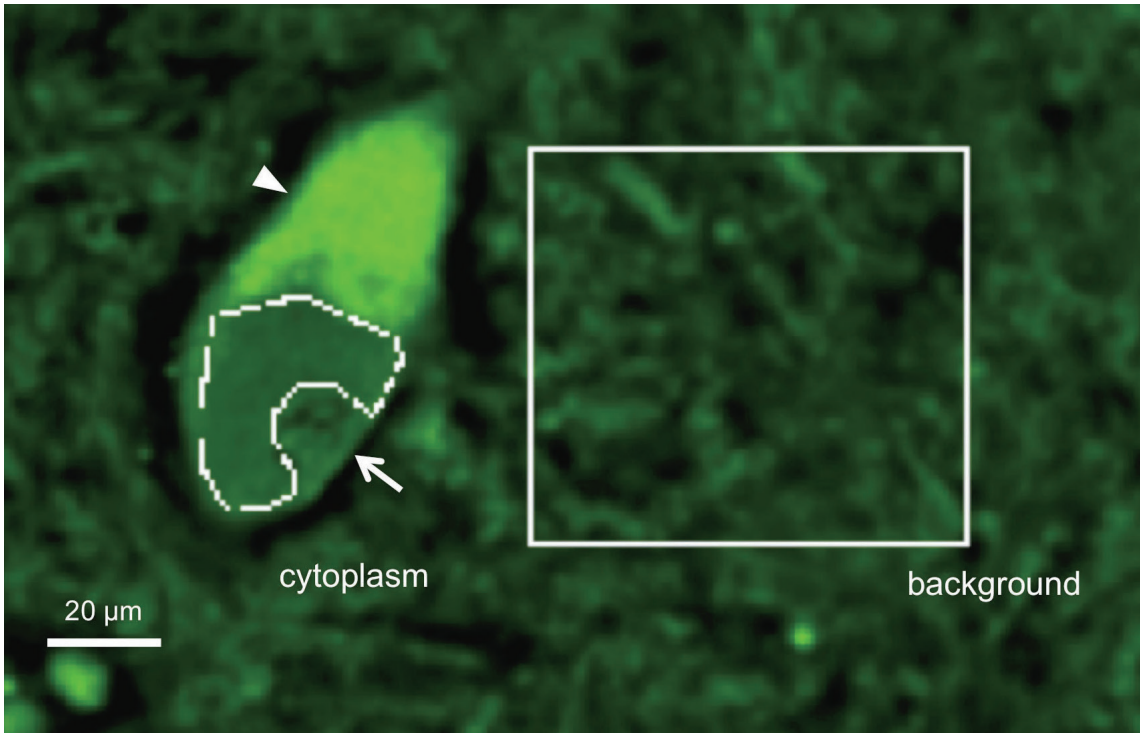

Fig. 2. Example image of Pin1 expression in the cytoplasm. We used a hybrid cell counting technique to detect Pin1 expression in the cytoplasm. Based on the intensities of Pin1 immunoluminescence in the cytoplasm and background, we used the BZ-X800 analyzer to classify the neurons into H-type and L-type cells. This image shows a spinal motor neuron with high Pin1 expression from a patient with sALS (Arrow, nucleus; Arrowhead, lipofuscin). H-type, high-Pin1-expression neuron type; L-type, low-Pin1-expression neuron type; Pin1, protein never in mitosis gene A interacting-1; sALS, sporadic amyotrophic lateral sclerosis.

or larger and did not contain spinal motor neurons. We subsequently classified each neuron into two categories according to Pin1 immunoluminescence intensity in the cytoplasm. Neurons for which the Pin1 immunoluminescence intensity in the cytoplasm was at least twice that in the background were classified as high-Pin1-expression neuron type (H-type), with other neurons classified as low-Pin1-expression neuron type (L-type). Since cytoplasmic TDP-43 inclusions are a pathological hallmark of sALS, we also examined the relationship between Pin1 expression and the presence of cytoplasmic TDP-43 inclusions in sALS motor neurons.

In addition to the Pin1 expression in spinal motor neurons obtained from controls and sALS patients, we performed immunohistochemical staining with anti-TDP-43 and anti-ADAR2 antibodies to investigate the correlation between the expression levels of TDP-43 and ADAR2 (Fig. 3). Formalin-fixed paraffin-embedded spinal cord sections from sALS patients and controls were double immunostained with anti-TDP-43 monoclonal antibody (1:1,000 dilution, Abnova, Taipei, Taiwan) and anti-ADAR2 antibody (1:100 dilution, Exalpha Biologicals, Watertown, MA, USA). The secondary antibodies used were labeled goat antirabbit IgG antibody (1:1000 dilution of Alexa 488, Molecular Probes, Eugene, OR, USA) and labeled goat antimouse IgG antibody (1:1000 dilution of Alexa 594, Molecular Probes, Eugene, OR, USA).

For statistical analyses, the χ^2 test or Mann-Whitney U test was used in SPSS Statistics software (version 27, IBM Corporation, Armonk, NY, USA). The significance criterion was set as a probability of 0.05% or lower.

RESULTS

The mean age did not differ significantly between sALS patients and controls in the Mann-Whitney U test. Totals of 254 and 422 spinal motor neurons were obtained from eight sALS patients and five control subjects, respectively. The number of spinal motor neurons in each spinal cord section was significantly lower for sALS patients (6.8±4.8 per section) than for controls (18.3±5.8 per section) (p<0.05 in Mann-Whitney U test).

Immunofluorescence analysis was used to assess the presence and localization of ADAR2 expression in the spinal motor neurons of sALS patients and controls. We observed ADAR2 expression in the cytoplasm and nucleus of spinal motor neurons obtained from controls. In sALS patients there was less ADAR2 expression in the spinal motor neurons with cytoplasmic TDP-43 inclusions (Fig. 3).

To clarify the relationship between the existence of cyto-

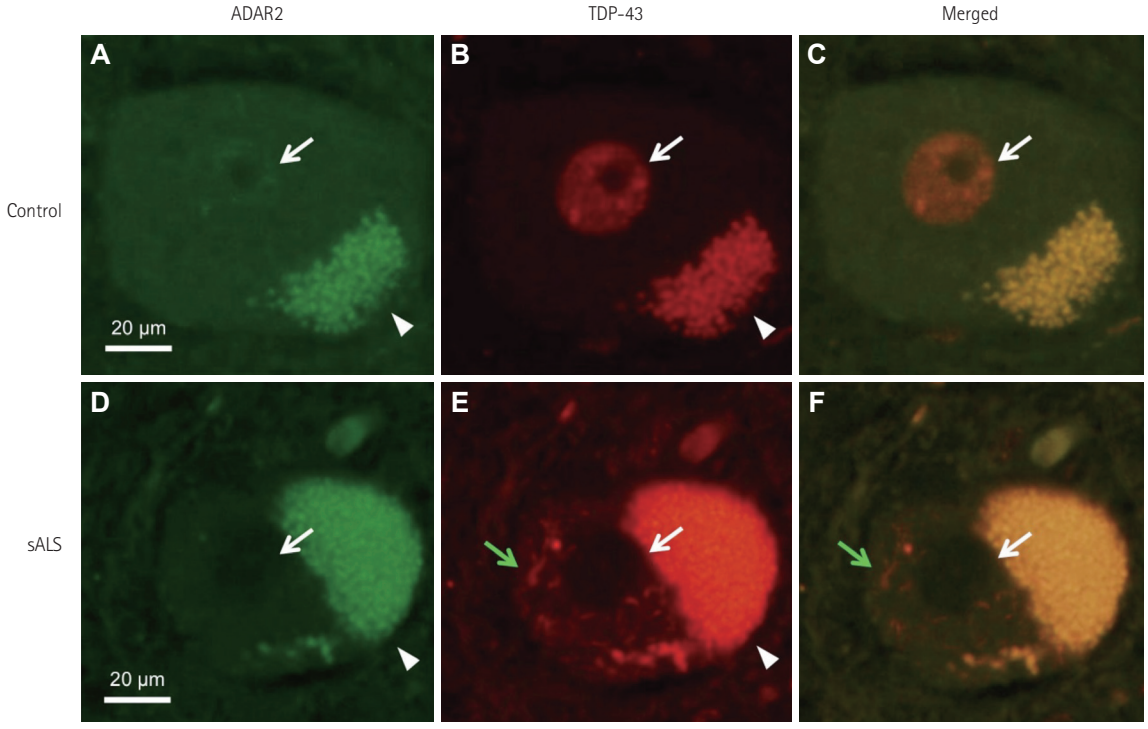

Fig. 3. ADAR2 and TDP-43 expression levels in spinal motor neurons of controls and sALS patients. Spinal motor neurons from controls (A–C) and sALS patients (D–F) are shown. ADAR2 was expressed in the spinal motor neurons obtained from controls. In contrast, ADAR2 expression was not observed in the spinal motor neurons with cytoplasmic TDP-43 inclusions obtained from sALS patients (Green arrows, cytoplasmic TDP-43 inclusions; White arrows, nuclei; Arrowheads, lipofuscin). ADAR2, adenosine deaminase acting on RNA 2; sALS, sporadic amyotrophic lateral sclerosis; TDP-43, TAR DNA-binding protein of 43 kDa.

plasmic TDP-43 inclusions and Pin1 expression in sALS motor neurons, we divided neurons into two groups: those with and without cytoplasmic TDP-43 inclusions. We calculated the H-type cell ratio in each participant as (number of H-type cells)/(total number of spinal motor neurons)×100. In sALS patients, we also calculated the H-type cell ratio according to the existence of cytoplasmic TDP-43 inclusions. We found variations in the frequencies of H-type spinal motor neurons in individual sALS patients and controls; in sALS, this tendency was seen in both spinal motor neurons with and without cytoplasmic TDP-43 (Table 2). However, H-type cells appeared to be more common among cells with cytoplasmic TDP-43 inclusions in sALS patients (Supplementary Fig. 1 in the online-only Data Supplement). Due to the relatively small number of participants in this study, the total values for spinal motor neurons from controls, spinal motor neurons without cytoplasmic TDP-43 inclusions from sALS patients, and spinal motor neurons with cytoplasmic TDP-43 inclusions from sALS patients were analyzed. We classified 85 (33.5%) neurons from sALS patients and 114 (27.0%) neurons from controls as H-type cells, and 169 (66.5%) neurons from sALS patients and 308 (73.0%) neurons from controls as L-type cells. The frequency of H-type cells did not significantly differ between sALS patients and controls in the χ^2 test.

We found that 29 (43.9%) and 37 (56.1%) neurons with cytoplasmic TDP-43 inclusions in sALS patients presented as H-type and L-type cells, respectively, as did 56 (29.8%) and 132 (70.2%) neurons without cytoplasmic TDP-43 inclusions, respectively. The frequency of H-type cells did not differ significantly between neurons without TDP-43 inclusions from sALS patients and neurons from controls. However, the frequency of H-type cells with cytoplasmic TDP-43 inclusions in sALS patients was higher than that of those without cytoplasmic TDP-43 inclusions in sALS patients ($p<0.05$ in χ^2 test) and controls ($p< 0.01$ in χ^2 test) (Fig. 4).

Table 2. H-type cell ratio of motor neurons from sALS patients and controls

Case no.	Spinal motor neurons with or without cytoplasmic TDP-43 inclusion	H-type cell ratio (%)*
A1	Without	87.0
	With	100.0
A2	Without	43.8
	With	75.0
A3	Without	100.0
	With	95.0
A4	Without	5.3
	With	0
A5	Without	100.0
	With	ND
A6	Without	6.3
	With	4.8
A7	Without	5.9
	With	12.5
A8	Without	11.1
	With	ND
C1	-	4.8
C2	-	48.6
C3	-	4.3
C4	-	80.0
C5	-	19.6

*Varying frequencies of H-type spinal motor neurons were found in case-by-case comparisons.
H-type, high-Pin1-expression neuron type; ND, not detected; Pin1, protein never in mitosis gene A interacting-1; sALS, sporadic amyotrophic lateral sclerosis; TDP-43, TAR DNA-binding protein of 43 kDa.

DISCUSSION

This study found that the level of Pin1 expression was higher in spinal motor neurons with cytoplasmic TDP-43 inclusions in sALS patients than in those without cytoplasmic TDP-43 inclusions in sALS patients and controls. In sALS spinal cord specimens, more motor neurons with high Pin1 expression levels were observed with cytoplasmic TDP-43 inclusions, indicating that this occurs predominantly in degenerating motor neurons. We found that the H-type cell ratio of spinal motor neurons varied between the participants (Table 2, Supplementary Fig. 1 in the online-only Data Supplement). Since the degree of degeneration of spinal motor

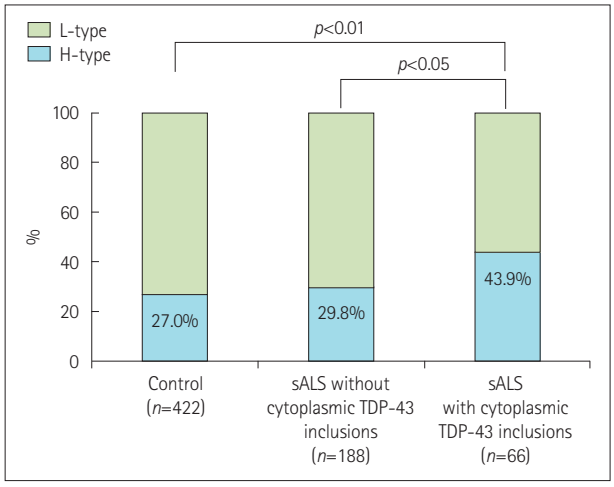

Fig. 4. Pin1 expression in specimens from controls and sALS patients. The frequency of H-type cells with cytoplasmic TDP-43 inclusions in sALS patients was higher than that of those without cytoplasmic TDP-43 inclusions in both sALS patients ($p < 0.05$ in χ^2 test) and controls ($p<0.01$ in χ^2 test). H-type, high-Pin1-expression neuron type; L-type, low-Pin1-expression neuron type; Pin1, protein never in mitosis gene A interacting-1; sALS, sporadic amyotrophic lateral sclerosis; TDP-43, TAR DNA-binding protein of 43 kDa.

neurons varies between individual sALS patients, and there were relatively few participants in this study, the total number of spinal motor neurons in each group was used for the analysis.

Spinal motor neurons with TDP-43 pathology invariably lose ADAR2 expression,[8] and Pin1 is reportedly required to stabilize ADAR2 and exert its editing activity.[10] Since spinal motor neurons in sALS patients present varying degrees of TDP-43 pathological changes, higher Pin1 expression levels in motor neurons with TDP-43 pathology from sALS patients may represent a compensatory mechanism, although the existence of a feedback mechanism between Pin1 and ADAR2 has not been demonstrated.

Pin1 is an enzyme that catalyzes peptide binding[11] and can affect the cell cycle, motility, and apoptosis of neurons.[12] Pin1 is expressed on neurons and also during neurodevelopmental stages.[13] The role of Pin1 has been investigated in several neurodegenerative diseases. In Alzheimer's disease, Pin1 expression depends on the presence of neurofibrillary tangles.[14,15] Since pyramidal cells without neurofibrillary tangles tended to exhibit higher levels of Pin1 expression, a neuroprotective effect of Pin1 has been speculated.[14] Moreover, Pin1 could facilitate the formation of alpha-synuclein inclusions of Lewy bodies in Parkinson's disease, and thus may be a factor in the pathogenesis of Parkinson's disease.[16] Iridoy et al.[17] recently found using proteomic analysis that Pin1 was one of the proteins associated with ALS. They also noted that Pin1 was downregulated in the anterior horn of the spinal cord in ALS patients compared with in controls, based on Southern blotting. We found that spinal motor neurons with cytoplasmic TDP-43 inclusions in sALS exhibited higher Pin1 expression levels than did those without cytoplasmic TDP-43 inclusions in the neurons of sALS patients and controls. Considering the observations of Iridoy et al.[17] and our findings related to sALS, although overall Pin1 activity may decrease in the anterior horn of the spinal cord, the reduction might be smaller in spinal motor neurons with TDP-43. Since high Pin1 expression was observed more frequently in TDP-43-positive neurons than in TDP-43-negative neurons, from a viewpoint of ADAR2 downregulation, Pin1 may exert a neuroprotective effect in spinal motor neurons in sALS.

TDP-43 pathology is also found in patients with frontotemporal lobar degeneration.[1,2] However, we could not determine if the relationship between Pin1 and TDP-43 pathology is common or specific to sALS, and so such an investigation is warranted in the future.

In conclusion, among sALS patients, TDP-43 inclusions were more common in spinal motor neurons with higher Pin1 expression levels. Although the existence of a feedback mechanism between Pin1 and ADAR2 has not been demonstrated, since Pin1 regulates ADAR2 positively, our results suggest that higher Pin1 expression levels in motor neurons with TDP-43 pathology from sALS patients act as a compensatory mechanism. Therefore, high Pin1 expression levels in spinal motor neurons with cytoplasmic TDP-43 inclusions may be closely related to the pathophysiology of sALS.

Supplementary Materials

The online-only Data Supplement is available with this article at https://doi.org/10.3988/jcn.2022.18.4.463.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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