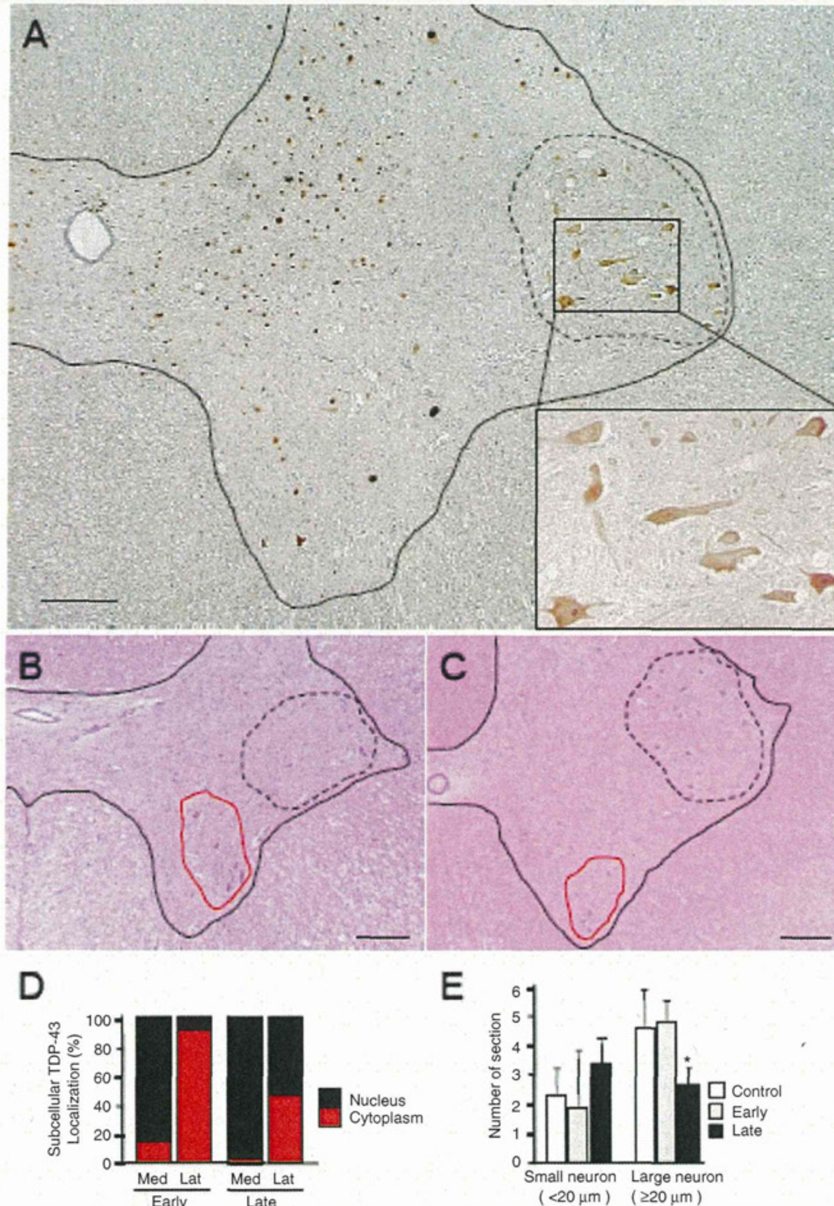

Figure 4 Regional mislocalization of TDP-43 and cell death in monkey spinal cords. (**A**) Sections from the injected side of the eighth cervical segment of the cord taken at the early stage and immunostained with an anti-Flag antibody. Most neurons in the lateral nuclear group (area encircled by broken line) showed cytoplasmic mislocalization of TDP-43 (inset), but almost all neurons in other areas expressed exogenous TDP-43 in the nucleus. Scale bars: 200 μm. (**B** and **C**) The eighth cervical level of cord from monkeys injected with TDP-43–expressing (**B**) and control (**C**) AAV, taken at the late stage and stained with haematoxylin and eosin. The number of large motoneurons decreased in the lateral nuclear group (areas encircled by broken line), but not in ventromedial nuclear group (areas encircled by red solid line). Scale bars: 200 μm. (**D**) Percentage of neurons with nuclear (black) or cytoplasmic (red) localization of exogenous TDP-43 in the lateral nuclear groups on the injected side. Neurodegeneration affects the lateral nuclear group more than the ventromedial nuclear group. (**E**) Cell count of neurons in the lateral nuclear group on haematoxylin and eosin staining. Mean ± SEM. $n = 3$, $*P < 0.05$. Lat = lateral nuclear group; Med = ventromedial nuclear group.

diagnosis of ALS and FTLD (Geser *et al.*, 2010). The classification of TDP-43 proteinopathy is based on a combination of neuronal cytoplasmic inclusions and dystrophic neurites (Mackenzie *et al.*, 2011). The morphological features in our monkeys are close to type B TDP-43 proteinopathy, which is usually observed in the brains of patients with ALS. The only difference between the pathology of our monkeys and type B TDP-43 proteinopathy is that mislocalized cytoplasmic TDP-43 was usually diffuse and neuronal cytoplasmic inclusions were less frequent in our monkeys. Since this monkey is an acute model for TDP-43 pathology, it possibly

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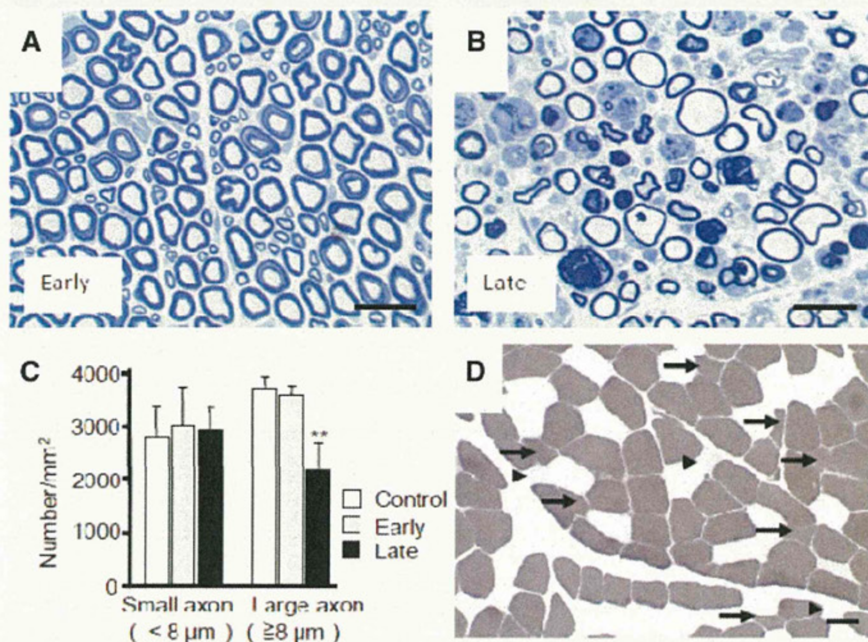

Figure 5 Pathological finding of monkey anterior root and skeletal muscle. Toluidine blue staining of the eighth cervical anterior roots on the injected side in the early (**A**) and late (**B**) stages, and their myelinated axon densities (**C**). Mean ± SEM, $n = 3$, $^{**}P < 0.01$. (**D**) Transverse section of the biceps brachii muscle from a TDP-43–expressing monkey 4 weeks after injection, stained with ATPase (pH 10.6). Small angulated atrophic changes of type I (arrowheads) and type II (arrows) fibres, with predominant involvement of type II fibres, can be seen. Scale bar: 50 μm.

takes more time for diffusely mislocalized TDP-43 to be aggregated. Moreover, in the spinal cords of patients with ALS, diffuse cytoplasmic TDP-43 staining is more common, and neuronal cytoplasmic inclusions are less frequent than in the brain and may even be absent (Giordana *et al.*, 2010). Thus, our monkey model shows the key features of TDP-43 proteinopathy as seen in the ALS spinal cord.

Interestingly, despite the diffuse expression of exogenous TDP-43 in the spinal cord, TDP-43 mislocalization and neuron loss predominantly occurred in the lateral nuclear group in Rexed lamina IX, in which large neurons are mostly α-motoneurons (Carpenter *et al.*, 1983). The sensory neurons and interneurons in laminae III–VIII rarely showed TDP-43 mislocalization, and large motoneurons in the ventromedial nuclear group, most of which are also α-motoneurons, showed much less TDP-43 mislocalization and neuron loss. Within lamina IX, the lateral nuclear group innervates the distal, fast-contracting muscles of the extremities, and the ventromedial nuclear group innervates the posture-related, continuously contracting muscles attached to the axial skeleton (Carpenter *et al.*, 1983). This regional vulnerability among α-motoneurons is consistent with the distal hand or foot muscles being the first involved in 73% of patients with non-bulbar ALS (Harverkamp *et al.*, 1995; Körner *et al.*, 2011) and might be related to axon length, which affects axonal transport (Bilsland *et al.*, 2010), or to the preferential susceptibility of fast-fatigue rather than slow motoneurons (Dengler *et al.*, 1990; Pun *et al.*, 2006). Furthermore, in nine patients with ALS, more TDP-43 mislocalization was observed in the lateral nuclear group than in the ventromedial nuclear group of the eighth cervical cord segments. Taking these results together, we think that the tropism of TDP-43 mislocalization was similar to that of ALS pathology. However, expression levels of exogenous wild-type TDP-43 in our monkey and rat models were very high (~20-fold higher than that of endogenous TDP-43), which was partly due to lack of 3′-untranslated region in our TDP-43 expression construct. This is probably because TDP-43 controls its own expression through a negative feedback loop by binding to 3′-untranslated region sequences in its own messenger RNA (Ayala *et al.*, 2010; Polymenidou *et al.*, 2010). The unphysiologically high level of TDP-43 expression in our animal models should be taken into consideration when interpreting our findings.

Since Flag TDP-43 messenger RNA was detected in the spinal cord contralateral to the injected side by real-time polymerase chain reaction analysis, the AAV virus was shown to spread contralaterally through the spinal cord causing motor paresis and reduction of compound muscle action potential size in the opposite forelimb. However, it is still possible that there was concomitant cell-to-cell or trans-synaptic propagation of Flag TDP-43 protein in the spinal cord. Moreover, it is interesting that the Flag-TDP-43 signal was selectively extended into Betz cells in the forelimb area of precentral gyrus contralateral to the injection side, which can be explained by a retrograde progression from α-motoneuron in the cervical cord. More sophisticated experimental paradigms are necessary to distinguish whether it is the AAV vector itself,

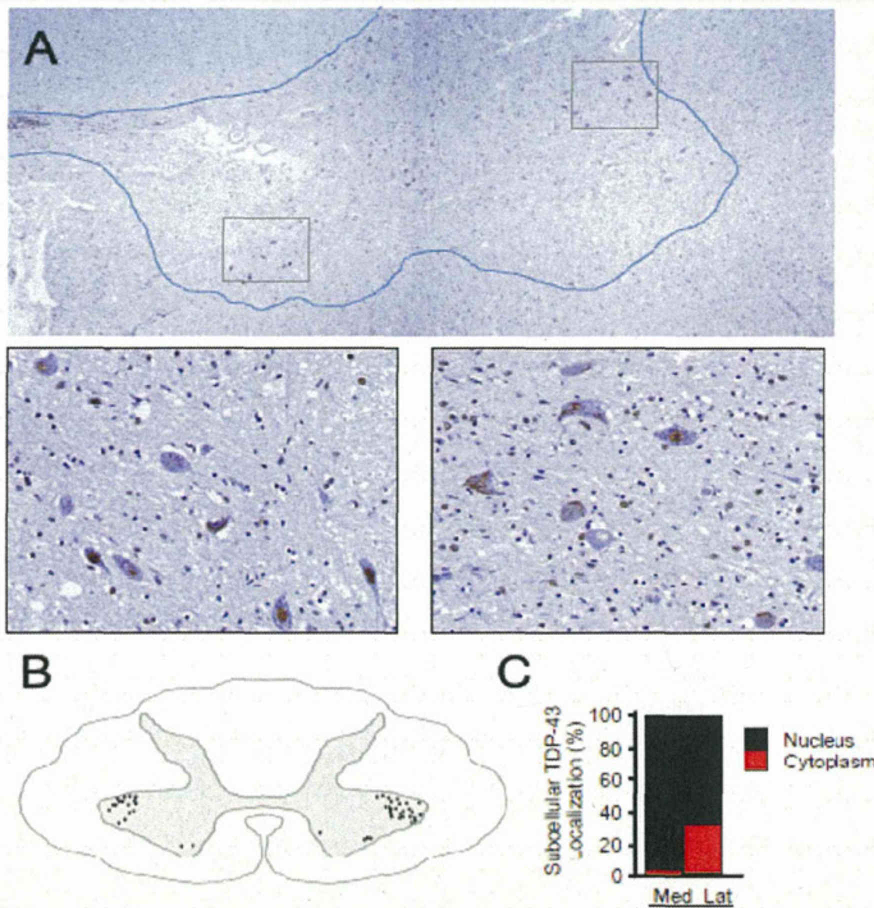

Figure 6 Pan-TDP-43 staining of spinal cords of patients with ALS. (**A**) Autopsied eighth cervical segment of spinal cord immunostained with pan-TDP-43 antibody. Scale bars: 200 μm in **A** and 50 μm in window insets. (**B**) Schematic illustration of the distribution of neurons with TDP-43 mislocalization, made by summing data from five sections (at 20-μm intervals). (**C**) Percentage of neurons with nuclear or cytoplasmic localization of exogenous TDP-43 in the lateral and medial nuclear groups. More frequent TDP-43 mislocalization in the lateral nuclear group in spinal cords of patients with ALS than in the medial nuclear group. Mean ± SEM, $n = 10$, $P < 0.01$. Scale bars: 50 μm. Lat = lateral nuclear group; Med = ventromedial nuclear group.

transcribed messenger RNA or Flag-TDP protein that is the molecule responsible for this progression, which is a prime objective for our future study.

Bunina bodies are small, cystatin C-positive, eosinophilic cytoplasmic inclusions and are generally considered a specific hallmark of sporadic ALS (Okamoto *et al*., 1993; Mitsumoto *et al*., 1998). Importantly, Bunina bodies are absent in familial ALS that is due to the SOD1 mutation (Tan *et al*., 2007) or FUS/TLS mutation (Tateishi *et al*., 2010), but they have been detected in familial ALS with the TDP-43 mutation (Yokoseki *et al*., 2008) as well as in sporadic ALS. These imply an association between Bunina bodies and TDP-43 pathology in sporadic ALS. From this point of view, the generation of cystatin C-positive cytoplasmic aggregates in our monkeys might strengthen their pathological value as a model of sporadic ALS.

Biochemically, TDP-43 proteinopathy is characterized by decreased solubility, phosphorylation and the generation of 25-kDa C-terminal fragment (Arai *et al*., 2006; Neumann *et al*., 2006; Hasegawa *et al*., 2008). In TDP-43-overexpressing monkeys, the exogenous TDP-43 became much more insoluble than endogenous TDP-43 of control monkeys, indicating that expression of large amounts of exogenous wild-type TDP-43 can render it insoluble. Unexpectedly, the solubility of endogenous monkey TDP-43 did not become insoluble in TDP-43-overexpressing monkeys. The expectation would be that exogenous insoluble TDP-43 would recruit endogenous monkey TDP-43 and alter its solubility. In this biochemical aspect of TDP-43 solubility, our monkey model differs from patients with ALS.

The pathological role of phosphorylated TDP-43 is still unclear; TDP-43 phosphorylation in culture cells enhances its oligomerization (Hasegawa *et al*., 2008), but experiments with a phosphorylation-resistant mutant TDP-43 indicated that phosphorylation is not required for inclusion formation or cellular toxicity (Zhang *et al*., 2009). In our monkeys, phosphorylation of TDP-43 was a late event but not observed at 4 days after symptom onset. This finding suggests that TDP-43 phosphorylation is not

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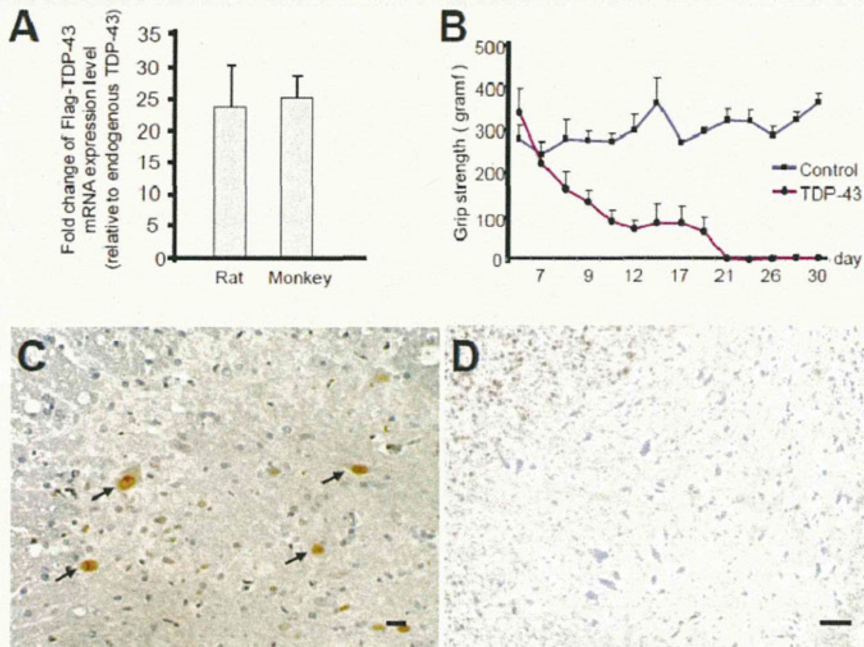

Figure 7 Effect of TDP-43-expressing AAV in rat spinal cords. (**A**) Ratio of exogenously expressed Flag-TDP-43 messenger RNA level to endogenous rat or cynomolgus TDP-43 messenger RNA level evaluated by quantitative real-time polymerase chain reaction. Mean ± SEM, rat, $n = 4$; cynomolgus, $n = 3$, $P = 0.74$. (**B**) Time course of grip strength. Mean ± SEM. (**C**) Nuclear staining of exogenous TDP-43 in cervical cord sections of AAV-injected rats by immunostaining with an anti-Flag antibody (arrows). (**D**) Immunostaining of cervical cord sections of TDP-43–expressing rat, 4 weeks after injection, with SMI31 did not show aberrant phosphorylated neurofilament in the neuronal cytoplasm. Scale bars: 20 μm.

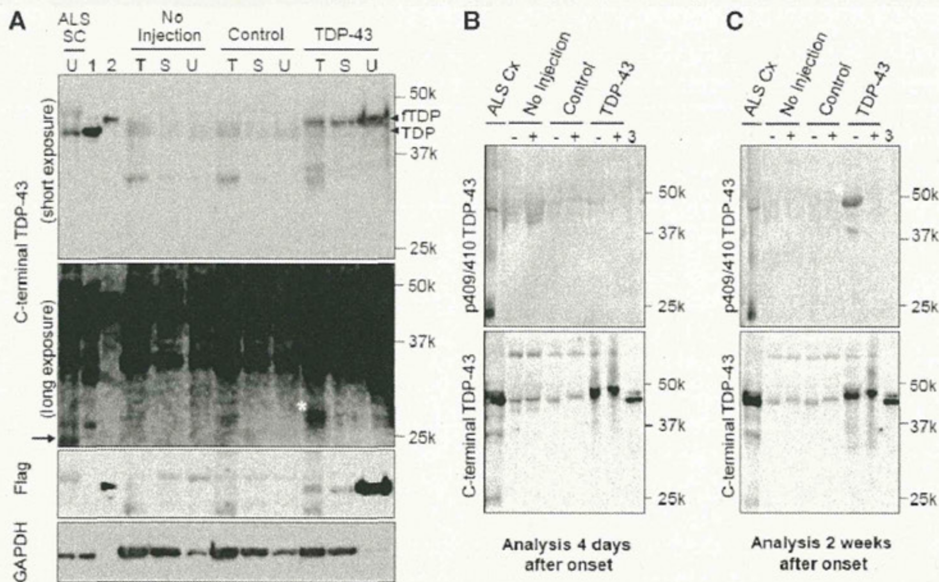

Figure 8 Biochemical analysis of monkey spinal cords. (**A**) Immunoblot of cervical spinal cord lysates from TDP-43–expressing monkeys (4 weeks after injection) and patients with ALS using antibodies recognizing the C-terminus of TDP-43 and Flag. SC = spinal cord of patient with ALS; 1 = TDP-43–expressing HEK 293 T cell lysate; 2 = Flag-TDP-43–expressing HEK 293T cell lysate; T = 1% Triton X-100-soluble; S = 1% sarkosyl-soluble; U = 8 mol/l urea-soluble fraction; fTDP = Flag-TDP-43. A longer exposure (second panel from *top*) revealed the 25-kDa C-terminal fragment in the spinal cord of a patient with ALS (arrow). The ~30-kDa band noted in the Triton-soluble fraction from the spinal cord of a TDP-43–expressing monkey (asterisk) was different from the 25-kDa C-terminal fragment (arrow). (**B** and **C**) Immunoblot of 8 mol/l urea-soluble fraction from the monkey spinal cord harvested 4 days (**B**) and 2 weeks (**C**) after onset of symptoms, using antibodies to pS409/410 TDP-43 (*top*) and C-TDP-43 (*bottom*) before (−) and after (+) treatment with lambda protein phosphatase (λPPase). 3 = Mixture of Flag-TDP-43- and TDP-43-expressing HEK 293T cell lysates. The phosphorylated TDP-43 was detected only in the late stage (asterisk).

necessary to initiate motor symptoms and is a late event in motoneuron degeneration.

In the spinal cords of our monkeys, neither a C-terminal nor a phosphospecific TDP-43 antibody detected the 25-kDa C-terminal fragment that is found in patients with ALS. Overexpressed 25-kDa C-terminal fragment in cultured cells is reported to be toxic (Igaz *et al.*, 2009; Zhang *et al.*, 2009), and the accumulation of 25-kDa C-terminal fragment in transgenic mouse brain correlates with disease progression (Xu *et al.*, 2010). Interestingly, unlike the FTLD/ALS brain, the 25-kDa C-terminal fragment is often absent in the ALS spinal cord (Neumann *et al.*, 2009). The absence of 25-kDa C-terminal fragment in ALS spinal cord does not necessarily preclude a primary role for this form; rather it can be pathologically crucial if its absence is due to the accelerated degeneration of motoneurons with 25-kDa C-terminal fragment. It is difficult to deny that small amounts of C-terminal truncated species are actually present, because mislocalization of TDP-43 was focal in the spinal cord of our monkeys. However, the failure to detect 25-kDa C-terminal fragment in our monkey spinal cord at the early stage may have an implication that full-length TDP-43 is sufficient to be toxic, because α-motor axonal excitability was impaired but their cell bodies were preserved at autopsy.

The results of studies on the relationship between TDP-43 mislocalization and neuron loss remain controversial. The overexpression of wild-type TDP-43 in the nuclei in a transgenic rodent model was sufficient to be toxic to spinal motoneurons (Li *et al.*, 2010; Shan *et al.*, 2010; Wils *et al.*, 2010; Xu *et al.*, 2010), which is consistent with our observations in the rat model. In this context, it can be interpreted that the cytoplasmic mislocalization of wild-type TDP-43 is an epiphenomenon and not a necessary condition for the disease. However, in our monkey model, TDP-43 mislocalization was detected in almost all of the large motoneurons of the lateral nuclear group at the early or even the presymptomatic stage, and these motoneurons later showed neuron loss. In contrast, overexpressed exogenous TDP-43 in the large motoneurons of the ventromedial nuclear group was restricted to the nucleus, but did not produce neuron loss. Mice with over-expression of human TDP-43 engineered to localize in the cytoplasm showed progressive neuronal loss and downregulation of endogenous nuclear mouse TDP-43 expression (Igaz *et al.*, 2011). These suggest that TDP-43 mislocalization is an upstream event in the cascade of motoneuronal degeneration. This finding is consistent with the observation that the highest percentage of neurons with TDP-43 mislocalization was found in the early stage of ALS in patients (Giordana *et al.*, 2010).

In conclusion, our monkey model is superior to rodent models in recapitulating the TDP-43 pathology and in the presence of Bunina body-like inclusion and is expected to be a powerful tool for investigating developing effective therapies as well as the disease pathogenesis of sporadic ALS.

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Supplementary material

Supplementary material is available at *Brain* online.

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Alteration of *POLDIP3* Splicing Associated with Loss of Function of TDP-43 in Tissues Affected with ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease caused by selective loss of motor neurons. In the ALS motor neurons, TAR DNA-binding protein of 43 kDa (TDP-43) is dislocated from the nucleus to cytoplasm and forms inclusions, suggesting that loss of a nuclear function of TDP-43 may underlie the pathogenesis of ALS. TDP-43 functions in RNA metabolism include regulation of transcription, mRNA stability, and alternative splicing of pre-mRNA. However, a function of TDP-43 in tissue affected with ALS has not been elucidated. We sought to identify the molecular indicators reflecting on a TDP-43 function. Using exon array analysis, we observed a remarkable alteration of splicing in the polymerase delta interacting protein 3 (*POLDIP3*) as a result of the depletion of TDP-43 expression in two types of cultured cells. In the cells treated with TDP-43 siRNA, wild-type *POLDIP3* (variant-1) decreased and *POLDIP3* lacking exon 3 (variant-2) increased. The RNA binding ability of TDP-43 was necessary for inclusion of *POLDIP3* exon 3. Moreover, we found an increment of *POLDIP3* variant-2 mRNA in motor cortex, spinal cord and spinal motor neurons collected by laser capture microdissection with ALS. Our results suggest a loss of TDP-43 function in tissues affected with ALS, supporting the hypothesis that a loss of function of TDP-43 underlies the pathogenesis of ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease accompanied by loss of motor neurons. A pathological hallmark of ALS is cytoplasmic inclusions in neurons and glia consisting of TAR DNA-binding protein of 43 kDa (TDP-43) [1,2,3]. Although TDP-43-positive inclusions have been identified in several neurodegenerative disorders, the discovery of mutations in the *TARDBP* gene in patients with familial and sporadic ALS (OMIM 612069; ALS10) indicates that an alteration of TDP-43 causes selective motor neuron degeneration [4,5]. The similarity between the pathological findings in ALS10 and sporadic ALS indicate that the alteration of TDP-43 may play an important role in the pathogenesis of sporadic ALS [3,6]. However, the way in which the molecular mechanism of this alteration of TDP-43 causes ALS is still obscure.

TDP-43 is a ubiquitously expressed nuclear protein and the clearance of nuclear TDP-43 in the affected neurons and glia is another pathological hallmark of ALS, suggesting that a loss of function of TDP-43 may underlie the pathogenesis of the ALS [2,3,7]. TDP-43 has two RNA recognition motifs (RRM1 and RRM2) and has been speculated as a heterogeneous nuclear ribonucleoprotein (hnRNP) [8,9]. TDP-43 binds to hnRNPs and functions in RNA metabolism through regulation of transcription, mRNA stability, and alternative splicing of pre-mRNA [10,11]. TDP-43 takes an important role for cell proliferation, neurite outgrowth and neuronal cell viability [12,13]. In animal models, several lines of evidence have revealed that TDP-43 is essential for normal embryogenesis, ES survival and takes an important role for motor neuron function [14,15,16,17]. However, a function of TDP-43 in the human tissues has not been well evaluated.

Controversy centers on whether a loss of TDP-43 function or an adverse effect of inclusions of TDP-43 results in motor neuron death in ALS [7]. The disappearance of nuclear TDP-43 in the affected motor neurons with TDP-43 inclusions supports the hypothesis that a loss of TDP-43 function may result in the motor neuron death in ALS [1,2,18]. However, TDP-43 function in tissues affected with ALS has not been evaluated. To explore the possibility that a loss of TDP-43 function may result in the motor neuron death in ALS, a function of TDP-43 in tissues affected with ALS should be elucidated. In this report, we first attempted to

identify the molecular indicators of a function of TDP-43 in human cells, with special attention to pre-mRNA splicing. Then we evaluated a function of TDP-43 in the affected tissues with ALS by using the identified molecular indicator, splicing variants of *POLDIP3*.

Results

Comprehensive screening of TDP-43 functions in pre-mRNA splicing

For investigating the function of TDP-43 in several tissues, ideal molecular indicators are the genes that are ubiquitously expressed and influenced by the depletion of TDP-43. TDP-43 has been reported to bind the intronic or noncoding sequences, which are markedly different between species, and affect pre-mRNA splicing [19,20]. Therefore we attempted to identify the genes which are influenced by TDP-43 by comparing the results of two differential human cells, HeLa and SH-SY5Y. siRNA treatment completely suppressed the expression of TDP-43 in HeLa cells, in contrast to <30% in SH-SY5Y cells compared to control siRNA (Fig. 1A). We obtained the cDNAs from mRNA of these cells and applied them to GeneChip Human Exon 1.0 ST Arrays, subsequently identifying genes that are significantly altered in their splicing and amount of expression. We found 892 genes in HeLa cells and 103 genes in SH-SY5Y cells that altered the pattern of splicing under the depletion of TDP-43 (Fig. 1B). In contrast, we found 123 genes, 98 genes upregulated and 25 genes down regulated, in HeLa cells and 10 genes, 3 genes upregulated and 7 genes downregulated, in SH-SY5Y cells in which the amounts of mRNA were significantly altered more than 2-fold by TDP-43 siRNA compared to control siRNA.

POLDIP3 exon 3 is excluded by depletion of TDP-43

Among the genes that altered their splicing, we found that only 15 genes overlapped between both cell lines (Fig. 1B and Table 1). By comparing the results of the exon array to the exon structure information in the Ensemble (http://asia.ensembl.org/index.html), we found candidate exon cassettes in six genes (polymerase delta interacting protein 3 [*POLDIP3*]; methyl malonic aciduria cblB type [*MMAB*]; heterochromatin protein 1, binding protein 3 [*HP1BP3*]; glutaminase [*GLS*]; stimulated by retinoic acid gene 6 homolog [*STRA6*]; and dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit [*DPM2*]) for further experiments to validate the exon array results (Fig. 1B and Table 1). Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis using primers specific for splicing variants revealed that only the *POLDIP3* and *HP1BP3* genes showed altered splicing consistent with the results of an exon array in both HeLa cells and in SH-SY5Y cells (Fig. 1C and D, Fig. S1A and B).

We were interested in *POLDIP3* because *POLDIP3* showed the highest fold change by qRT-PCR in both cell lines (Fig. 1E). The mean gene-normalized probe-set signals for *POLDIP3* revealed the lack of exon 3 as a result of the depletion of TDP-43 (Fig. 2A). RT-PCR analysis using primers spanning exon 3 revealed that the cells treated with TDP-43 siRNA showed only one product; subsequent sequence analysis showed that it was from the transcripts excluding *POLDIP3* exon 3 (Fig. 2B and C). We designated the *POLDIP3* gene lacking exon 3 as *POLDIP3* variant-2 and wild-type as variant-1.

We also quantified the amounts of *POLDIP3* splicing variants mRNA in various human cultured cells, HeLa, SH-SY5Y, and U87-MG, by qRT-PCR using primers that specifically amplified each splice variant. Under treatment with TDP-43 siRNA, the total amount of *POLDIP3* in these cells was not significantly altered, whereas variant-1 was significantly reduced to <5% and variant-2 was increased to more than 4 to 6 times compared to the control (Fig. 2D and E). Similar results were obtained using two other mixtures of TDP-43 siRNAs (Fig. 2F and G).

We then performed Western blot analysis using anti-POLDIP3 antibodies that predominantly react with POLDIP3 variant-1 or POLDIP3 variant-2 (Fig. 2H). In the cells treated with TDP-43 siRNA, POLDIP3 variant-1 decreased and variant-2 increased remarkably (Fig. 2I). Next, we performed immunohistochemical analysis using POLDIP3 variant-1 antibody. This antibody clearly showed immunoreactivity for POLDIP3 variant-1 in the nucleus in cells with TDP-43 as previously reported [21] (Fig. 2J). However, the immunoreactivity for nuclear POLDIP3 variant-1 was decreased in the cells with decreased TDP-43 expression (Fig. 2J).

RNA binding ability of TDP-43 is necessary for including *POLDIP3* exon 3

To investigate whether TDP-43 directly regulates the inclusion of exon 3 of *POLDIP3*, we first examined whether TDP-43 binds to the *POLDIP3* mRNA by RNA-immunoprecipitation (RIP) assay using TDP-43 antibody. *POLDIP3* mRNA was amplified in the material immunoprecipitated with anti-TDP-43 antibody (Fig. 3A, lane 4, top panel).

Next, we applied a supplementation assay by introducing the exogenous mutant TDP-43 in the cell depleted of endogenous TDP-43 by using siRNA targeted to the 3′-UTR of *TDP-43*. TDP-43 has two RNA recognition motifs (RRM1 and RRM2) and the substitutions of F147L and F149L in the RRM1 motif decreased the binding ability to RNA, but the substitutions of F194L, F229L, and F231L in the RRM2 motif did not (Fig. 3B) [8]. We treated the HeLa cells by siRNA targeted to the 3′-UTR of *TDP-43* then transfected the various TDP-43 cDNAs and quantified the amount of *POLDIP3* variants by real-time qRT-PCR. Although exogenous expression of TDP-43 wild-type and each mutant did not affect the expression of *POLDIP3* total and variant-2 mRNA (Fig. 3E and F), wild-type TDP-43 restored the amount of *POLDIP3* variant-1 (Fig. 3C and D, lane 3). In contrast, TDP-43 RRM1 mutant, but not RRM2 mutant, failed to restore the amount of *POLDIP3* variant-1 (Fig. 3D, lanes 4 and 5). These results suggest that TDP-43 includes exon 3 of *POLDIP3* by binding to its pre-mRNA.

Alteration of *POLDIP3* splicing in the CNS of patients with ALS

To investigate the function of TDP-43 in tissues affected with ALS, we analyzed the amount of each *POLDIP3* splicing variant mRNA. We isolated total RNAs from the thalamus, motor cortex and spinal cord of patients with ALS and control individuals (detailed information about subjects is summarized in Table S1) and quantified the amounts of mRNA of *POLDIP3* splicing variants using real-time qRT-PCR. The total amounts of *POLDIP3* and *POLDIP3* variant-1 mRNA were not significantly different between control and ALS tissues (Fig. 4A). In contrast, the amount of *POLDIP3* variant-2 was significantly increased in ALS tissues compared with controls (Fig. 4A). An increase of *POLDIP3* variant-2 mRNA was also observed in the thalamus, which has not been thought to be involved in ALS. However, consistent with the previous report, TDP-43 pathology was observed in four out of six ALS cases used in this experiment [22] (Fig. S2).

Next we investigated the amounts of *POLDIP3* mRNA in the spinal motor neurons of patients with ALS. We isolated at least

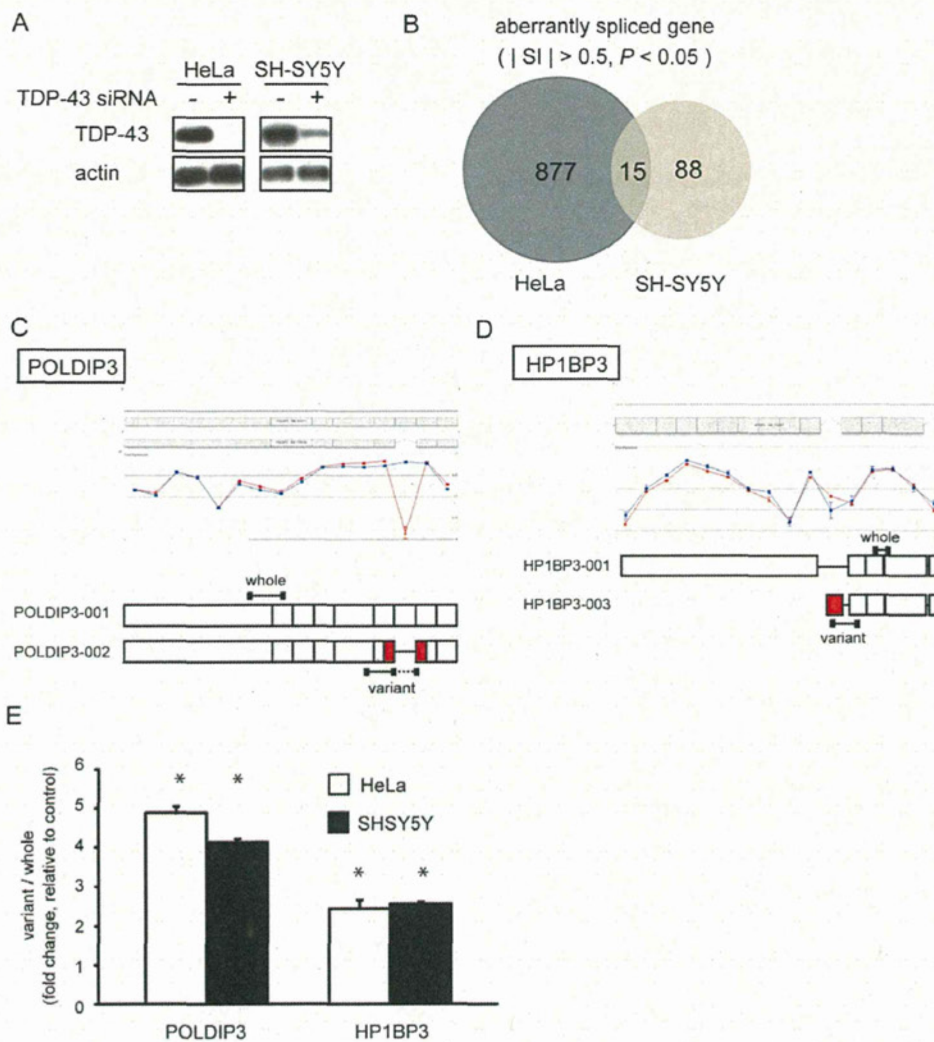

Figure 1. Genes with altered splicing by depletion of TDP-43 in culture cells. (**A**) Western blot analysis of TDP-43 in HeLa and SH-SY5Y cells with or without TDP-43 siRNA. TDP-43 and actin were shown by immunoblotting with anti-TDP-43 (top) and anti-actin (bottom) antibody, respectively. (**B**) Microarray analyses were performed in control (transfected with siControl non-targeting pool) and TDP-43 knockdown HeLa and SH-SY5Y cells in triplicate. Venn diagram shows overlapping of altered splicing genes between HeLa and SH-SY5Y cells. Only 15 genes overlapped between them. From the array data, we calculated the *P* value and the splicing index (|SI|). Both $P<0.05$ and $|SI|>0.5$ were used as thresholds to identify the genes with altered splicing. (**C**) Exon structure diagram of the main isoform (top) and candidate variant (bottom) for *POLDIP3* and *HP1BP3*. From the exon array result, we selected the candidate splicing variant that was induced by the depletion of TDP-43. The name of each transcript is labeled on the side of the exon structure. The red box indicates the exons that are expected to be altered by TDP-43 depletion. The black bars indicate the position of the primers we used in this experiment. The gene views show the expression of exons as determined by analyzing the results of exon array in HeLa cells using Genespring GX. The expression levels are shown on a log2 scale; the error bars show standard errors of means. TDP-43 siRNA, red circle; control siRNA, blue square. (**D**) qRT-PCR analysis revealed that the splicing alteration of *POLDIP3* and *HP1BP3* gene were validated in both HeLa and SH-SY5Y cells. *RPLP1* and *RPS18* were used as reference genes. Data represent the mean with standard error from three independent experiments. Asterisk indicates significant difference (*$P<0.01$, Student *t* test).
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100 spinal motor neurons at L2 in each individual with ALS and controls by laser capture microdissection. The extracted RNA was retrotranscribed, pre-amplified, and subsequently analyzed by real-time qRT-PCR. To assess the purity of the motor neuron-enriched material, we quantified the amount of choline acetyltransferase (*ChAT*) mRNA, one of the markers for motor neurons, and compared it to neighboring cells. Similar expression levels of ribosomal protein, large P1 (*RPLP1*), which was one of the internal controls used in this experiment, was detected between them. In contrast, the expression of *ChAT* mRNA was only detected in the motor neuron-enriched material (Fig. 4B). The amount of expression of *POLDIP3* variant-1 in spinal motor neurons was similar between ALS and controls (Fig. 4C). However, the amounts of expression of *POLDIP3* variant-2 in ALS tissues was statistically increased over 4 times compared to controls (Fig. 4C).

The function of POLDIP 3 variant-2

Finally, we were interested in the function of POLDIP3 variant-2. POLDIP3 has been reported to enhance mammalian target of rapamycin (mTOR)/S6 protein kinase 1 (S6K1)-mediated translation efficiency of mRNA [23]. mTOR/S6K1 signal functions in cell proliferation and growth [24]. Indeed, the depletion of

Table 1. Aberrantly spliced genes overlapping between HeLa and SH-SY5Y cells.

Gene Symbol	Gene Name	Splicing Index (HeLa)	Splicing Index (SH-SY5Y)	Splicing ANOVA Corrected p-value (HeLa)	Splicing ANOVA Corrected p-value (SH-SY5Y)	RefSeq
POLDIP3	polymerase (DNA-directed), delta interacting protein 3	−5.061	−1.967	3.57E-36	8.85E-09	NM_032311
SMC1A	structural maintenance of chromosomes 1A	−1.664	−1.552	8.60E-28	2.99E-12	NM_006306
MMAB	methylmalonic aciduria (cobalamin deficiency) cblB type	−1.177	−1.149	0.00134	7.85E-04	NM_052845
HP1BP3	heterochromatin protein 1, binding protein 3	1.132	1.116	0.00906	0.03201	NM_016287
GLS	glutaminase	−1.062	−1.037	4.12E-09	3.42E-07	NM_014905
STRA6	stimulated by retinoic acid gene 6 homolog (mouse)	1.053	1.077	2.91E-06	4.10E-05	NM_022369
RNFT2	ring finger protein, transmembrane 2	1.028	−0.692	2.40E-06	0.00511	NM_001109903
DPM2	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	0.993	0.929	0.00204	0.02473	NM_003863
JAGN1	jagunal homolog 1 (Drosophila)	0.941	0.991	6.54E-04	2.25E-04	NM_032492
BPNT1	3′(2′), 5′-bisphosphate nucleotidase 1	0.933	1.108	0.02682	7.85E-04	NM_006085
CDCA3	cell division cycle associated 3	0.931	0.745	0.03150	0.00220	NM_031299
LOC729927	similar to 40S ribosomal protein S20	−0.827	0.913	0.00721	0.03092	-
OLFM1	olfactomedin 1	0.783	0.552	1.62E-04	0.00220	NM_014279
CPNE1	copine I	0.635	0.573	0.01643	0.04548	NM_152927
VEGFA	vascular endothelial growth factor A	0.614	0.627	0.01974	0.01099	NM_001025366

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POLDIP3 results in decreased cell size [25]. Therefore, to estimate the function of POLDIP3 variant-2, we investigated the cell size in a neuronal cell line, SH-SY5Y, with or without TDP-43 siRNA. In SH-SY5Y cells treated with TDP-43 siRNA, cell size was significantly decreased and was comparable to depletion of POLDIP3 (Fig. 5A and B) [25]. In contrast, in HeLa and HEK293T cells treated with TDP-43 siRNA, cell size was significantly increased, which is consistent with a previous report (Fig. S3A and B) [26]. In addition, although there was no statistical significance, cell size in U87-MG cells treated with TDP-43 siRNA showed a tendency to be larger (Fig. S3C).

To further elucidate the functional difference between POLDIP3 variant-1 and POLDIP3 variant-2, we investigated whether these POLDIP3 variants can restore the size of SH-SY5Y cells treated with TDP-43 siRNA. We transfected SH-SY5Y cells, which stably expressed the GFP-tagged POLDIP3 variant-1 or variant-2, with control or TDP-43 siRNA. Then we calculated the cell size recovery rate by comparing the cell size between the cells with or without the expression of GFP-tagged POLDIP3 variants (Fig. 5C; Fig. S4). TDP-43 siRNA treatment suppressed the expression of TDP-43 to $<20\%$ in both POLDIP3 stable cell lines compared to control siRNA (Fig. 5D). Although both POLDIP3 variants improved the cell size reduction caused by depletion of TDP-43, the recovery rate of cell size was higher in variant-1-expressing cells than in variant-2-expressing cells and reached statistical significance, indicating that the POLDIP3 variant-2 is less effective at maintaining the size of SH-SY5Y cells than variant-1 (Fig. 5E).

Discussion

Here, we show the increasing the amounts of the *POLDIP3* variant-2 mRNA in the affected tissues with ALS, including spinal motor neuron. The result suggests that a function of TDP-43 decreases in the affected tissues with ALS. The finding that *POLDIP3* variant-2 increased in the cultured human cells depleted of TDP-43 is consistent with the previous reports [19,20]. In addition, we and Fiesel *et al.* found that RNA binding ability of TDP-43 is necessary for inclusion of exon 3 of *POLDIP3*, indicating that TDP-43 directly regulates splicing of *POLDIP3* [26]. Therefore we conclude that the alteration of splicing of *POLDIP3* represents the dysfunction of TDP-43. The finding that the TDP-43 function decreases in the affected tissue with ALS supports the hypothesis that a loss of function of TDP-43 underlies the pathogenesis of ALS.

We were not able to show the decreasing the amounts of *POLDIP3* variant-1 in the affected tissues with ALS. There is a possibility that the increasing the amount of *POLDIP3* variant-2 might be resulted from the mechanism which is not associated with the TDP-43 function. However, the reason why the decreasing the amounts of *POLDIP3* variant-1 was not observed in affected tissues with ALS is related to the number of the neurons and glial cells in which *POLDIP3* splicing altered. The alteration of *POLDIP3* splicing may occur only in the cells with disappearance of nuclear TDP-43. The number of these cells is relatively small even though in the spinal motor neurons in ALS [27]. In addition, the amount of *POLDIP3* variant-1 is abundant in normal cells, thus it would be difficult to detect the decreasing the amounts of *POLDIP3* variant-1. In contrast, the *POLDIP3* variant-2 is nearly absent in normal cells (Fig. 2B), thus increasing the amounts of *POLDIP3* variant-2 is detected sensitively. The immunohistochemical analysis to compare the amount of variant-1 between the cells with or without nuclear TDP-43 should clarify this issue; however, the POLDIP3 antibodies evaluated in this study were not suitable for immunohistochemical analysis in human autopsied tissues (data not shown). Further study by using the specific antibodies for POLDIP3 variant-1 is necessary to elucidate this issue.