wild-type TDP-43 when a large amount of TDP-43 was loaded (data not shown), suggesting that this antiserum may not be entirely phosphorylation-dependent.

3.2. Immunohistochemical characterization of mAb pS409/410

MAb pS409/410 strongly stained ubiquitin-positive inclusions in both FTLD-U and ALS brains (Fig. 2). It recognized NCIs in the dentate gyrus (Fig. 2A) and DNs in the temporal cortex (Fig. 2B) of sporadic FTLD-U cases, and skein-like inclusions (Fig. 2C), round inclusions (Fig. 2D) and glial inclusions (Fig. 2E) in the spinal cord of ALS cases. These inclusions were unambiguously identified by mAb pS409/410, with no nuclear staining (Fig. 2). In some cases, pAb pS409/410 weakly stained ghost tangles and dot-like structures in the hippocampal region of brains from patients with AD or other related diseases, but mAb pS409/410 did not recognize these structures (data not shown).

3.3. Immunoreactivity of TDP-43 deposited in FTLD-U and ALS brains and TDP-43 extracted rapidly from rat tissues

MAb pS409/410 strongly stained 45 kDa TDP-43 band, ~25 kDa fragments and smearing substances in the Sarkosyl-insoluble fractions of FTLD-U and ALS brains. Lambda protein phosphatase (λPPase) treatment of Sarkosyl-insoluble TDP-43 resulted in loss of mAb pS409/410 immunoreactivity (Fig. 3A). The patterns of the bands of ~25 kDa fragments detected with mAb 409/410 were different between sporadic cases

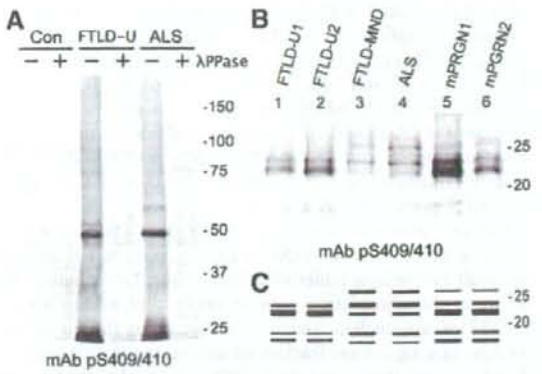

Fig. 3. (A) Immunoblot analyses of the Sarkosyl-insoluble, urea-soluble fractions from human control, FTLD-U and ALS brains with anti-TDP-43 (ProteinTech) and mAb pS409/410 antibodies before (−) and after (+) treatment with lambda protein phosphatase (λPPase). MAb pS409/410 specifically labels the ~45 kDa full-length TDP-43, as well as ~25 kDa fragments and the smearing substances, in FTLD-U and ALS. The labeling is abolished after dephosphorylation. Normal 43 kDa TDP-43 in control and diseased brains is not stained by mAb pS409/410. (B) Immunoblots of the Sarkosyl-insoluble, urea-soluble fractions from sporadic FTLD-U, FTLD-MND, ALS and mPGRN cases with the mAb pS409/410. The samples were loaded on 15% polyacrylamide gel. (C) Schematic diagram of the band pattern of the C-terminal fragments of phosphorylated TDP-43.

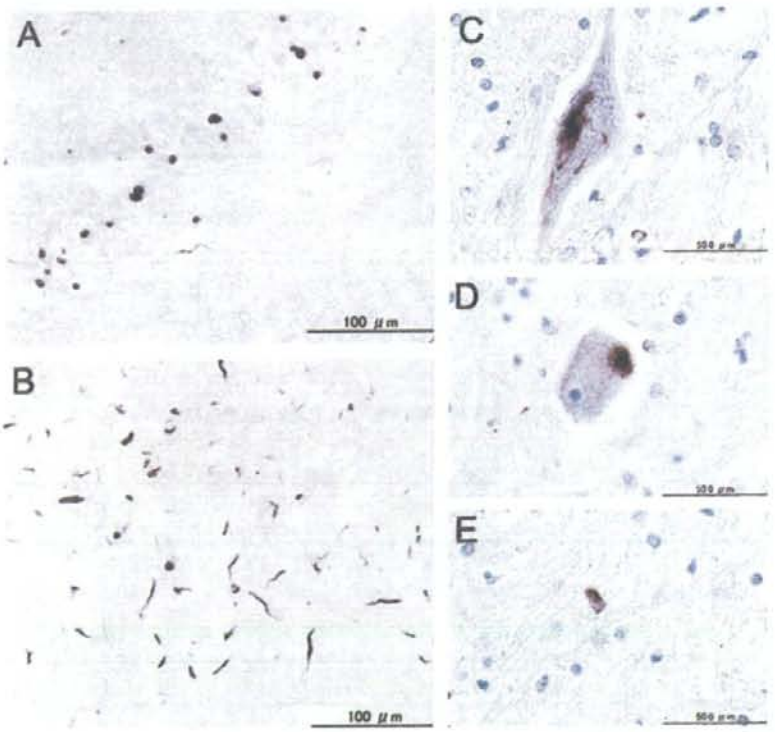

Fig. 2. Immunohistochemistry of TDP-43 lesions. Thirty-micrometer-thick free floating sections of the dentate gyrus of hippocampus (A) and temporal cortex (B) from FTLD-U and of spinal cords from ALS (C–E) were immunostained with mAb pS409/410. TDP-43-positive NCIs in dentate gyrus and DNs in cortex are specifically stained. Note the absence of nuclear staining in A–E. Bars 100 μm (A,B) and 50 μm (C–E).

of FTLD-U, ALS and familial cases of FTLD-U with progranulin mutation (Fig. 3B), as previously observed with pAb pS409/410 [14]. Sporadic FTLD-U cases showed two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa, while FTLD-MND and ALS cases showed three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa (Fig. 3B and C). The band pattern of mPGRN cases was intermediate between those of FTLD-U, FTLD-MND and ALS cases (Fig. 3B and C).

We next examined whether the phosphorylation of Ser409/410 can be detected in TDP-43 rapidly extracted from fetal or adult rats with a buffer containing phosphatase inhibitors, because phosphorylation seen at many sites of tau and at Ser129 of α-synuclein under pathological conditions is also present in a significant fraction of normal tau [15] or α-synuclein [16], and these sites are rapidly dephosphorylated postmortem [17,18].

Anti-TDP-43 antibody strongly labeled the TDP-43 band at 43 kDa in Tris-soluble fractions of brains from fetal to 6-week-old adult rats (Fig. 4A). In contrast, mAb pS409/410 failed to detect any of the TDP-43 bands in these fractions (Fig. 4A), though the antibody strongly stained recombinant TDP-43 phosphorylated with CK1. On the other hand, an antibody specific for phosphorylated tau at Ser396 (pS396) strongly recognized phosphorylated tau bands in fetal and adult rat brains (Fig. 4B). Similar results were obtained with pAb pS409/410 (data not shown). These findings indicate that phosphorylation of Ser409/410 does not occur in rat brain during normal development. Immunoblot analysis of extracts from various tissues revealed that TDP-43 is broadly expressed in various tissues (Fig. 4C). The 43 kDa band was strongly immunostained in cerebellum, brain stem, spinal cord, liver, lung, spleen and ovary, but only weakly in heart and kidney (Fig. 4C). In addition to the 43 kDa band, some low-molecular bands at 30–35 kDa in most of the tissues and a doublet at ~30 kDa in kidney were detected with anti-TDP-43 (Fig. 4C). In contrast, no apparent immunoreactivity was observed with mAb pS409/410, which was confirmed to strongly immunostain recombinant TDP-43 that had been phosphorylated with CK1.

3.4. Changes of TDP-43 during postmortem incubation

Rapid postmortem dephosphorylation occurs in tau and α-synuclein, and the Cdk5 activator p35 is degraded to p25 during postmortem incubation [19]. Therefore, we investigated

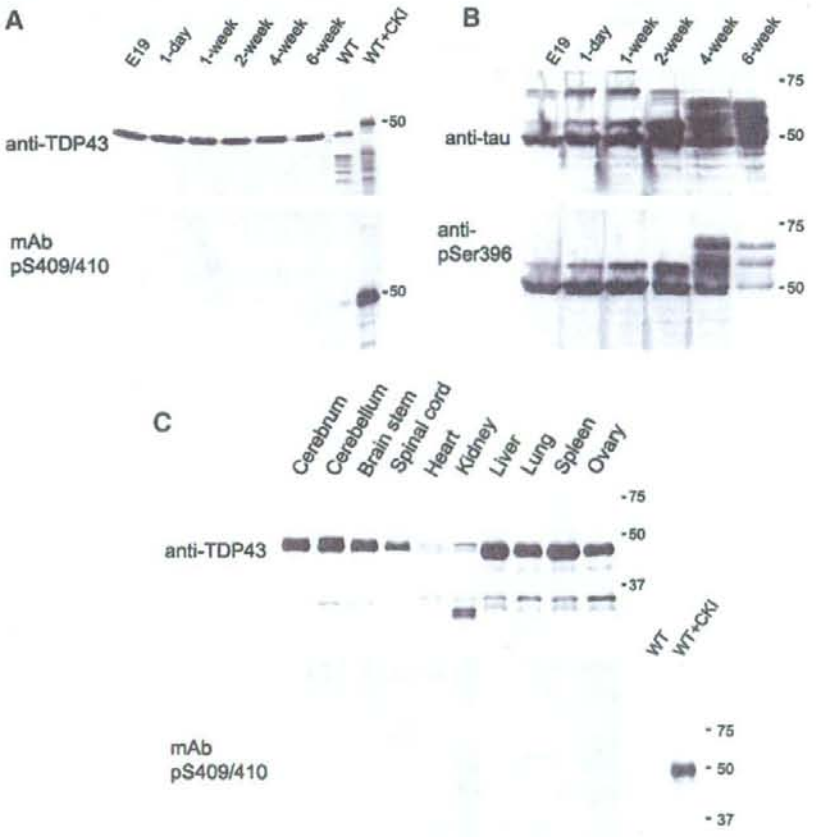

Fig. 4. (A) Immunoblot analyses of TDP-43 differentially extracted with Tris–HCl, Triton-X100, Sarkosyl and 8 M urea, from rat brains at various developmental stages, with anti-TDP43 antibody (ProteinTech), mAb pS409/410, an anti-tau antibody (T46: Zymed) and an anti-pS396 tau antibody (Calbiochem). (B) Immunoblots of Tris-soluble extracts from various tissues of 6-week-old rats with anti-TDP-43 and mAb pS409/410.

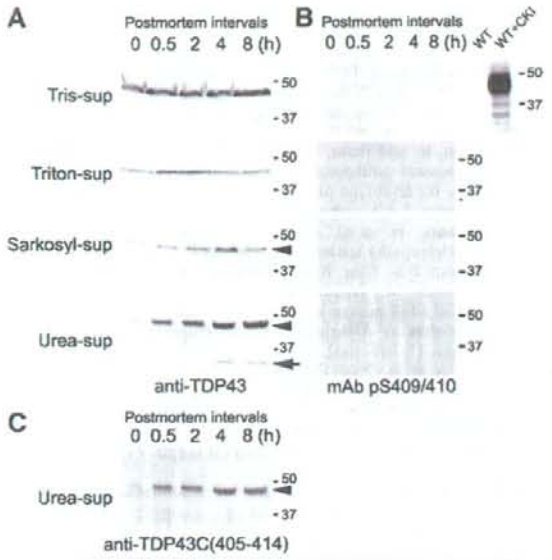

Fig. 5. Immunoblot analyses of brain extracts from rats after different postmortem intervals with anti-TDP43 antibody (ProteinTech) (A), mAb pS409/410 (B) and anti-TDP43C (405–414) (C).

the changes of phosphorylation and degradation of TDP-43 postmortem. One-year-old rats were lethally anethetized and the brains were excised 0, 0.5, 2, 4 or 8 h later, then homogenized differentially. The extracted fractions were analyzed by immunoblotting (Fig. 5). As shown in Fig. 5B, pS409/410 immunoreactivity was not detected in soluble or insoluble fractions of the brains, nor was it generated during postmortem incubation, indicating that phosphorylation of Ser409/410 does not occur even in aged rats. Interestingly, the levels of Sarkosyl-insoluble and urea-soluble TDP-43 of 43 kDa as well as Sarkosyl-soluble TDP-43 (Fig. 5A, arrowheads) and the levels of urea-soluble 35 kDa fragment (Fig. 5A, arrow) gradually increased during postmortem incubation at room temperature, although no apparent reduction was detected in TS-soluble TDP-43. The postmortem increase of Sarkosyl-insoluble and urea-soluble TDP-43 was also observed with antiserum directed against the C-terminus of TDP-43 (405–414) (Fig. 5C).

4. Discussion

Abnormal phosphorylation of TDP-43 has been suggested in the initial reports that identified TDP-43 as the major component of ubiquitin-positive inclusions in FTLD-U and ALS [3,4]. In the present study, we have produced a monoclonal antibody that distinguishes the disease-associated phosphorylation of TDP-43. By means of ELISA assay of phosphopeptides and a combination of site-directed mutagenesis and in vitro phosphorylation, we have shown that mAb pS409/410 is highly specific for TDP-43 phosphorylated at both Ser409 and Ser410. MAb pS409/410 strongly stained TDP-43 lesions in FTLD-U brains and ALS brains/spinal cords, as well as abnormal TDP-43 bands and smears on immunoblots, in agreement with previous observations using pAb pS409/410 [14]. In contrast, mAb pS409/410 did not recognize normal human TDP-43 of 43 kDa or TDP-43 rapidly extracted from brains of fetal to 1-year-old adult rats. It also did not react with TDP-43 expressed in other various tissues. These results indicate that phosphorylation of TDP-43 at Ser409/410 is a pathological event. This is different from the cases of tau or α-synuclein, where phosphorylation at most of the sites can be detected in normal fetal or adult brain. It remains unknown whether the abnormal phosphorylation of TDP-43 at Ser409/410 precedes assembly of TDP-43, or whether it is a reaction associated with aggregation or conformational change of TDP-43. However, it is clear that the phosphorylation is a specific biological marker for detection of abnormal TDP-43 deposits in FTLD-U and ALS brains/spinal cords.

In the course of characterization of mAb pS409/410, we found that the levels of Sarkosyl-insoluble and urea-soluble TDP-43 increased during postmortem incubation. A similar increase of Sarkosyl-insoluble TDP-43 was observed during the incubation of recombinant human TDP-43 at 37 °C for 24 h (data not shown). These results suggest that the sarkosyl-insoluble, urea-soluble TDP-43 of 43 kDa and the 35 kDa fragment detected in brains of human controls and patients with FTLD-U or ALS may have been artifacts generated postmortem. Zhang et al. reported that the amounts of the 35 and 25 kDa fragments were increased in cells treated with staurosporin or PGRN siRNA and in brains of patients of FTLD-U [20], whereas Shankaran found that these fragments of TDP-43 were generated independently of PGRN knock-down in cell lines and zebrafish [21]. Our results show that a 35 kDa N-terminal fragment of TDP-43 is generated during postmortem incubation and recovered in insoluble fractions, but this is irrelevant to the pathology of TDP-43 proteinopathy.

As had been found with pAb pS409/410, mAb pS409/410 revealed that the 18–26 kDa C-terminal fragments of TDP-43 are the major species of TDP-43 deposited in patients with FTLD-U and ALS, and the patterns of the fragments are closely related to the neuropathological subtypes of the inclusions. Although further studies are needed to clarify the molecular mechanisms of the fragmentation and aggregation of TDP-43, the results presented here suggest that aggregation of TDP-43 is implicated in FTLD-U and ALS, just as aggregation and phosphorylation of tau or α-synuclein may be involved in other neurodegenerative diseases. MAb pS409/410 may be a useful probe for detection of abnormal TDP-43 in tissues of patients and for evaluation of cellular or animal models of TDP-43 proteinopathy. Antibodies specific for the abnormal phosphorylation sites of TDP-43 may be useful for antibody therapy, or the antigens may be suitable for immunization against FTLD-U and ALS.

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Phosphorylated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis

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Objective: TAR DNA-binding protein of 43kDa (TDP-43) is deposited as cytoplasmic and intranuclear inclusions in brains of patients with frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). Previous studies reported that abnormal phosphorylation takes place in deposited TDP-43. The aim of this study was to identify the phosphorylation sites and responsible kinases, and to clarify the pathological significance of phosphorylation of TDP-43.

Methods: We generated multiple antibodies specific to phosphorylated TDP-43 by immunizing phosphopeptides of TDP-43, and analyzed FTLD-U and ALS brains by immunohistochemistry, immunoelectron microscopy, and immunoblots. In addition, we performed investigations aimed at identifying the responsible kinases, and we assessed the effects of phosphorylation on TDP-43 oligomerization and fibrillization.

Results: We identified multiple phosphorylation sites in carboxyl-terminal regions of deposited TDP-43. Phosphorylation-specific antibodies stained more inclusions than antibodies to ubiquitin and, unlike existing commercially available anti–TDP-43 antibodies, did not stain normal nuclei. Ultrastructurally, these antibodies labeled abnormal fibers of 15nm diameter and on immunoblots recognized hyperphosphorylated TDP-43 at 45kDa, with additional 18 to 26kDa fragments in sarkosyl-insoluble fractions from FTLD-U and ALS brains. The phosphorylated epitopes were generated by casein kinase-1 and -2, and phosphorylation led to increased oligomerization and fibrillization of TDP-43.

Interpretation: These results suggest that phosphorylated TDP-43 is a major component of the inclusions, and that abnormal phosphorylation of TDP-43 is a critical step in the pathogenesis of FTLD-U and ALS. Phosphorylation-specific antibodies will be powerful tools for the investigation of these disorders.

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Tau-negative and ubiquitin-positive inclusions (UPIs) that include neuronal cytoplasmic inclusions (NCIs), neuronal intranuclear inclusions (NIIs), and dystrophic neurites (DNs) are the pathological hallmarks of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) with or without clinical features of motor neuron disease (MND).[1] Recently, several genes and chromosomal loci, including the progranulin *(PGRN)* gene,[2,3] valosin-containing protein *(VCP)* gene,[4] and an unidentified site at chromosome 9p,[5,6] have been reported to be associated with familial FTLD-U. Ubiquitin-positive, tau-negative NCIs have also been recognized in patients with the classic type of MND, amyotrophic lateral sclerosis (ALS),[7] in which skein-like cytoplasmic inclusions are found in the lower motor neurons of the hypoglossal nucleus and spinal cord.[8,9] In both FTLD-U and ALS, understanding why these inclusions form may provide critical clues to the neurodegenerative process.

Recently, TAR DNA-binding protein of 43kDa

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(TDP-43), which functions in regulating transcription and alternative splicing,[10,11] was identified as a component of these UPIs.[12–14] TDP-43 appears to belong to the group of two RNA-binding domain-Gly RNA-binding proteins, which include the heterogeneous nuclear ribonucleoprotein (hnRNP) family and factors involved in RNA splicing and transport.[15] TDP-43 binds hnRNP A/B and hnRNP A1 through its C-terminal region, inhibiting premessenger RNA splicing.[16] Several disorders, including FTLD-U, FTLD-MND, and ALS are now referred to as TDP-43 proteinopathies.[12–14] Immunoblot analysis of the sarkosyl-insoluble fraction extracted from brains of patients afflicted with these disorders shows an abnormal TDP-43–immunoreactive band at 45kDa. The electric mobility of this band changes after dephosphorylation, suggesting that abnormal phosphorylation takes place in accumulated TDP-43.[12,13] However, the phosphorylation sites, responsible kinases, and pathological significance of phosphorylation are still unknown.

In this report, we demonstrate that multiple antibodies raised against TDP-43 phosphopeptides label UPIs in histological sections from FTLD-U and ALS brains. These antibodies may offer advantages over previous antibodies used to identify these structures because they appear to be more sensitive than anti-ubiquitin antibodies and, unlike commercially available anti–TDP-43 antibodies, do not stain normal neuronal nuclei. In addition, these antibodies specifically recognize abnormal TDP-43 species on immunoblots of sarkosyl-insoluble fractions extracted from FTLD-U and ALS brains. Furthermore, we show that the multiple phosphorylation epitopes identified in aggregated TDP-43 are generated by casein kinase-1 (CK1), and that oligomerization or fibril formation of TDP-43 is promoted by phosphorylation with CK1 in vitro. These results suggest that phosphorylated TDP-43 is a critical component of UPIs in FTLD-U and ALS, and that phosphorylation of TDP-43 by CK1 may be involved in the accumulation of the protein.

Subjects and Methods

Materials

Human brain tissue was obtained from the Brain Donation Program at Sun Health Research Institute (Sun City, AZ), Aichi Medical University (Japan), Jichi Medical University (Japan), National Shimofusa Mental Hospital (Japan), and Tokyo Metropolitan Matsuzawa Hospital (Japan). Small blocks of brain tissue were dissected at autopsy and frozen rapidly at −70 to 80°C or fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 2 days. Brain tissue from sporadic FTLD-U, familial FTLD-U with PGRN mutations (mPGRN), sporadic ALS, and sporadic FTLD-MND was compared with brain tissue from Alzheimer's disease (AD) and neurologically normal control subjects. The age, sex, brain regions examined, diagnosis, and histopathological subtyping for these cases are given in Table 1. Neuropathological diagnoses of FTLD-U, FTLD-MND, ALS, and AD were made in accordance with published guidelines.[1,9,17–19]

Table 1. **Subjects, Brain Regions, Pathological Diagnosis, and Subtypes Examined**

Case No.	Age (yr)	Sex	Region	Diagnosis	Subtype
1	67	M	Hip, T, F	FTLD-U (sporadic)	1
2	59	M	Hip, T	FTLD-U (sporadic)	1
3	68	F	Hip, T	FTLD-U (sporadic)	1
4	49	F	T	FTLD-MND	2
5	76	M	Prec	FTLD-MND	2
6	66	M	F, SC	ALS	2
7	70	M	Prec, SC	ALS	2
8	69	M	Prec	ALS	2
9	53	M	Hip, T, F	FTLD-U (mRGRN)	3
10	56	F	Hip, T, F	FTLD-U (mRGRN)	3
11	54	M	Hip, T, F	FTLD-U (mRGRN)	3
12	68	F	Hip, T	AD	—
13	83	F	Hip, T	AD	—
14	65	M	Hip, T	Control	—
15	72	M	Hip, T	Control	—

Hip = hippocampus; T = temporal; F = frontal; FTLD-U = frontotemporal lobar degeneration with ubiquitinated inclusions; MND = motor neuron disease; Prec = precentral; SC = spinal cord; ALS = amyotrophic lateral sclerosis; mPGRN = mutations of progranulin gene; AD = Alzheimer's disease.

Although none of the three ALS cases had a documented history of dementia, all had immunohistochemical evidence of pathology in the neocortex.

Preparation of Antibodies

Immunogens consisted of 39 synthetic phosphopeptides representing 36 of the 64 Ser/Thr/Tyr sites in the human TDP-43 molecule. All peptides were conjugated at the amino or carboxyl terminal by a cysteine linkage to synthetic thyroglobulin using m-maleimidobenzoyl-*N*-hydroxysuccinimide ester as a coupling reagent.[20] The rabbit antisera were purified by obtaining flow-through fractions from a Toyopearl AF-Tresyl-650M (TOSOH, Tokyo, Japan) or SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL) precoated with the nonphosphorylated synthetic peptide. The specificities of the antibodies were verified by enzyme-linked immunosorbent assay and immunoblot. A phosphorylation-independent rabbit polyclonal antibody to TDP-43 was also produced using a C-terminal peptide of TDP-43 (405-414) as immunogen.

Immunohistochemistry

After cryoprotection in 15% sucrose in 0.01M phosphate-buffered saline (pH 7.4), paraformaldehyde-fixed tissue blocks were cut on a freezing microtome at 30μm thickness. The free-floating sections were immunostained with an anti-ubiquitin antibody (DF2, a gift from Dr Mori, 1:200),[21] a commercially obtained phosphorylation-independent anti–TDP-43 antibody (10782-1-AP; ProteinTech Group, Chicago, IL; 1:2,000), and a panel of phosphorylation-dependent anti–TDP-43 antibodies including pS409/410, using methods previously described.[13] Double-labeled immunofluorescence was performed using fluorescein isothiocianate– and tetramethylrhodamine isothiocyanate–conjugated secondary antibodies; sections were examined with a confocal laser microscope (LSM5 PASCAL; Carl Zeiss MicroImaging GmbH, Jena, Germany).

Immunoelectron Microscopy

Tissue blocks of ALS lumber spinal cord were fixed in paraformaldehyde and embedded in LR White Resin (London Resin, Reading, United Kingdom). Ultrathin sections were incubated with pS409/410 (1:1,000), and the immunoreaction products were probed using colloidal gold particles (1:10 dilution; BBinternational, Cardiff, United Kingdom) according to a standard immunogold-based postembedding electron microscopic procedure.[22]

Immunoblotting

Sarkosyl-insoluble, urea-soluble fractions were extracted from the frontal and the temporal regions of control, FTLD-U, and ALS brains as previously described.[23] The samples before (−) and after (+) the treatment with lambda protein phosphatase (λPPase) were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins in the gel were then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 3% gelatin in tris(hydroxymethyl)aminomethane (Tris)–buffered saline (50mM Tris-HCl, pH 7.5, 150mM NaCl), membranes were incubated overnight with the primary antibodies. After incubation with an appropriate biotinylated secondary antibody, labeling was detected as described previously.[13,23]

In Vitro Phosphorylation and Fibrillization of TDP-43

Human TDP-43 complementary DNA were subcloned into pRK172 expression vectors and transformed into *Escherichia coli* BL21(DE3). For in vitro phosphorylation, crude extracts from *E. coli* that expressed human TDP-43 were used to prepare partially purified TDP-43 using heparin-Toyopearl column chromatography and elution with 0.5M NaCl. The elutes were phosphorylated with CK1 (10,000U/ml; New England Biolabs, Beverly, MA), casein kinase-2 (CK2) (10,000U/ml; New England Biolabs), or glycogen synthase kinase-3β (GSK3β) (10,000U/ml; New England Biolabs) at 30°C for 14 hours. To study fibrillization, we incubated partially purified TDP-43 aliquots in 30mM Tris-HCl at pH 7.5 containing 4mM magnesium chloride, 2mM ATP, with or without CK1 (10,000U/ml) at 30°C for 3 days. A few drops of reaction solution was then applied to a carbon-coated copper grid and allowed to air-dry. The grid was placed on a drop of blocking solution (10mg/ml bovine serum albumin in phosphate-buffered saline) for 10 minutes and then placed on a drop of primary antibody (pS409/410, 1:200) for 2 hours at room temperature. After washing with 10mg/ml bovine serum albumin in phosphate-buffered saline, the grid was placed on a drop of the secondary antibody conjugated to 10nm colloidal gold particles (1:50; Sigma, St. Louis, MO) for 1 hour at room temperature. Finally, after another round of washing, the grid was negatively stained with 2% sodium phosphotungstate and examined with the electron microscopy (JEM-1230; JEOL, Akishime, Japan).

Results

Multiple Sites within TDP-43 Are Abnormally Phosphorylated in Frontotemporal Lobar Degeneration with Ubiquitinated Inclusions and Amyotrophic Lateral Sclerosis

There are multiple potential phosphorylation sites within human TDP-43, including 41 serine (Ser), 15 threonine (Thr), and 8 tyrosine (Tyr) residues. To identify the critical phosphorylation sites of TDP-43, we raised antibodies against 39 different synthetic phosphopeptides, representing 36 of 64 candidate phosphorylation sites (Table 2). The major strategy was to choose Ser and Thr residues that cover known protein kinase consensus phosphorylation motifs, including R-X-pSer/Thr for protein kinase A, pSer/Thr-X-X-Ser/Thr for CK1, pSer/Thr-X-X-E/D for CK2, and pSer/Thr-X-X-X-Ser for GSK3 and CK1. In addition, Ser/Thr residues in C-terminal region of TDP-43 were chosen because they are analogous to abnormal phosphorylation sites found in tau or α-synuclein.

Of the generated antibodies, pS379, pS403/404, pS409, pS410, and pS409/410 intensely immunostained the UPIs in FTLD-U and ALS, and demonstrated, on immunoblots of sarkosyl-insoluble fractions

Table 2. **Antigen Peptides for Immunization of Rabbits**

	Site	Antigen Peptide
1	pT8	EYIRVT(p)EDENDEC
2	pS20	PIEIPS(p)EDDGTC
3	pS29	GTVLLS(p)TVTAC
4	pT88	CNYPKDNKRKMDET(p)D
5	pS91	CDETDAS(p)SAVKVKR
6	pS92	CDETDASS(p)AVKVKR
7	pS91/92	DETDAS(p)S(p)AVKVC
8	pT103	CKRAVQKT(p)SDLIVLG
9	pS104	CKRAVQKTS(p)DLIVLG
10	pT116	PWKTT(p)EQDLKEC
11	pT141	KKDLKT(p)GHSKGC
12	pT153	CGFVRFT(p)EYETQVK
13	pS180	CKLPNS(p)KQSQDE
14	pS183	CPNSKQS(p)QDEPLR
15	pS190	CKQSQDEPLRS(p)RK
16	pT199	CT(p)EDMTEDLE
17	pT203	RCTEDMT(p)EDELR
18	pT233	CRAFADVT(p)FADDQ
19	pS254	IIKGIS(p)VKISC
20	pS258	CISVHIS(p)NAEPK
21	pS266	CPKHNS(p)NRQLER
22	pS273	CRQLERS(p)GRFGGN
23	pS292	CGFGNS(p)RGGGA
24	pS305	CNNQGS(p)NMGGG
25	pS317	CFGAFS(p)INPAM
26	pS332	CAALQS(p)SWGMM
27	pS350	CQSGPS(p)GNNQN
28	pS375	GNNSYS(p)GSNSGC
29	pS379	CSGSNS(p)GAAIG
30	pS389	CGWGSAS(p)NAGS
31	pS393	CASNAGS(p)GSGF
32	pS395	CAGSGS(p)GFNGG
33	pS403	CGFNGGFGS(p)SMD
34	pS404	CNGGFGSS(p)MDSK
35	pS403/404	CNGGFGS(p)S(p)MDSK
36	pS407	CGSSMDS(p)KSSGW
37	pS409	CMDSKS(p)SGWGM
38	pS410	CMDSKSS(p)GWGM
39	pS409/410	CMDSKS(p)S(p)GWGM

extracted from brains of the FTLD-U and ALS cases, an abnormal band of 45kDa but not the 43kDa band corresponding to normal TDP-43. The results suggest that these sites are phosphorylated in the abnormal aggregates of TDP-43 present in FTLD-U and ALS.

Immunohistochemical Characterization of Phosphorylated TDP-43

Immunohistochemical staining showed that five of the phosphorylation-specific anti-TDP-43 antibodies identified UPIs in both FTLD-U and ALS brains. Of the phosphorylation-specific antibodies, the immunoreactivity of pS409/410 was particularly robust (Fig 1). In comparison, the commercially obtained, phosphorylation-independent, anti–TDP-43 antibody labeled NCIs, DNs, and neuronal nuclei in the dentate gyrus (see Fig 1A) and the temporal cortex (see Fig 1B) of the sporadic FTLD-U cases, and skein-like inclusions and neuronal nuclei in the spinal cord of ALS cases (see Fig 1C). It was particularly difficult to distinguish the staining of NCIs from that of neuronal nuclei in the dentate gyrus of the sporadic FTLD-U cases (see Fig 1A). By contrast, NCIs and DNs were unambiguously identified by the phosphorylation-specific antibody pS409/410, with no nuclear staining (see Figs 1D, E). Similarly, pS409/410 clearly labeled skein-like (see Fig 1F) and glial inclusions (see Fig 1G) in the spinal cord, and NCIs in the frontal and precentral cortices of the FTLD-MND and ALS cases (see Fig 1H). Glial inclusions in the frontal and precentral regions of the FTLD-MND and ALS cases were also immunopositive (data not shown). In the cases of familial FTLD-U with PGRN mutations, pS409/410 intensely stained NCIs, DNs, and NIIs in the cerebral cortex (see Figs 1I–K) and abundant positive structures in the cerebral white matter (see Fig 1L). The results of double-immunofluorescence staining showed that pS409/410 identified more NCIs than the ubiquitin antibody (see Figs 1M–O).

Based on morphological aspects, TDP-43 proteinopathies have been classified into four subtypes.[24] Type 1 is characterized by DNs with few NCIs and no NIIs, type 2 has numerous NCIs with few DNs and no NIIs, type 3 has numerous NCIs and DNs and an occasional NII, and type 4 has numerous NIIs and DNs with few NCIs. Type 4 is specific for familial FTLD-U with mutations of valosin-containing protein *(VCP)* gene. In this study, using the commercial anti–TDP-43 antibody, all of the sporadic FTLD-U cases showed type 1 pathology, all FTLD-MND and ALS cases showed type 2 pathology, and all FTLD-U with mPGRN showed type 3 pathology, in agreement with previous reports.[24,25]

The staining patterns obtained with our phosphorylation-dependent antibodies were similar to those seen

with the commercial phosphorylation-independent antibody, suggesting that all of the inclusion types previously described contain phosphorylated TDP-43. Similar staining patterns were obtained using pS379 (Figs 2A–C), pS403/404 (see Figs 2D–F), pS409 (see Figs 2G–I), and pS410 (see Figs 2J–L). Preabsorption of the antibodies with phosphopeptide immunogens abolished the labeling of these structures (data not shown).

Immunoelectron microscopic examination of the

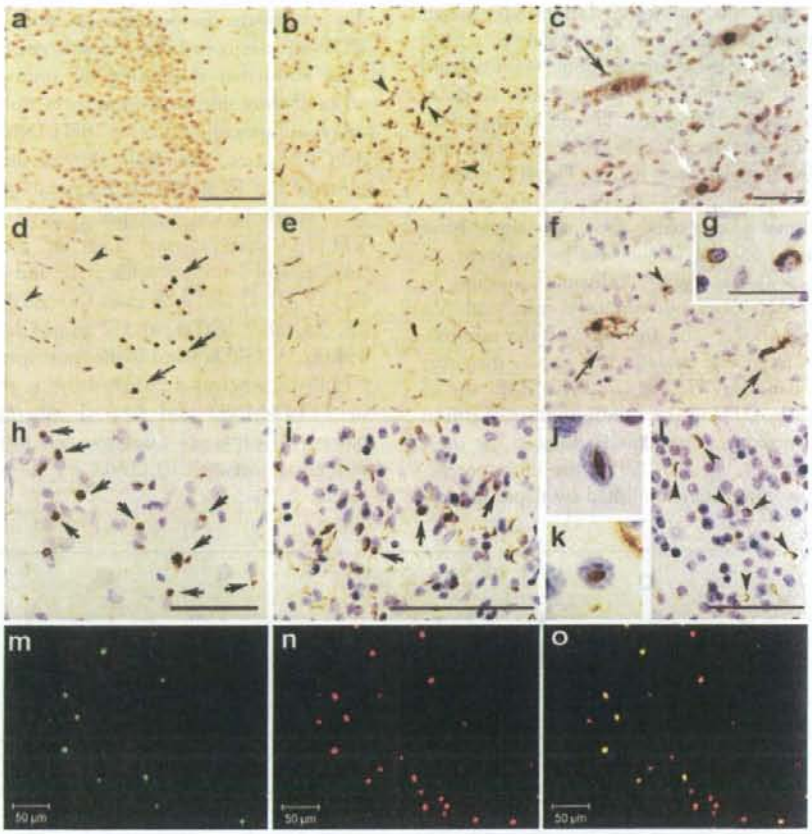

Fig 1. Immunohistochemical comparison of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) brains using the phosphorylation-independent anti–TAR DNA-binding protein of 43kDa (TDP-43) antibody (ProteinTech) (A–C) and the phosphorylation-dependent anti–TDP-43 antibody (pS409/410) (D–L), in the dentate gyrus (A, D) and temporal cortex (B, E) of the sporadic FTLD-U cases, in the lumbar spinal cord (C, F, G) and the frontal cortex (H) of the ALS cases, and in the frontal cortex (I–K) and the frontal white matter (L) of the familial FTLD-U cases with progranulin (PGRN) mutations. (A) Because most of the nuclei of dentate gyrus granular neurons are immunopositive with the phosphorylation-independent antibody, it is difficult to identify neuronal cytoplasmic inclusions (NCIs). (B) TDP-43–positive dystrophic neurites (DNs) are recognizable (arrowheads) *in addition to the nuclei. (C) The* black arrow *indicates a cell with skein-like inclusions.* White arrows *and* arrowheads *indicate the normal nuclei of anterior horn cells and glial nuclei, respectively. Photomicrographs (D–F) illustrate the corresponding areas to (A–C), respectively. Note the absence of nuclear staining in (D–G) with the phosphorylation-dependent antibody pS409/410. (D) NCIs* (arrows) *and DNs* (arrowheads) *are clearly seen. (E) More abundant DNs are seen than in (B). (F)* Arrows *indicate skein-like inclusions;* arrowheads *indicate glial inclusions. (G, insert) Glial inclusions at a higher magnification. (H) NCIs in the frontal cortices of the ALS case are immunopositive. In the cases with PGRN mutations, pS409/410 clearly stains NCIs* (arrows), *DNs (I), and neuronal intranuclear inclusions (NIIs) (J, K) in the superficial cortical layers, and abundant immunopositive structures in the white matter (L,* arrowheads), *with no nuclear staining. Sections are counterstained with hematoxylin to show nuclei in (C, F–L). (M–O) Antiubiquitin (DF2) and pS409/410 double-label immunofluorescence histochemistry of the dentate gyrus in the FTLD-U case. Only some of the pS409/410-positive NCIs are also ubiquitin positive. (M) DF2; (N) pS409/410; (G) merge. Cell nuclei are stained with TO-PRO-3 (Invitrogen, Tokyo, Japan), producing a blue color. Scale bars = 100μm (A, B, D, E, I); 50μm (C, F, H, L); 25μm (G); 10μm (J, K).*

spinal cord motoneuron inclusions of an ALS patient with the pS409/410 antibody showed immunopositive abnormal fibers of 15nm in diameter (Figs 3A, B).

Immunoblot Analysis of Phosphorylated TDP-43

Immunoblot analyses of sarkosyl-insoluble fractions extracted from the brains of control, AD, FTLD-U, and ALS cases with the phosphorylation-independent TDP-43 antibody (ProteinTech) always showed a band of 43kDa and also showed an additional 45kDa band that was present only in FTLD-U and ALS cases, as described previously[12,13] (Fig 4A). The phosphorylation-dependent antibodies specific for pS409/410 (see Fig 4B), pS409 (see Fig 4C), pS410 (see Fig 4D), pS403/404 (see Fig 4E), and pS379 (see Fig 4F) did not recognize the normal 43kDa band, showing a single band at approximately 45kDa, several smaller fragments at approximately 25kDa, and indistinct smears in FTLD-U and ALS cases but not in control and AD cases (see Figs 4B–F). The intensity of the approximately 25kDa fragments tended to be greater than that of the 45kDa band in FTLD-U (see Figs 4B–E) and in ALS (see Figs 4B, D). As for the immunohistochemical findings, the antibody to pS409/410 showed the most intense labeling (see Fig 4B). All of the immunoreactive bands were completely abolished by dephosphorylation, which was performed with lambda protein phosphatase (8,000U/ml; New England Biolabs) at 30°C for 2 hours.

Immunoblot Distinction between Clinicopathological Subtypes of TDP-43

To investigate the biochemical basis of the different TDP-43 clinicopathological subtypes, we have carefully compared the results of immunoblots of the sarkosyl-insoluble, urea-soluble fractions from cerebral cortex of sporadic FTLD-U, FTLD-MND, ALS, and mPGRN cases. The results showed that the band patterns of the 18 to 26kDa fragments differed between clinicopathological subtypes (Figs 5A, B). Sporadic FTLD-U cases showed 2 major bands at 23 and 24kDa, and 2 minor bands at 18 and 19kDa, whereas FTLD-MND and ALS cases showed 3 major bands at 23, 24, and 26kDa, and 2 minor bands at 18 and 19kDa. A 23kDa band is the most intense in sporadic FTLD-U, whereas a 24kDa band is the most intense in FTLD-MND and ALS. Furthermore, the band pattern of mPGRN cases was not distinctive but intermediate between FTLD-U, FTLD-MND, and ALS cases.

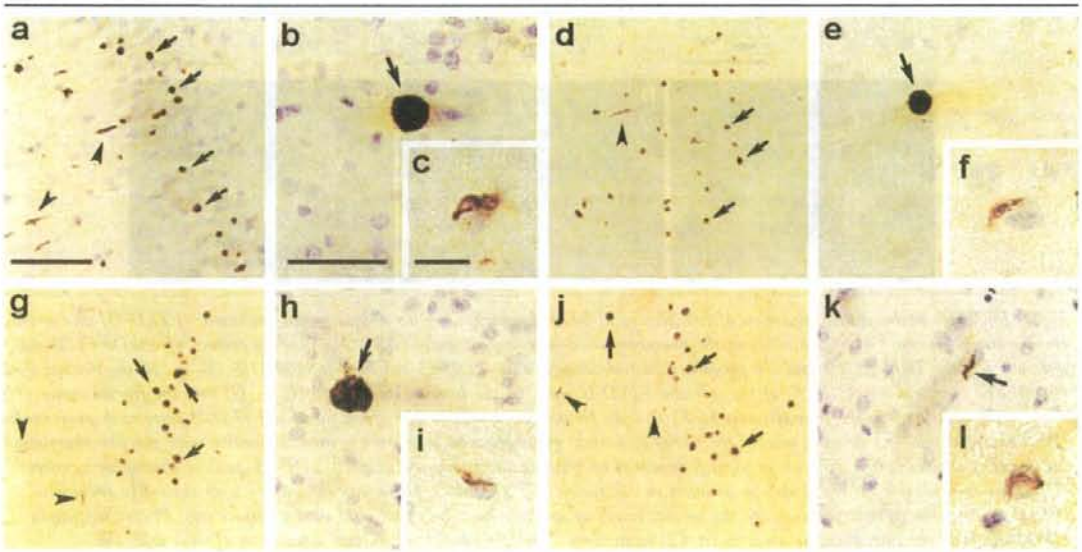

Fig 2. Immunohistochemistry of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) brains and amyotrophic lateral sclerosis (ALS) spinal cords using the phosphorylation-dependent anti–TAR DNA-binding protein of 43kDa (TDP-43) antibodies specific for pS379 (A–C), pS403/404 (D–F), pS409 (G–I), and pS410 (J–L). These antibodies recognize neuronal cytoplasmic inclusions (NCIs) (arrows *in A, D, G, J) and dystrophic neurites (DNs)* (arrowheads *in A, D, G, J) in the dentate gyrus of the sporadic FTLD-U cases and motoneuronal round inclusions* (arrow *in B, E, H), skein-like inclusion (K,* arrow*), and glial inclusions (C, F, I, L) in the lumber spinal cord of the ALS cases. Note the absence of nuclear staining. Sections are counterstained with hematoxylin to show nuclei in (A–C, E, F, H, I, K, L). Scale bars = 100μm (A, D, G, J); 25μm (B, E, H, K); 12.5μm (C, F, I, L).*

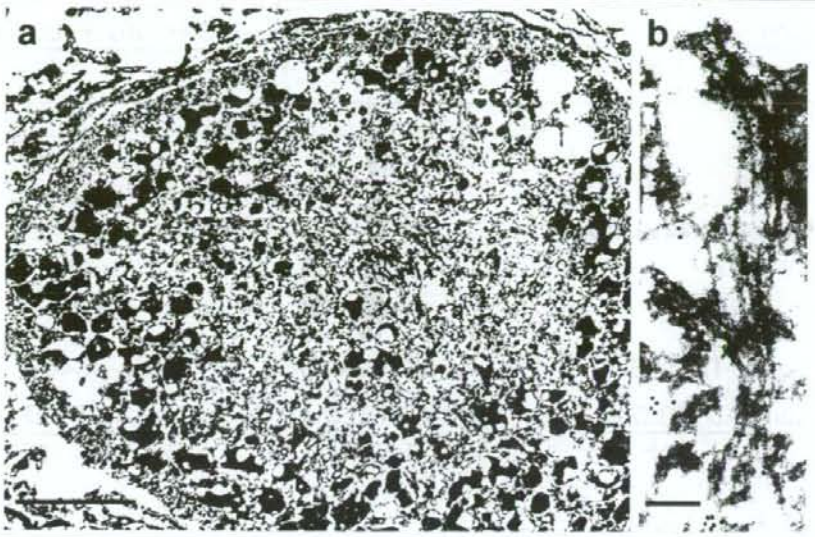

Fig 3. (A) A low-power immunoelectron micrograph of a phosphorylated TAR DNA-binding protein of 43kDa (TDP-43)–positive motoneuronal inclusion in the spinal cord of an amyotrophic lateral sclerosis (ALS) patient. The irregularly shaped structure surrounded by lipofuscins (arrowheads) *is the inclusion. (B) At higher magnification, abnormal filaments of 15nm in diameter are immunopositive. Immunoreaction with pS409/410, probed with immunogold particles (diameter 10nm), appears as* black dots. *Bars = 5μm (A); 500nm (B).*

Phosphorylation Epitopes Are Generated by Casein Kinase-1

To investigate the kinase responsible for the abnormal phosphorylation of TDP-43, we treated recombinant TDP-43 in vitro with CK1, CK2, and GSK3β. Immunoblot analyses of the recombinant TDP-43 showed that phosphorylation by CK1 caused a reduction in gel mobility of TDP-43 to approximately 45kDa and strong immunoreactivity to the phosphorylation-specific antibodies (Fig 6A). TDP-43 phosphorylated by CK2 was only weakly immunoreactive for these antibodies (see Fig 6A), and that phosphorylated by GSK3β was negative (data not shown). Kinase activity capable of generating the approximately 45kDa TDP-43 with pS409/410 epitopes was also detected in crude rat brain extracted with a high concentration (10–20mM) of $MgCl_2$ (data not shown). This kinase activity was not inhibited by the CK2 inhibitor heparin, suggesting that CK1 may be the major kinase in brain extract. Interestingly, increased levels of sodium dodecyl sulfate–stable TDP-43 oligomers were observed after phosphorylation by CK1 (see Fig 6B). Furthermore, based on immunoelectron microscopic analysis, recombinant TDP-43 phosphorylated by CK1 formed abundant filaments when applied on a carbon-coated copper grid (see Fig 6C), whereas nonphosphorylated recombinant TDP-43 formed few filaments (data not shown).

Discussion

We show here that antibodies generated to multiple TDP-43 phosphorylation sites stain the pathological structures in FTLD-U and ALS. These structures include NCIs, NIIs, and DNs in the cerebral cortex and hippocampus, as well as skein-like, round, and glial inclusions in the spinal cord. The phosphorylation-dependent antibodies stain these structures more extensively than an anti-ubiquitin antibody and do not stain normal neuronal nuclei. Furthermore, on immunoelectron microscopy, the phosphorylation-dependent antibodies label abnormal filaments in the motoneuronal inclusion of the ALS case, although these findings may not be the same as for other types of cytoplasmic and intranuclear inclusions.[26] Immunoblot analysis of sarkosyl-insoluble fractions from FTLD-U and ALS brains shows that these antibodies specifically stain abnormal TDP-43 species. These findings are therefore analogous to previous discoveries of phosphorylation-specific epitopes for tau and α-synuclein in tauopathies and α-synucleinopathies.[27–29]

At least five sites on TDP-43 are phosphorylated (Ser 379, Ser 403/404, Ser 409/410) in subjects with FTLD-U and ALS. These results suggest that abnormal phosphorylation takes place mainly near the carboxyl (C)-terminal region of TDP-43. This again is similar to tauopathies and synucleinopathies,[27,28] where multiple Ser residues in the C-terminal region, including

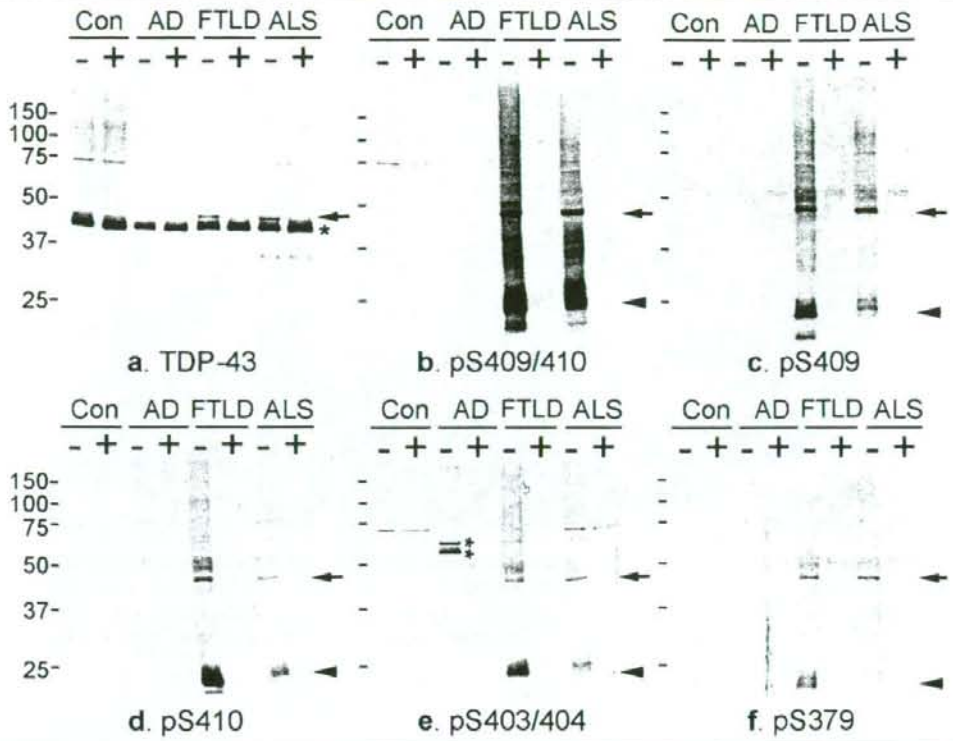

Fig 4. (A) Immunoblot analyses of sarkosyl-insoluble, urea-soluble fractions from control, Alzheimer's disease (AD), frontotemporal lobar degeneration (FTD; frontotemporal lobar degeneration with ubiquitinated inclusions [FTLD-U]), and amyotrophic lateral sclerosis (ALS) brains with phosphorylation-independent anti–TAR DNA-binding protein of 43kDa (TDP-43) antibody (Protein-Tech) (A) and phosphorylation-dependent anti–TDP-43 antibodies specific for pS409/410 (B), pS409 (C), pS410 (D), pS403/404 (E), and pS379 (F) before (−) and after (+) the treatment with lambda protein phosphatase (λPPase). (A) With the phosphorylation-independent antibody, a positive band of 43kDa is commonly seen (asterisk), *whereas an additional band of 45kDa is observed only in FTD and ALS* (arrow), *the labeling of which is abolished after dephosphorylation. (B–F) The phosphorylation-dependent antibodies specifically label the approximately 45kDa band* (arrow) *and the approximately 25kDa fragment* (arrowhead), *as well as a smear, only in FTD and ALS. These immunoreactivities are abolished after dephosphorylation. Normal 43kDa TDP-43 in control and diseased brains is not stained by these phosphorylation-dependent antibodies. The two bands recognized by the antibody specific for pS403/404 in AD (E,* double asterisk*) disappear after dephosphorylation, suggesting a cross-reaction of the antibody to other phosphorylated proteins.*

Ser422 in tau and C-terminal Ser129 in α-synuclein, are abnormally phosphorylated. It has been established that hyperphosphorylated tau and α-synuclein represent the earliest detectable molecular change in the brain in these neurodegenerative diseases.[29,30] Thus, the results of this study suggest that abnormally phosphorylated TDP-43 is a critical component of UPIs in FTLD-U and ALS.

There is a close relation between the pathological subtypes of TDP-43 proteinopathy and the immunoblot pattern of C-terminal fragments of phosphorylated TDP-43. These findings confirm and extend Sampathu and colleagues'[31] and Neumann and coworkers'[12] previous reports that showed C-terminal fragment composition varied between cases with type 1 and 2 pathology. Furthermore, we have shown that cases with type 3 pathology have a band pattern that is mixed or intermediate. These results parallel our earlier findings of differing C-terminal tau fragments in progressive supranuclear palsy and corticobasal degeneration, despite identical composition of tau isoforms.[32] Taken together, these results suggest that elucidating the mechanism of C-terminal fragment origination may shed light on the pathogenesis of several neurodegenerative disorders involving TDP-43 proteinopathy and tauopathy.

These phosphorylation-specific antibodies are a new and powerful tool for the investigation of TDP-43 pro-

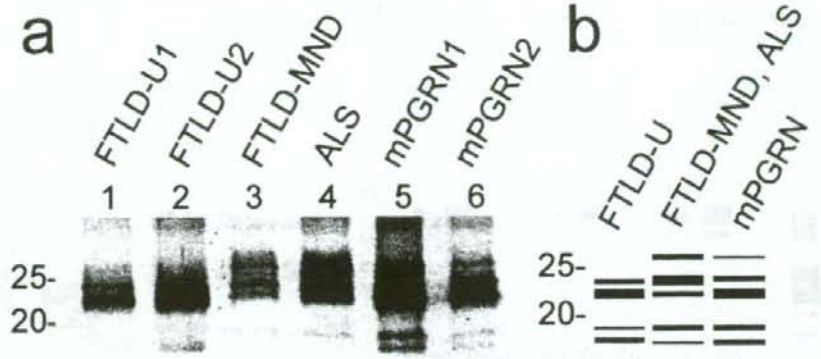

Fig 5. A relation between the clinicopathological subtypes of TAR DNA-binding protein of 43kDa (TDP-43) proteinopathies and the band pattern of the C-terminal fragments of phosphorylated TDP-43. (A) Immunoblots of the sarkosyl-insoluble, urea-soluble fractions from sporadic frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U), FTLD-motor neuron disease (MND), amyotrophic lateral sclerosis (ALS), and progranulin mutations (mPGRN) cases with the pS409/410 antibody. The samples are loaded on 15% polyacrylamide gel. Sporadic FTLD-U cases (lanes 1, 2) show a band pattern with 2 major bands at 23 and 24kDa, and 2 minor bands at 18 and 19kDa. A band of 24kDa is weaker than that of 23kDa, and a 19kDa band is weaker than an 18kDa band. FTLD-MND (lane 3) and ALS (lane 4) cases show a pattern with 3 major bands at 23, 24, and 26kDa, and 2 minor bands at 18 and 19kDa. A 24kDa band is the most intense, and an 18kDa band is weaker than a 19kDa band. mPGRN (lanes 5, 6) cases show 3 major bands at 23, 24, and 26kDa, and 2 minor bands at 18 and 19kDa. A 23kDa band is the most intense, and a band of 18kDa and that of 19kDa show similar intensity. The band pattern of mPGRN cases is therefore a composite of that seen in FTLD-U, FTLD-MND, and ALS. (B) Schematic diagram of the band pattern of the C-terminal fragments of phosphorylated TDP-43.

teinopathies. Because phosphorylation-dependent antibodies to TDP-43 react only with abnormally deposited TDP-43, they offer advantages over existing commercially available antibodies for the pathological diagnosis and subtyping of TDP-43 proteinopathies. In addition, and again in analogy with tauopathies, these antibodies may be useful for detecting abnormal TDP-43 in biological fluids such as cerebrospinal fluid.[33]

The results suggest that CK1 is involved in the abnormal phosphorylation and accumulation of TDP-43. In this study, the treatment of recombinant TDP-43 by CK1 generates the same phosphorylation epitopes that are recognized by phosphorylation-dependent antibodies. In addition, phosphorylation at these epitopes facilitates filament formation. In comparison, several protein kinases have been reported to be responsible for phosphorylating tau and α-synuclein. They include, for tau phosphorylation,[34–37] GSK3β, cyclin-dependent kinase 5, mitogen-activated protein kinase, and mitogen-activated protein/microtubule affinity-regulating kinase, and for α-synuclein phosphorylation,[38–40] CK1, CK2, and G-protein–coupled receptor kinase 5.

The pathological significance of phosphorylation of TDP-43 is not clear. It is well known that protein phosphorylation plays an important role in regulating transcription and premessenger RNA splicing. Several splicing factors including hnRNPs, small nuclear ribonucleoproteins, and serine/arginine-rich protein family are known to be phosphorylated in vivo. Various kinases including CK1 have been implicated in phosphorylating these factors.[41–43] Phosphorylation of these factors modulates protein-protein and protein-RNA interactions, and affects their subcellular localization and physiological functions.[41] For instance, Habelhah and colleagues[44] showed that phosphorylation of hnRNP-K by extracellular-signal–regulated kinase results in its cytoplasmic accumulation and also inhibits messenger RNA translation. van der Houven van Oordt and coauthors reported that stress-induced activation of the mitogen-activated protein kinase kinase$_{3/6}$-p38 pathway causes hyperphosphorylation and cytoplasmic accumulation of hnRNP A1, affecting alternative splicing regulation.[45] Thus, phosphorylation of TDP-43 may lead to its cytoplasmic accumulation and influence various physiological functions. Currently, however, it is unclear whether TDP-43 is physiologically phosphorylated in brain. Although in HeLa cells, Ser91 and Ser92 of TDP-43 were reported to be phosphorylated,[46] the antibody specific to pS91/pS92 we made in this study did not stain any structures in normal brains (data not shown). Despite the normal nuclear location of TDP-43, none of the our five phosphorylation-

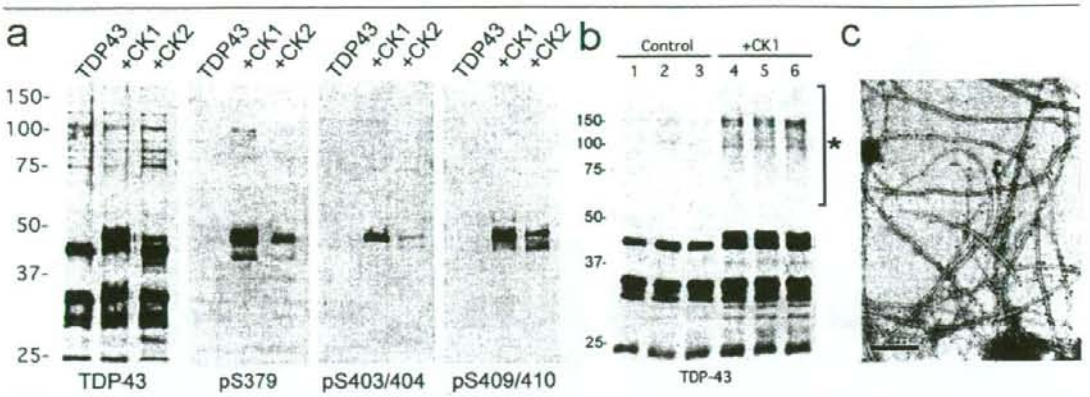

Fig 6. (A) Immunoblot analyses of recombinant TAR DNA-binding protein of 43kDa (TDP-43) phosphorylated in vitro. The crude extract from E. coli *that expressed human TDP-43 is treated with casein kinase-1 (CK1) and CK2 at 30°C for 14 hours, and probed with a phosphorylation-independent antibody against a C-terminal peptide of TDP-43 (405–414), and with phosphorylation-dependent antibodies pS379, pS403/404, and pS409/410. Phosphorylation by CK1 causes the mobility shift to approximately 45kDa and induction of intense immunoreactivity to the phosphorylation-dependent antibodies. (B) Immunoblot analyses of recombinant TDP-43 phosphorylated by CK1. The recombinant TDP-43, which is partially purified by heparin-Toyopearl column chromatography, is incubated with (lanes 4–6) or without (lanes 1–3) CK1 in the presence of adenosine triphosphate at 37°C for 14 hours, and probed with the phosphorylation-independent TDP-43 antibody (ProteinTech). Results in three independent, representative experiments are shown. Note the sodium dodecyl sulfate (SDS)–stable TDP-43 oligomers at approximately 100 to 200kDa* (asterisk) *are detected after phosphorylation by CK1. (C) Positive immunolabeling by pS409/410 of filaments assembled from recombinant TDP-43 phosphorylated by CK1 (10nm colloidal gold). Scale bar = 200nm.*

dependent antibodies stained normal nuclei, suggesting that phosphorylation of these sites is a disease-specific phenomenon.

Our in vitro studies suggest that phosphorylation of TDP-43 facilitates the formation of sodium dodecyl sulfate–stable oligomers and filaments of TDP-43. These abnormal structures may be neurotoxic, as suggested previously for tauopathies and α-synucleinopathies.[30] Thus, abnormal phosphorylation of TDP-43 may be pathological through either a loss of function or a toxic gain of function, or both, leading to the characteristic neuronal degeneration and clinical syndromes.

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ORIGINAL PAPER

Phosphorylated TDP-43 in Alzheimer's disease and dementia with Lewy bodies

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Abstract Phosphorylated and proteolytically cleaved TDP-43 is a major component of the ubiquitin-positive inclusions in the most common pathological subtype of frontotemporal lobar degeneration (FTLD-U). Intracellular accumulation of TDP-43 is observed in a subpopulation of patients with other dementia disorders, including Alzheimer's disease (AD) and dementia with Lewy bodies (DLB). However, the pathological significance of TDP-43 pathology in these disorders is unknown, since biochemical features of the TDP-43 accumulated in AD and DLB brains, especially its phosphorylation sites and pattern of fragmentation, are still unclear. To address these issues, we performed immunohistochemical and biochemical analyses of AD and DLB cases, using phosphorylation-dependent anti-TDP-43 antibodies. We found a higher frequency of pathological TDP-43 in AD (36–56%) and in DLB (53–60%) than previously reported. Of the TDP-43-positive cases, about 20–30% showed neocortical TDP-43 pathology resembling the FTLD-U subtype associated with progranulin gene (*PGRN*) mutations. Immunoblot analyses of the sarkosyl-insoluble fraction from cases with neocortical TDP-43 pathology showed intense staining of several low-molecular-weight bands, corresponding to C-terminal fragments of TDP-43. Interestingly, the band pattern of these C-terminal fragments in AD and DLB also corresponds to that previously observed in the FTLD-U subtype associated with *PGRN* mutations. These results suggest that the morphological and biochemical features of TDP-43 pathology are common between AD or DLB and a specific subtype of FTLD-U. There may be genetic factors, such as mutations or genetic variants of *PGRN* underlying the co-occurrence of abnormal deposition of TDP-43, tau and α-synuclein.

Keywords Phosphorylation · Fragmentation · Frontotemporal lobar degeneration · Progranulin · Tau · Alpha-synuclein · TDP-43

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Introduction

TAR DNA-binding protein of M_r 43 kDa (TDP-43) is a major component of the tau-negative and ubiquitin-positive inclusions that characterize the most common pathological subtype of frontotemporal lobar degeneration (FTLD-U) and amyotrophic lateral sclerosis (ALS) [2, 9, 24, 31, 32, 38]. Several genes and chromosomal loci, including the progranulin gene (*PGRN*) [4, 8], valosin-containing protein gene (*VCP*) [42] and an unidentified gene at chromosome 9p [28, 41], have been reported to be associated with familial forms of FTLD-U. Recent findings of various missense mutations of TDP-43 gene (*TARDBP*) in familial and sporadic ALS cases prove the essential role of abnormal TDP-43 in neurodegeneration [12, 20, 37, 40, 43]. These disorders are now collectively referred to as TDP-43 proteinopathies [2, 9, 31, 32].

Ubiquitin- and TDP-43-positive pathological inclusions found in FTLD-U include neuronal cytoplasmic inclusions (NCIs), dystrophic neurites (DNs), neuronal intranuclear inclusions (NIIs), and glial cytoplasmic inclusions [2, 25, 26, 32, 35]. Based on the cerebral ubiquitin immunohistochemistry, FTLD-U was classified into three subtypes by Sampathu et al. [35] and Mackenzie et al. [25]. Unfortunately, the numbering schemes used in these two systems do not match. Type 1 by Sampathu et al. or Type 2 by Mackenzie et al. is characterized by DNs with few NCIs and no NIIs. Type 2 by Sampathu et al. or Type 3 by Mackenzie et al. has numerous NCIs with few DNs and no NIIs. Type 3 by Sampathu et al. or Type 1 by Mackenzie et al. has numerous NCIs and DNs and occasional NIIs. This is the pattern found in all cases of FTD caused by mutations in *PGRN* [7, 25]. Recently, Cairns et al. [7] drew these two systems together into a unified scheme, and added familial FTLD-U with *VCP* mutations as Type 4, which has numerous NIIs and DNs with few NCIs. Since they adopted the numbering system by Sampathu et al. in their consensus paper, we will use that for the rest of this paper.

Biochemical analyses of the detergent-insoluble fraction extracted from brains of patients afflicted with FTLD-U showed that TDP-43 accumulated in these pathological structures is composed of abnormal C-terminal fragments that are phosphorylated and ubiquitinated [2, 32]. Using antibodies specific for phosphorylated TDP-43 (pTDP-43), made by ourselves, we previously identified several phosphorylation sites in the C-terminal region of the TDP-43 that accumulates in FTLD-U brains [14]. Furthermore, we found a close relationship between the pathological subtypes of FTLD-U and the immunoblot pattern of phosphorylated C-terminal fragments of TDP-43, suggesting that proteolytic processing may be crucial in TDP-43 proteinopathy [14].

Recently, immunohistochemical examination, using commercially available phosphorylation-independent anti-TDP-43 antibodies, has demonstrated abnormal intracellular accumulation of TDP-43 in neurodegenerative disorders other than FTLD-U and ALS. These include Alzheimer's disease (AD), dementia with Lewy bodies (DLB), Pick's disease, hippocampal sclerosis, corticobasal degeneration, Huntington disease and argyrophilic grain disease [1, 11, 15, 17, 19, 23, 30, 36, 39]. However, the pathological significance of TDP-43 accumulation in these disorders is unclear, since it takes place only in a subpopulation of the patients with most of these disorders. Moreover, although the morphology of the TDP-43 positive structures has been described, the biochemical features of accumulated TDP-43, especially its phosphorylation sites and fragmentation, are still unclear in these disorders. To address these issues, in the present study, we performed detailed immunohistochemical and biochemical analyses of TDP-43 in cases of AD and DLB, using our phosphorylation-dependent anti-TDP-43 antibodies. We find a relatively higher frequency of TDP-43 deposition in AD and DLB than previously reported. When TDP-43 pathology occurs in the neocortex of cases with AD and DLB, the pattern is Type 3. In these cases, the accumulated TDP-43 demonstrates abnormal C-terminal phosphorylation and fragmentation. These results suggest the presence of a common mechanism underlying the abnormal processing and accumulation of TDP-43 in AD, DLB and a specific subtype of FTLD-U.

Materials and methods

Materials

We studied two independent series of cases (Table 1). The first was comprised of 53 AD cases and 15 DLB cases from the institutional collections at the Department of Psychogeriatrics, Tokyo Institute of Psychiatry in Japan. The second series included 25 AD cases and 10 DLB cases from the Canadian Collaborative Cohort of Related Dementia (ACCORD) study, a well-characterized memory clinic population, prospectively followed to death [10]. The second series provided validation of the findings from the first series, included examination of some additional neuroanatomical regions not available in the first series and tested whether similar results could be obtained by using more traditional immunohistochemical methodology.

Neuropathological diagnoses of AD and DLB were made in accordance with published guidelines [27, 33] for both series. Two cases from the first series and one case from the second series had little AD pathology, corresponding to the pure form of diffuse Lewy body disease (DLBD) [21].

Table 1 Demographics and pathology of all cases employed in this study

	Alzheimer's disease						Dementia with Lewy bodies					
	First series ($N = 53$)			Second series ($N = 25$)			First series ($N = 15$)			Second series ($N = 10$)		
	TDP-43 positive	TDP-43 negative	*P* value	TDP-43 positive	TDP-43 negative	*P* value	TDP-43 positive	TDP-43 negative	*P* value	TDP-43 positive	TDP-43 negative	*P* value
Number of cases (%)	19 (36%)	34 (64%)		14 (56%)	11 (44%)		8 (53%)	7 (47%)		6 (60%)	4 (40%)	
Mean age at death ± SD (years)	82.8 ± 7.5	78.8 ± 10.7	0.16	81.2 ± 7.0	72.7 ± 9.2	0.015*	73.5 ± 12.8	81.7 ± 6.5	0.15	76.3 ± 7.4	71.0 ± 9.5	0.35
Sex, M:F	11:8	20:14	0.95	8:6	5:6	0.56	6:2	5:2	>0.99	4:2	3:1	>0.99
Median Braak NFT stage (25th, 75th percentile)	5 (5.0, 6.0)	5 (4.0, 5.0)	0.027*	6 (6.0, 6.0)	6 (6.0, 6.0)	0.7	4 (3.0, 6.0)	4 (3.25, 4.0)	0.54	4.5 (2.0, 6.0)	6 (6.0, 6.0)	0.11
Brain weight ± SD (g)	1,117 ± 162	1,116 ± 137	0.98	NA	NA		1,123 ± 151	1,144 ± 146	0.79	NA	NA	

Immunohistochemistry

For the first series, small blocks of brain were dissected at autopsy and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 2 days. Following the cryoprotection in 15% sucrose in 0.01 M phosphate-buffered saline (PBS, pH 7.4), blocks were cut on a freezing microtome at 30 μm thickness. The free floating sections were incubated with 0.5% H_2O_2 for 30 min to eliminate endogenous peroxidase activity in the tissue. After washing with PBS containing 0.3% Triton X-100 (Tx-PBS) for 30 min, sections were blocked with 10% normal serum, and then incubated with the primary antibody for 72 h in the cold. Following treatment with the appropriate secondary antibody, labeling was detected using the avidin–biotinylated HRP complex (ABC) system (Vector Laboratories, Burlingame, CA) coupled with a diaminobenzidine (DAB) reaction to yield a brown precipitate, or with a DAB reaction intensified with nickel ammonium sulfate to yield a dark purple precipitate, as previously described [2, 3, 13, 14]. For the second series, immunohistochemistry was performed on 5-μm-thick sections of formalin-fixed, paraffin-embedded tissue, using the Ventana BenchMark® XT automated staining system (Ventana, Tuscon, AZ), as previously described [25]. Prior to immunostaining, sections underwent microwave antigen retrieval for 13 min in citrate buffer, pH 6.0. Immunoreactions were developed with aminoethylcarbizole (AEC).

In the first series, the presence and severity of pTDP-43 immunoreactivity was assessed in amygdala (where available), hippocampus, entorhinal cortex and temporal neocortex. More extensive anatomical sampling was available in the second series allowing pathology to be assessed in the amygdala, hippocampus, entorhinal cortex, cingulate gyrus, temporal neocortex, frontal neocortex and parietal neocortex. pTDP-43 pathology was semiquantitatively scored based on a five-point grading scale (−, none; ±, rare; +, mild; ++, moderate; +++, severe). The primary antibodies used in this study and their dilutions are summarized in Table 2. Only pTDP-43-specific antibodies (pS409/410 and pS403/404) were employed in the first series, while both phosphorylation-independent commercial anti-TDP-43 antibody and pTDP-43-specific antibodies were employed in the second series. In the second series, all the same cases were stained with both antibodies but the pTDP-43-specific antibodies often stained a greater amount of pathology.

Statistical analyses

Unpaired Student's *t* tests were used to analyze differences between groups for age and brain weights, whereas the Braak stage score was analyzed with Mann–Whitney

Table 2 Antibodies used in this study

Antibody	Type	Source	Dilution
Phosphorylation-independent anti-TDP-43			
Anti-TDP-43	Rabbit polyclonal (affinity purified)	ProteinTech, Chicago, IL	1:1,000 (IB, IHC-P)
Phosphorylation-dependent anti-TDP-43			
pS409/410	Rabbit serum	[a]	1:1,000 (IB, IHC-P), 1:10,000 (IHC-F), 1:5,000 (IF)
pS403/404	Rabbit serum	[a]	1:1,000 (IB, IHC-P), 1:10,000 (IHC-F), 1:5,000 (IF)
Anti-tau			
AT8	Mouse monoclonal	Innogenetics, Gent, Belgium	1:2,000 (IHC-P), 1:100 (IF)
Anti-α-synuclein			
pα#64	Mouse monoclonal	Wako Chemical, Osaka, Japan	1:3,000 (IF)
Anti-α-synuclein	Mouse monoclonal	Invitrogen, Burlington, ON, Canada	1:10,000 (IHC-P)
Anti-amyloid β protein			
6F3D	Mouse monoclonal	DAKO, Mississauga, ON, Canada	1:100 (IHC-P)

IB immunoblotting, *IHC-P* immunohistochemistry in paraffin-embedded sections, *IHC-F* immunohistochemistry in free-floating sections, *IF* immunofluorescence

[a] Made by ourselves [14]

U test. χ^2 and Fisher's exact test were used to analyze the difference between groups for sex.

Confocal microscopy

For double labeling immunofluorescence for pTDP-43 and phosphorylated tau in AD or for pTDP-43 and phosphorylated α-synuclein in DLB, 4% PFA-fixed and free floating sections from the first series were used. The sections were incubated overnight at 4°C in a cocktail of pS409/410 or pS403/404 and AT8 or pα#64. After washing with Tx-PBS for 30 min, sections were incubated for 2 h at room temperature in a cocktail of Fluorescein isothiocianate (FITC)-conjugated goat anti-mouse IgG (1:100, Millipore, Temecula, CA) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (1:100, Millipore). After washing, sections were incubated in 0.1% Sudan Black B for 10 min at room temperature and washed with Tx-PBS for 30 min. Sections were coverslipped with Vectashield (Vector Laboratories) and observed with a confocal laser microscope (LSM5 PASCAL; Carl Zeiss MicroImaging gmbh, Jena, Germany).

Immunoblotting

Sarkosyl-insoluble, urea-soluble fractions were extracted from the temporal lobe of an autopsied case with no neurological abnormality as a normal control and cases with AD, DLB and FTLD-U, as previously described [13, 14]. For SDS-PAGE of the samples, 15% polyacrylamide gel was used to visualize low-molecular weight fragments of accumulated TDP-43 clearly as previously reported [14]. Proteins in the gel were then electrotransferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). After blocking with 3% gelatin in Tris-buffered saline (20 mM Tris–HCl, pH 7.5, 500 mM NaCl), membranes were incubated overnight with pS409/410 or pS403/404. Following incubation with an appropriate biotinylated secondary antibody, labeling was detected using the ABC system coupled with a DAB reaction intensified with nickel chloride.

Results

Immunohistochemical analyses

Accumulation of phosphorylated TDP-43 in AD

As we have described previously, antibodies against pTDP-43 demonstrated abnormal structures only and did not show the diffuse nuclear staining pattern typical of normal TDP-43 [14]. The two primary antibodies (pS409/410 and pS403/404) labeled similar pathological structures with similar sensitivity. pTDP-43-positive structures were present in 36% (19/53) of the first AD series (Tables 1, 3) and in 56% (14/25) of the second AD series (Tables 1, 4) with highly variable severity and regional distribution among these cases. The frequency of pTDP-43 immunoreactivity in the two series was not significantly different ($\chi^2 = 2.826$; 1 *df*; $P = 0.093$). pTDP-43-positive NCIs and DNs were variably present in the amygdala, hippocampus, parahippocampal

Table 3 TDP-43-positive structures in the first series of Alzheimer's disease

Case no.	Age	Sex	SP (CERAD)	NFT (Braak)	Amyg	DG	CA4	CA2/3	CA1	Sub	EC	Temp	TDP-43 path
F-AD1	86	M	NA	NA	+++	NA	NA	NA	NA	++	+++	+++	Diffuse
F-AD2	85	F	C	V	NA	+++	++	++	++	+	+++	+++	Diffuse
F-AD3	80	F	C	V	NA	++	+	+	++	++	+++	+++	Diffuse
F-AD4	67	F	C	VI	NA	±	−	−	+	++	+++	+++	Diffuse
F-AD5	82	M	NA	NA	NA	++	−	±	++	++	++	−	Limbic
F-AD6	85	F	C	VI	NA	+	−	±	±	+	++	−	Limbic
F-AD7	86	M	C	V	+	+	−	−	±	+	++	−	Limbic
F-AD8	86	M	NA	NA	−	−	−	−	±	+	+	−	Limbic
F-AD9	75	M	C	VI	+	+	±	±	±	+	+	±	Limbic
F-AD10	77	M	C	V	NA	NA	NA	NA	NA	++	−	−	Limbic
F-AD11	78	M	C	VI	NA	±	−	−	±	+	−	−	Limbic
F-AD12	96	M	NA	NA	NA	−	−	−	−	+	−	−	Limbic
F-AD13	91	F	C	IV	NA	−	−	−	−	+	−	−	Limbic
F-AD14	89	F	C	V	NA	−	−	−	−	+	−	−	Limbic
F-AD15	81	F	C	V	++	±	−	−	−	+	+	−	Limbic
F-AD16	93	M	C	V	NA	−	−	−	−	+	−	−	Limbic
F-AD17	75	M	C	VI	−	−	−	−	+	+	+	−	Limbic
F-AD18	89	M	C	V	+	−	−	−	−	−	+	−	Limbic
F-AD19	72	F	NA	NA	+	NA	NA	NA	NA	NA	NA	NA	NA

SP Senile plaque, *NFT* neurofibrillary tangle, *Amyg* amygdala, *DG* dentate gyrus, *Sub* subiculum, *EC* entorhinal cortex, *Temp* temporal cortex, *path* pathology, *NA* not available

−, None; ±, slight; +, mild; ++, moderate; +++, severe

Table 4 TDP-43-positive structures in the second series of Alzheimer's disease

Case no.	Age	Sex	SP (CERAD)	NFT (Braak)	Amyg	DG	CA4	CA2/3	CA1	Sub	EC	Cing	Temp	Front	Par	TDP-43 path
S-AD1	72	F	C	VI	+++	++	+	+	+++	+++	+++	+	+++	+	−	Diffuse
S-AD2	85	M	C	V	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	Diffuse
S-AD3	84	M	C	V	+++	+++	+	++	++	+++	+++	++	+++	+++	+	Diffuse
S-AD4	89	M	C	VI	+++	++	±	+	+++	+++	+++	+	++	−	−	Diffuse
S-AD5	81	F	C	VI	+++	++	−	±	+++	+++	+++	+	++	−	−	Diffuse
S-AD6	80	M	C	VI	+++	+	±	+	+++	++	++	±	−	−	−	Limbic
S-AD7	80	F	C	VI	++	±	−	−	++	++	++	−	−	−	−	Limbic
S-AD8	89	F	C	VI	±	+	±	+	+++	+++	+	−	−	−	−	Limbic
S-AD9	84	F	C	VI	++	−	−	−	±	+	+	−	−	−	−	Limbic
S-AD10	95	M	C	VI	++	−	−	−	±	±	+	−	−	−	−	Limbic
S-AD11	74	F	C	VI	±	−	−	−	−	−	−	−	−	−	−	Amygdala
S-AD12	72	M	C	VI	±	−	−	−	−	−	−	−	−	−	−	Amygdala
S-AD13	79	M	C	VI	+	−	−	−	−	−	−	−	−	−	−	Amygdala
S-AD14	73	M	C	VI	±	−	−	−	−	−	−	−	−	−	−	Amygdala

SP Senile plaque, *NFT* neurofibrillary tangle, *Amyg* amygdala, *DG* dentate gyrus, *Sub* subiculum, *EC* entorhinal cortex, *Cing* cingulate cortex, *Temp* temporal cortex, *Front* frontal cortex, *Par* parietal cortex, *path* pathology

−, None; ±, slight; +, mild; ++, moderate; +++, severe

gyrus and neocortex (Fig. 1). In cases where the neocortex was involved, NCIs and DNs were predominantly distributed in the upper layers, most closely resembling FTLD-U type 3. Moreover, most of these cases also had a few NIIs with a lentiform shape in the dentate gyrus or the neocortex, similar to those characteristic of cases with *PGRN* mutations [26] (Fig. 1k, inset). Neurofibrillary tangle-like pTDP-43-positive structures were occasionally found in the CA1 region (Fig. 1f). Small round, short thread-like or coiled body-like structures were sometimes observed in the white