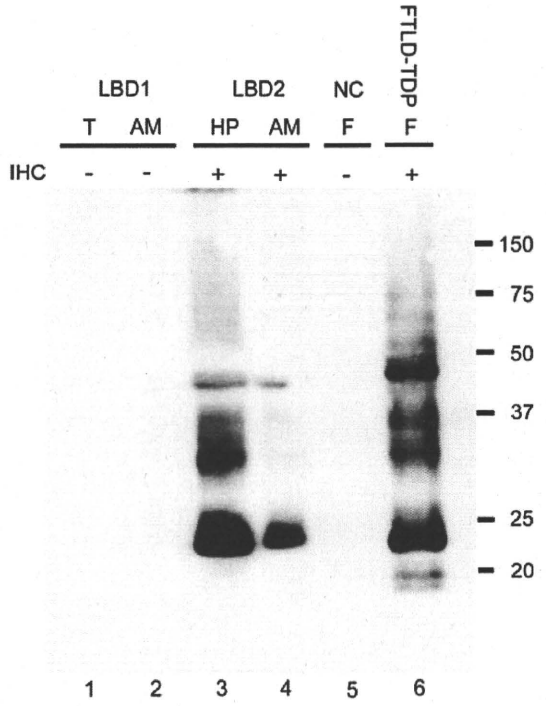

Fig. 6 Immunoblot analysis of the sarkosyl-insoluble fraction in representative LBD cases with phosphorylation-dependent monoclonal anti-TDP-43 antibody (mAb pS409/410). The approximately 45 and 25 kDa fragments, as well as smears are strongly labeled in a LBD case with TDP-43 pathology (*lanes 3* and *4*) and a FTLD-TDP case (*lane 6*). These 45 and 25 kDa bands and smears were not labeled in any other cases without detectable TDP-43 pathology by immunohistochemistry (*lanes 1*, *2*, and *5*). Normal 43 kDa TDP-43 is not stained by this phosphorylation-dependent antibody in any cases. *LBD* Lewy body disease, *NC* normal control, *AM* amygdala, *HP* hippocampus, *F* frontal cortex, *T* temporal cortex, *IHC* pAb pS409/410 immunohistochemistry

Nakashima-Yasuda series was about 50% (91 of 180 LBD cases), this being smaller than that in our series (66.1%). Considering that several studies have suggested a possible relationship between TDP-43 accumulation and the severity of tau pathology in several tauopathies [2, 8, 28], it is plausible that the differences regarding the degree of tau pathology might have influenced the overall frequency of TDP-43 pathology in LBD series. More recently, Arai et al. [2], using the same phosphorylation-dependent TDP-43 antibody employed in the present study, reported TDP-43 pathology in up to 56% of DLB and DLB + AD cases. Although Arai et al. did not present detailed data regarding tau and α-synuclein pathologies, the degree of tau pathology, at least, in their TDP-43-positive LBD cases tended to be more severe than that in our TDP-43-positive cases: 43% of their TDP-43-positive LBD cases was classified as having severe tau pathology of Braak NFT stages V–VI (compared to 20% in the present TDP-43-positive cases). Because of the relative paucity of cases having severe tau pathology of Braak NFT stages V–VI in our series (2 of 5 cases, 40%), it is difficult to discuss about the significance of the frequency of TDP-43 pathology in this subpopulation of LBD cases. However, similar frequencies have been observed in some subgroups in Nakashima-Yasuda et al. [28] series where the pathological background may be similar to that in our series. These authors reported that TDP-43 pathology in 47% of LBD cases of Braak NFT stages V–VI (the severity of α-synuclein pathology in this group was not shown), and in 31% of DLB + AD cases (a high and intermediate likelihood for both DLB and AD pathology [27, 31]). We could not fully examine the differences of the severity and distribution of TDP-43 pathology between diffuse neocortical type of LBD cases with and without severe tau pathology, because the number of cases having severe tau pathology was small. The data regarding the difference of TDP-43 pathology between these two groups, including the presence or absence of TDP-43 accumulation in the frontal and occipital cortices, may provide clues to understand the impacts of not only α-synuclein but also tau accumulations on TDP-43 accumulation in LBD cases.

Whereas the degree of α-synuclein pathology is highly variable among LBD cases, there was few previous data available regarding the relationship between the severity of α-synuclein pathology and the development of TDP-43 pathology. In the light of present results, the severity of α-synuclein pathology may be a potential factor for the development of TDP-43 pathology. We suggest that the severity of α-synuclein pathology should be considered when interpreting the frequency of TDP-43 pathology in LBD cases, and probably, in other pathological conditions as well. It is notable that TDP-43 pathology was frequently found in our LBD cases even when severe tau pathology did not coexist, especially in cases of the diffuse neocortical type. Although inconsistent with findings of an early study in which none of ten LBD-Ltau cases had TDP-43 pathology [28], Higashi et al. [14] reported TDP-43 pathology in 3/7 LBD-Ltau cases using a phosphorylation-independent antibody and Arai et al. [2] reported that all of four LBD-Ltau cases in their series had variable degrees of TDP-43 pathology. Our present results agree with these findings and suggest a possible association of α-synuclein and TDP-43 accumulations in LBD-Ltau cases.

On the other hand, in a study by Josephs et al. [20] in which 84 AD cases were examined, multivariate analysis did not demonstrate any significant effect of the presence of α-synuclein pathology on the development of TDP-43 accumulation in AD. In their AD series, although the prevalence of α-synuclein pathology was only 25%, the frequency of α-synuclein pathology in TDP-43-positive cases was significantly higher than in TDP-43-negative cases (38 vs. 18%). No detailed data about the degree of α-synuclein pathology in this series was presented. In the

context of present findings, if the degree of α-synuclein pathology in this series [20] was mild, the effect of α-synuclein pathology on the development of TDP-43 accumulation would not likely be demonstrated.

Although multivariate analysis in our study failed to demonstrate a significant association between tau pathology and TDP-43 accumulation, the result does not necessarily deny the possible effect of tau pathology in LBD-Ltau cases. Since our study was conducted to mainly examine the effect of α-synuclein pathology, the proportion of subjects having severe tau pathology of Braak NFT stages V–VI was low, less than 10%. The low proportion of this subgroup might therefore have influenced our results. Nevertheless, it is notable that tau burden in the hippocampal dentate gyrus tended to be more severe in TDP-43-positive LBD cases, and that tau was often colocalized with TDP-43 in the amygdala. The independent effects on the development of TDP-43 accumulation of tau pathology, as well as that of α-synuclein pathology, need to be further examined in future studies using a multivariate model in a larger number of cases with various degrees of tau and α-synuclein pathologies.

The pathophysiological mechanism underlying the coexistence of α-synuclein and TDP-43 accumulations in the same LBD case remains unclear. It has been reported that some FTLD-TDP cases with progranulin gene mutations had concomitant α-synuclein pathology [5, 24], although the frequency is not high: in a previous study, only one of 18 cases (5.5%) of FTLD with ubiquitin-positive inclusion (FTLD-U) had Lewy pathology [18]. It was also reported that one case of familial PD (α-synuclein A53T heterozygote) had TDP-43 pathology [26]. To our knowledge, the frequency of TDP-43 pathology in familial LBD cases has not been examined. Colocalization of TDP-43 and α-synuclein in DLB cases was demonstrated in two studies [2, 14], being consistent with our findings. An ultrastructural study also demonstrated that filaments and granular material associated with α-synuclein filaments in Lewy bodies were labeled with anti-TDP-43 antibodies [25].

It is difficult to draw any definite conclusions regarding the biological mechanism underlying the coexistence of α-synuclein and TDP-43 in the same neuron or the same case. However, the results presented in this paper suggest that the limbic system, and in particular the amygdala, is vulnerable to the deposition of TDP-43 in LBD, as well as other degenerative diseases including AD [1, 2, 15], AGD [8], and PSP [34]. Therefore, TDP-43 deposition in the amygdala may be a region-specific rather than disease-specific phenomenon. In LBD, α-synuclein deposition in the limbic region may be primary, and TDP-43 secondarily deposits upon pre-existing LBs, generating some colocalization. This hypothesis seems to be supported by observations that some TDP-43-positive inclusions in our cases showed typical morphological features of LBs. However, the existence of TDP-43 deposited separately from α-synuclein accumulation may also suggest that TDP-43 accumulation cannot only be explained by some direct biological synergy between the proteins. Further, severe neurodegeneration associated with α-synuclein deposition might indirectly lead TDP-43 to accumulate in the vulnerable regions, in particular the amygdala. In addition, the possibility that the accumulation of TDP-43 might be associated with aging should not be excluded. Indeed, some previous studies have demonstrated that aging influences the accumulation of TDP-43 in AD [2, 20] and PDD [28], with age at death being later in cases with TDP-43 changes than in those without. Recently, Geser et al. [10] proposed that TDP-43 proteinopathies could be divided into two categories, major TDP-43 proteinopathies (e.g., ALS and FTLD-TDP [3, 6, 7, 29]) and disorders with secondary TDP-43 pathologies (e.g., AD [1, 2, 15], LBD [2, 14, 28], AGD [8], CBD [32], and PSP [34]). Potential mechanisms regarding TDP-43 accumulation (as mentioned above) could be associated with the pathophysiology in the latter category.

TDP-43 pathology is known to be strongly associated with the development of hippocampal sclerosis. For example, it was reported that 59 of 75 cases (79%) of FTLD with ubiquitin-positive inclusions (FTLD-U) had hippocampal sclerosis [19], 8 of 11 cases (73%) of pure hippocampal sclerosis had TDP-43-positive inclusions [1]. With respect to neuronal loss in the hippocampus in LBD cases with TDP-43 pathology, Nakashima-Yasuda et al. [28] reported that the frequency of hippocampal sclerosis was 60% of 25 TDP-43-positive DLB + AD cases, and 50% of 4 TDP-43-positive PDD cases, and none of 5 TDP-43-positive PD cases. Why none of the present LBD cases with TDP-43 pathology had hippocampal sclerosis is unclear. However, one plausible explanation is that TDP-43 pathology in our LBD series might less severe than that in LBD series by Nakashima-Yasuda: all of their TDP-43-positive LBD cases had the labeled inclusions in the hippocampal dentate gyrus, while only 30% of TDP-43-positive cases in our series had the lesions in the site. As with the relationship with hippocampal sclerosis, it is also plausible that the severity of TDP-43 pathology might have an impact on clinical presentation in LBD cases. However, the fact that no clear association between TDP-43 pathology and presence or absence of dementia was noted in our LBD cases might be explained by the relatively mild TDP-43 pathology in our series. The relationship between the severity of TDP-43 pathology and the development of hippocampal sclerosis, and the effects of these pathological parameters on clinical presentation in LBD cases should be examined in the future.

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ORIGINAL PAPER

Phosphorylated TDP-43 pathology and hippocampal sclerosis in progressive supranuclear palsy

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Abstract TDP-43 is characteristically accumulated in TDP-43 proteinopathies such as frontotemporal lobar degeneration and motor neurone disease, but is also present in some tauopathies, including Alzheimer's disease, argyrophilic grain disease, and corticobasal degeneration (CBD). However, several studies have suggested that cases of progressive supranuclear palsy (PSP) lack TDP-43 pathology. We have therefore examined limbic regions of the brain in 19 PSP cases, as well as in 12 CBD cases, using phosphorylation-dependent anti-TDP-43 antibodies. We observed TDP-43-positive inclusions in five PSP cases (26%), as well as in two CBD cases (17%). The amygdala and hippocampal dentate gyrus were most frequently affected in PSP. Regional tau burden tended to be higher in TDP-43-positive PSP cases, and a significant correlation between tau and TDP-43 burden was noted in the occipitotemporal gyrus. Hippocampal sclerosis (HS) was found in 3/5 TDP-43-positive PSP cases, but HS was significantly more frequent in TDP-43-positive than TDP-43 negative PSP cases. Dementia was present in 13/19 (58%) of the PSP cases, in 4/5 TDP-43-positive cases, in all 3 TDP-43-positive cases with HS, in 1/2 TDP-43-positive cases without HS, and 7/14 cases lacking both. TDP-43 and tau were frequently colocalized in the amygdala, but not in the hippocampal dentate gyrus. Immunoblotting demonstrated the characteristic (for TDP-43 proteinopathies) 45 and 25 kDa bands and high molecular weight smear in the TDP-43-positive PSP case. These findings suggest that (1) although PSP is nominally a tauopathy, pathological TDP-43 can accumulate in the limbic system in some cases, and (2) TDP-43 pathology may be concurrent with HS.

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Introduction

Transactivation-responsive DNA-binding protein of M_r 43 kDa (TDP-43) is a nuclear protein involved in transcriptional repression and alternative splicing. It was originally identified as a major component of ubiquitin-positive and tau-negative inclusions in the frontotemporal cortex and motor neurons in frontotemporal lobar degeneration (FTLD-U), with or without progranulin gene mutations, and in amyotrophic lateral sclerosis (ALS) [3, 12, 31]. Subsequent studies revealed that TDP-43 is also abnormally accumulated in familial FTLD-U with mutations in the valosin-containing protein gene [32], in familial FTLD with motor neuron disease linked to chromosome 9p [10], and in ALS with TDP-43 gene mutations [25, 38, 41, 44]. TDP-43 is considered to play an essential pathogenic role in these diseases, now-called TDP-43 proteinopathies.

Although TDP-43 accumulation was originally considered to be a specific disease marker for FTLD-U and ALS, subsequent studies demonstrated that abnormal TDP-43 accumulation in some cases of other neurodegenerative diseases, such as Alzheimer's disease (AD) [2], Parkinson's disease with and without dementia [30], dementia with Lewy bodies (DLB) + AD [4, 30], ALS/parkinson-dementia complex of Guam (ALS/PDC of Guam) [15, 16], argyrophilic grain disease (AGD) [14], and Huntington disease [37]. However, the pathophysiological significance of concurrent TDP-43 accumulation, and its impact on clinical phenotype in these diseases remain unclear.

Several previous studies have suggested that cases of progressive supranuclear palsy (PSP) lack abnormal TDP-43 accumulation [3, 18, 40]. In these early studies, phosphorylation-independent antibodies were employed in TDP-43 immunohistochemistry and immunoblot analysis. We have made polyclonal and monoclonal antibodies specific for phophorylated TDP-43, which identify phosphorylation sites in the C-terminus of the TDP-43 accumulated in FTLD-TDP brains [17, 20], and selectively immunolabel pathological inclusions and dystrophic neurites without physiological nuclear staining in FTLD-TDP, ALS, AD with TDP-43 pathology, and in DLB with TDP-43 [4, 17]. They also recognize hyperphosphorylated TDP-43 at 45 kDa and additional 18–26 kDa fragments in sarkosyl-insoluble fractions on immunoblotting.

The principal aim of this study was to revisit the presence or absence, and the frequency, of TDP-43 pathology in PSP cases using a phosphorylation-dependent anti-TDP-43 antibody. In contrast to previous reports, we demonstrated that a significant proportion of PSP cases had variable degrees of TDP-43 pathology in the limbic system. We subsequently examined the relationships between TDP-43 pathology, tau pathology, and hippocampal sclerosis, as well as biochemical nature of the abnormally accumulated TDP-43, in PSP.

Materials and methods

Subjects

We investigated 19 pathologically confirmed PSP cases, 12 pathologically confirmed corticobasal degeneration (CBD) cases and 4 pathologically normal control subjects (Table 1). These cases were obtained from UK Parkinson's Disease Society Tissue Bank (7 PSP and 4 control cases), Department of Pathology, Northwestern University Feinberg School of Medicine Cognitive Neurology and Alzheimer Disease Center (5 PSP and 7 CBD cases), and Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (7 PSP and 5 CBD cases). All brains had been collected with Local Research Ethical Committee approval. All PSP cases showed characteristic tufted astrocytes, and all CBD cases astrocytic plaques, as revealed by Gallyas-Braak silver methods and tau immunohistochemistry.

Immunohistochemistry

Sections cut at 5-μm thickness to include the amygdala, entorhinal cortex, hippocampus, occipitotemporal cortex in all cases, as well as the substantia nigra in two cases for which tissue was available, were stained with antibodies against phosphorylated TDP-43 (pAb pS409/410, rabbit, polyclonal, 1:1,000 [17]), phosphorylated tau (AT8, mouse, monoclonal, 1:3,000, Innogenetics, Ghent, Belgium), phosphorylated α-synuclein (#1175, rabbit, polyclonal, 1:1,000, [33]), and Aβ (4G8, mouse, monoclonal, 1:2,000, Covance Research Products Inc., Dedham, MA, USA). Deparaffinized sections were incubated with 1% H_2O_2 in methanol for 20 min to eliminate endogenous peroxidase activity in the tissue. When using anti-α-synuclein and anti-TDP-43 antibodies, sections were pretreated to enhance immunoreactivity in a microwave oven for 5 min in 10 mM sodium citrate buffer, pH 6.0, at 100°C. After blocking with 10% normal serum, sections were incubated 1 h at room temperature with the primary antibody. After three 5-min washes in phosphate-buffered saline (PBS), sections were incubated in biotinylated secondary antibody for 30 min, and then in avidin-biotinylated horseradish peroxidase complex (ABC Elite kit, Vector, Burlingame, CA, USA) for

Table 1 Demographic data in PSP and CBD cases with and without TDP-43 pathologies

	PSP			CBD		
	All	TDP-43-positive PSP	TDP-43-negative PSP	All	TDP-43-positive CBD	TDP-43-negative CBD
N (%)	19	5 (26.3)	14 (73.7)	12	2 (16.7)	10 (83.3)
Male [*N* (%)]	16 (84.2)	4 (80.0)	12 (85.7)	7 (58.3)	1 (50.0)	6 (60.0)
Age at onset [mean (SD)]	68.3 (9.8)	75.0 (9.4)	65.7 (9.0)	55.2 (10.2)	49.0 (12.7)	56.6 (9.9)
Age at death [mean (SD)]	76.3 (10.7)	82.4 (11.7)	74.1 (9.8)	62.8 (11.2)	56.0 (15.6)	64.1 (10.7)
Duration [mean (SD)]	7.4 (4.4)	7.4 (4.6)	7.5 (4.6)	7.3 (2.9)	7.0 (2.8)	7.3 (3.1)
Dementia (%)	11 (57.9)	4 (80.0)	7 (50.0)	11 (91.7)	2 (100.0)	9 (90.0)
Brain weight [g, mean (SD)]	1,202 (142)	1,234 (180)	1,190 (132)	1,174 (146)	1,008 (152)	1,215 (120)
Argyrophilic grains [*N* (%)]	4 (21.1)	1 (20.0)	3 (21.4)	3 (25.0)	1 (50.0)	2 (20.0)
Hippocampal sclerosis [*N* (%)]	3 (15.8)	3 (60.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

30 min. The peroxidase labeling was visualized with 0.2% 3,3′-diaminobenzidine (DAB) as chromogen. Sections were lightly counterstained with hematoxylin.

Semiquantitative assessment

TDP-43, tau, and Aβ pathologies in the amygdala, anterior and posterior portions of the entorhinal cortex, hippocampal dentate gyrus, CA1, 2, 3, and 4 regions, subiculum, fusiform gyrus, occipitotemporal gyrus were semiquantitatively evaluated using the following grading system blinded to any clinical or pathological information:

1. The total number of TDP-43-positive neuronal cytoplasmic inclusions (NCIs) in each anatomical region was assessed as follows: − no lesion, + one inclusion, ++ two or three inclusions, +++ four or five inclusions, ++++ 6–10 inclusions, +++++ 11 or over inclusions. In addition, the presence or absence of neuronal intranuclear inclusions (NIIs) and dystrophic neurites was also assessed. Then, we classified the topographic distribution of TDP-43 pathological changes using following system, which is similar to that reported by Amador-Ortiz et al. [2]: the amygdala type: inclusions were present only in the amygdala; the limbic type: inclusions extend to the amygdala, hippocampal dentate gyrus, CA1-4, entorhinal cortex, and fusiform gyrus, but not in the occipitotemporal gyrus; the temporal type: inclusions are present in the limbic system and also the in the occipitotemporal gyrus.
2. Tau-positive neuronal inclusions were counted in low power microscopic fields: 0, no tau-positive lesions; 1, one neuronal inclusion per few microscopic fields; 2, one inclusion in every field; 3, 4–30 inclusions in every field; 4, over 30 inclusions associated with numerous neurites in every field.
3. Aβ deposits were counted in low power microscopic fields: 0, no Aβ deposits; 1, two to three Aβ plaques in each field; 2, 4–10 Aβ plaques in each field; 3, 11–20 Aβ plaques in each field; 4, more than 20 Aβ deposits in each field.

Hippocampal sclerosis (HS) was defined by neuronal loss with gliosis in the hippocampal CA1 and/or subiculum, with relatively preserved neurons in the CA4, 3, and two regions and absence of intracellular and extracellular NFTs, or ischaemic changes that might explain neuronal loss in the CA1 and subiculum. HS was assessed blind to any clinical or pathological information.

Statistical analysis

The Mann–Whitney *U* test and Fisher's exact test were used to compare the demographic and pathological data between TDP-43-positive and TDP-43-negative groups in PSP and CBD series, respectively. Correlations between ratings of TDP-43 pathology and demographic data, or ratings of tau and Aβ pathologies in each anatomical region were assessed with Spearman's rank-order correlation statistic. Statistical analysis was performed using StatView for Macintosh program, version J-4.5. A value of $p < 0.05$ was accepted as significant.

Confocal laser scanning microscopy

Double-labeling immunofluorescence was performed with the combination of phosphorylation-dependent anti-TDP-43 (pAb pS409/410, rabbit, polyclonal, 1:1,000 [17]) and anti-tau antibodies (AT8, mouse, monoclonal, 1:500, Innogenetics, Ghent, Belgium). Sections from the amygdala and hippocampus in some PSP cases with TDP-43 pathology were pretreated by heating in a microwave oven for 5 min in 10 mM sodium citrate buffer, pH 6.0, at

100°C, allowed to cool then permeabilized with 0.2% (v/v) Triton X-100 in PBS. Following washing in PBS, non-specific antibody binding was blocked with normal sera and sections were incubated with a mixture of the two primary antibodies for 1 h at room temperature. After washing in PBS, sections were incubated with fluorescence-labeled secondary antibodies [AlexaFluor 488 anti-rabbit IgG (1:200) and AlexaFluor 555 anti-mouse IgG (1:200), Molecular Probes, Invitrogen, Paisley. UK]. After washing with PBS, sections were incubated with Toto-3 Iodide (Molecular Probes, Invitrogen, Paisley, UK) with 1 mg/ml RNase (Roche Diagnostics GmbH, Manheim, Germany) at 37°C. To quench (lipofuscin) autofluorescence, sections were incubated in 0.1% Sudan Black B for 10 min at room temperature and washed with 0.1% Tx-PBS for 30 min. Sections were coverslipped with Vectashield mounting media (Vector Laboratories Inc., Burlingame, CA, USA). Images were collected on a Leica TCS SP5 AOBS upright confocal (Leica Microsystems, Milton Keynes, UK) using the 488 nm (19%), 543 nm (30%) and 633 nm (60%) laser lines, respectively. To eliminate cross-talk between channels, the images were collected sequentially.

Immunoblotting

Frozen tissue from the amygdala, hippocampus, and frontal, temporal, and occipital cortices in one PSP case with TDP-43 pathology, one FTLD-TDP case (as a positive control) and eight negative controls (six PSP, one LBD, and one pathologically normal case) were prepared for western blotting according to methods previously described by Neumann et al. [31]. Briefly, 1 g of fresh frozen brain was homogenized in 5 ml/g (w/v) of low salt (LS) buffer-containing 10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 1 mM DTT, 10% (w/v) sucrose and Roche complete EDTA-free protease inhibitor. Homogenates were sequentially extracted with increasing strength buffers [Triton X-100 buffer (LS buffer + 1% Triton X-100 + 0.5 M NaCl), Triton X-100 buffer with 30% sucrose to float myelin, Sarkosyl buffer (LS buffer + 1% *N*-lauroyl-sarcosine + 0.5 M NaCl)]. Detergent-insoluble pellets were extracted in 0.25 ml/g Urea buffer (7 M Urea, 2 M Thiourea, 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris–HCl pH 8.5, Roche complete EDTA free protease inhibitor. Prior to SDS-PAGE immunoblot analysis, urea fractions were added in 1:1 ratio to SDS sample buffer (10 mM Tris pH 6.8, 1 mM EDTA, pH 8.0, 40 mM DTT, 1% SDS, 10% Sucrose, 0.01% Bromophenol Blue). Protein was resolved on 12% Tris–Glycine SDS-PAGE gels along with size standard (Bio-Rad kaleidoscope broad-range marker; Bio-Rad, Hercules, CA, USA). Proteins were transferred onto nitrocellulose membrane (Hybond ECL, GE Life Sciences, UK) and blocked for 1 h at 4°C in 5% (w/v) milk solution [5% powdered milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T)]. Membranes were incubated in phosphorylation-dependent mouse monoclonal antibody (mAb pS409/410, mouse, 1:1,000 [20]) for 1 h at room temperature followed by HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology Inc, CA, USA). Antibodies were visualized by incubating in enhanced chemiluminescent reagent (ECL, GE Life Sciences) and imaged using the ImageQuant 350 system fitted with a F0,95 25 mm Fixed Lens (GE Healthcare, Life Sciences, UK). TDP-43 probed membranes were exposed for 5 min at different timeframes to obtain multiple images of differing intensity. Images were processed using ImageQuant TL software (GE Healthcare, Life Sciences, UK).

Results

Frequency and distribution of TDP-43 pathology

Clinical and pathological features for all subjects are shown in Table 1. TDP-43 pathology was noted in 5 of 19 PSP cases (26%) and in 2 of 12 CBD cases (17%). Disease duration, gender ratio and brain weight were not statistically different between PSP cases with and without TDP-43 pathology, or between CBD cases with and without TDP-43 pathology, respectively. Age at onset of disease (75 vs. 66 years) and age at death (82 vs. 74 years) tended to be higher, and dementia occurred more often, in PSP cases with TDP-43 pathology than in PSP cases without it (80 vs. 50%), although these differences did not reach statistical significance. One PSP case without TDP-43 pathology also had Lewy body pathology corresponding to brainstem-predominant type [26]. Ten PSP cases (3 TDP-43-positive and 7 TDP-43-negative cases) and four CBD cases (all were TDP-43-negative) had Aβ-positive diffuse plaques in the amygdala, hippocampus, and/or temporal cortex. Of the three TDP-43-positive PSP cases, one case had only a few neuritic plaques in the occipitotemporal gyrus. None of the PSP or CBD cases in our series fit the pathological criteria of AD [9, 28, 39].

In PSP cases, TDP-43-positive NCIs were most frequently noted in the amygdala and dentate gyrus granule cells in the hippocampus (5 cases, 100% of TDP-43-positive PSP cases), followed by the anterior portion of the entorhinal cortex (4 cases, 80%), subiculum (3 cases, 60%), posterior portion of the entorhinal cortex (3 cases, 60%), occipitotemporal gyrus (2 cases, 50%), fusiform gyrus (2 cases, 40%), and CA1 region (2 cases, 20%) (Table 2, Fig. 1a–d). In addition to the rounded inclusions noted in FTLD-TDP, all PSP cases had many irregular shaped

Table 2 Distribution of TDP-43 pathology in PSP and CBD cases

TDP-43 pathology												Hippocampal sclerosis (CA1/Subiculum)	Argyrophilic grains[b]
No.	Amygdala	ant.EC	DG	CA3/4	CA2	CA1	SB	post.EC	FG	OTG	TDP-43 distribution[a]		
PSP cases													
PSP1	++++	−	+	−	−	−	−	−	−	−	Limbic	−	−
PSP2	+++++	+++++	+++++	−	−	−	+	−	−	n	Limbic	+	−
PSP3	+++++	+++++	+++	−	−	−	++	++++	−	−	Limbic	−	−
PSP4	+++++	+++++	++	−	−	+	+++	+++++	+++++	+	Temporal	+	−
PSP5	+++++	+++	+++++	−	−	++	−	++	+++	+++	Temporal	+	Stage III
%	100.0	80.0	100.0	0.0	0.0	20.0	60.0	60.0	40.0	50.0		60.0	20.0
CBD cases													
CBD1	+++	++	−	−	−	−	−	n.a.	n.a.	n.a.	Limbic	−	−
CBD2	+++++	++	++++	++	−	+++++	+++++	++	−	−	Limbic	−	Stage II
%	100.0	100.0	50.0	50.0	0.0	50.0	50.0	50.0	0.0	0.0		0.0	50.0

The stages of TDP-43 pathology: −, no lesion in the anatomical region; +, 1 inclusion in the anatomical region; ++, 2–3 inclusions in the anatomical region; +++, 4–5 inclusion in the anatomical region; ++++, 6–10 inclusions in the anatomical region; +++++, 11 or over inclusions in the anatomical region. The stage of hippocampal sclerosis: −, no; +, mild; ++, moderate; +++, severe. The stage of argyrophilic grains: −, absent; +, present. ant.EC, the anterior portion of the entorhinal cortex; DG, hippocampal dentate gyrus; SB, subiculum; post.EC, the posterior portion of the entorhinal cortex; FG, fusiform gyrus; OTG, occipitotemporal gyrus

[a] The amygdala type: inclusions were present only in the amygdala; the limbic type: inclusions extend to the limbic system, but not in the occipitotemporal gyrus; the temporal type: inclusions are present in the limbic system and occipitotemporal gyrus as well

[b] The distribution of argyrophilic grains are assessed using a staging system proposed by Saito et al. [35]

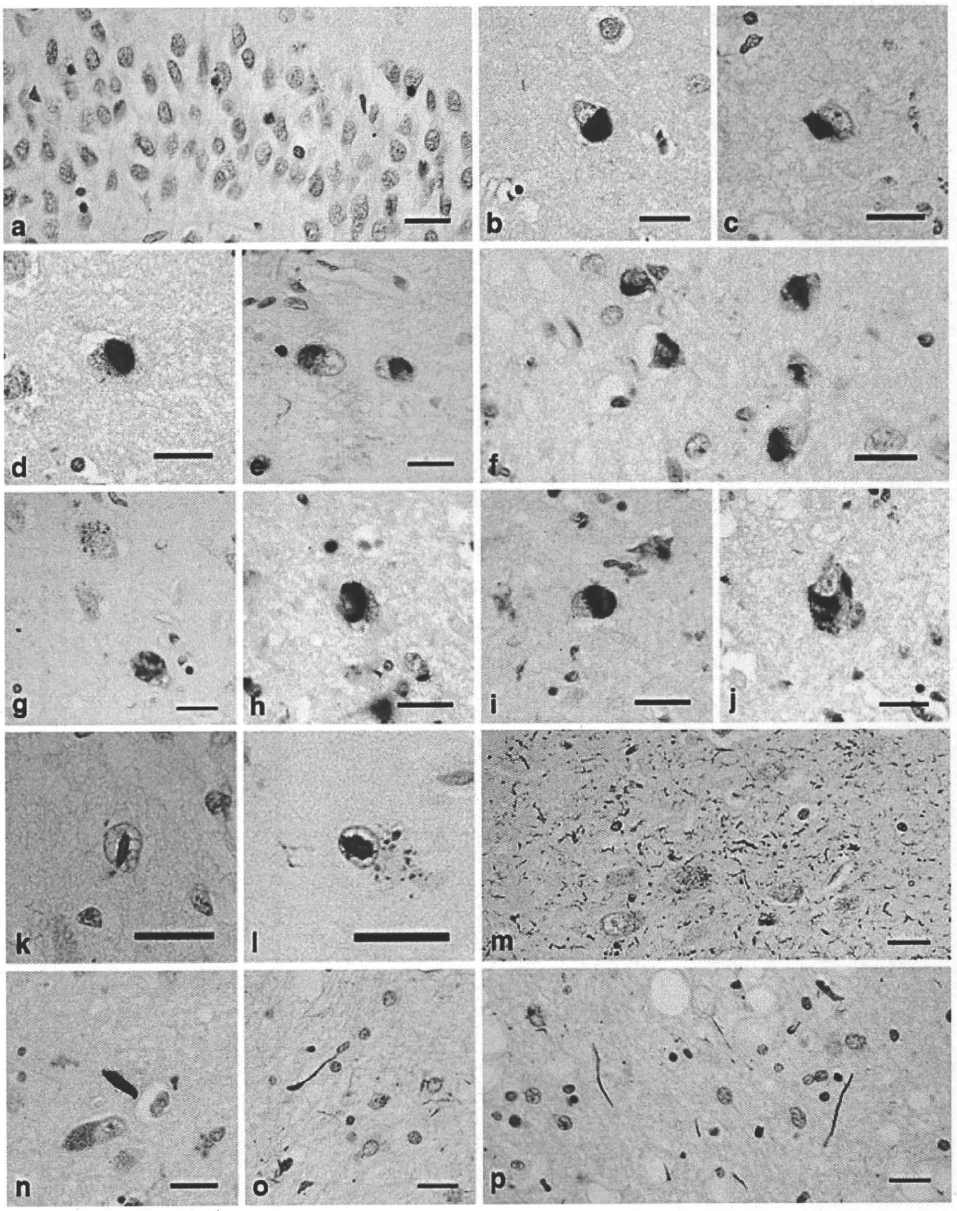

Fig. 1 TDP-43-positive lesions in PSP. **a** Neuronal cytoplasmic inclusions (NCIs) in the hippocampal dentate gyrus. **b–d** NCIs in the entorhinal cortex. Irregular shaped NCIs in the entorhinal cortex (**e**), fusiform gyrus (**f**), and subiculum (**g**). These inclusions have weakly stained or unstained regions. Small dot-like structures are also seen in the neuronal cytoplasm (**g**). Horseshoe-shaped (**h, i**) and NFT-like (**j**) NCIs in the entorhinal cortex. Intranuclear inclusions in the amygdala (**k**) and in the subiculum (**l**), cases PSP3 and PSP2, respectively. **m** Massive short threads-like structures in the subiculum, case PSP3. **n** Thick, thread-like structures in the amygdala. **o, p** Long, thin thread-like structures in the amygdala. pAb pS409/410 immunohistochemistry. All *scale bars* 20 μm

NCIs, such as flame-shape NFT-like, globose-type NFT-like, and horseshoe-like inclusions (Fig. 1e–j). One PSP case (PSP 2 in Table 2) showed a few NII in the subiculum (Fig. 1k, l). Two cases (PSP4 and PSP5) had abundant fine, short, thread-like structures immunopositive for TDP-43 from the CA1 to subiculum (Fig. 1m). TDP-43-positive thread-like structures were also observed in the amygdala (3 cases), entorhinal cortex (2 cases), CA1 (one case), and subiculum (one case) (Fig. 1n–p).

In two CBD cases, TDP-43-positive NCIs were observed in the amygdala, entorhinal cortex, hippocampal dentate gyrus, CA1, CA3/4, and subiculum (Table 2, Fig. 2a). The distribution of TDP-43 pathology was roughly consistent with that observed in PSP cases. NIIs were found in the subiculum and amygdala in one CBD case with severe TDP-43 pathology (Fig. 2b, c). Short thread-like structures immunopositive for TDP-43 were found in the amygdala, entorhinal cortex, CA1, CA3, and/or subiculum in both CBD cases with TDP-43 pathology. One CBD case had TDP-43-positive coiled body-like structures and thread-like structures in the alveus in the subiculum (Fig. 2d–f). Abnormal accumulation of TDP-43 was not found in the white matter of the temporal lobe and substantia nigra in any of the TDP-43-positive PSP or CBD cases.

Relationship between TDP-43 pathology and tau or Aβ burden

The ratings for tau burden in the TDP-43-positive PSP cases tended to be higher (but not significantly so) than

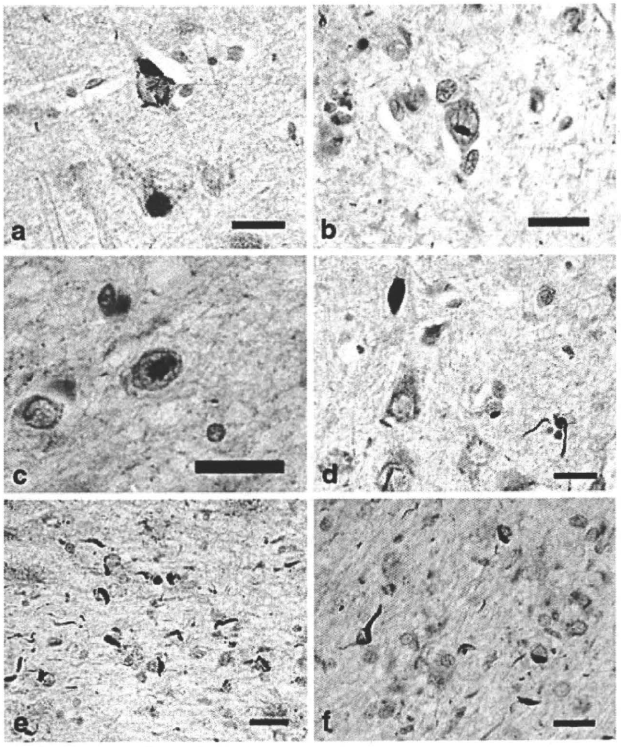

Fig. 2 TDP-43-positive lesions in CBD. **a** Neuronal cytoplasmic inclusions (NCIs) in CA3 region of hippocampus. **b, c** Neuronal intranuclear inclusions in the amygdala. **d** A thick neurite and thin, thread-like structures in the amygdala. **e** Short thread-like structures and glial cytoplasmic inclusions (GCIs) in the alveus in the entorhinal cortex. **f** Coiled body-like structures and GCis in the alveus in the entorhinal cortex. pAb pS409/410 immunohistochemistry. All *scale bars* 20 μm

those in the TDP-43-negative PSP cases, in almost all regions examined (i.e., including amygdala, entorhinal cortex, hippocampal dentate gyrus, CA1-4, fusiform gyrus, and occipitotemporal gyrus) (Fig. 3). In the PSP cases overall, rating for tau pathology in the occipitotemporal gyrus was significantly correlated with that of TDP-43 pathology ($r = 0.504$, $p < 0.05$), but no significant correlations between tau and TDP-43 ratings were found in any other regions. There were no significant differences in the degree of Aβ burden in any region between TDP-43-positive and TDP-43-negative PSP cases, and ratings for TDP-43 pathology did not correlate with those for Aβ burden in any region. Of three TDP-43-positive PSP cases having Aβ deposits, only one case had a few neuritic plaques in the occipitotemporal gyrus; however, this case did not have any TDP-43-positive inclusions in the region.

In the CBD cases, there were no significant differences in tau or Aβ burden in any region between TDP-43-positive and TDP-43-negative cases, and ratings for TDP-43 pathology did not correlate with those for tau or Aβ burden in any region.

Relationship of HS, argyrophilic grains, TDP-43 accumulation, and dementia

In 3 of 19 PSP cases (16%), evident neuronal loss in the CA1 and subiculum consistent with HS was noted (Fig. 4a, b). No CBD case showed HS. All three PSP cases with HS had a various degrees of TDP-43 pathology in the CA1 and/or subiculum (Fig. 4e–h), and two had extensive TDP-43

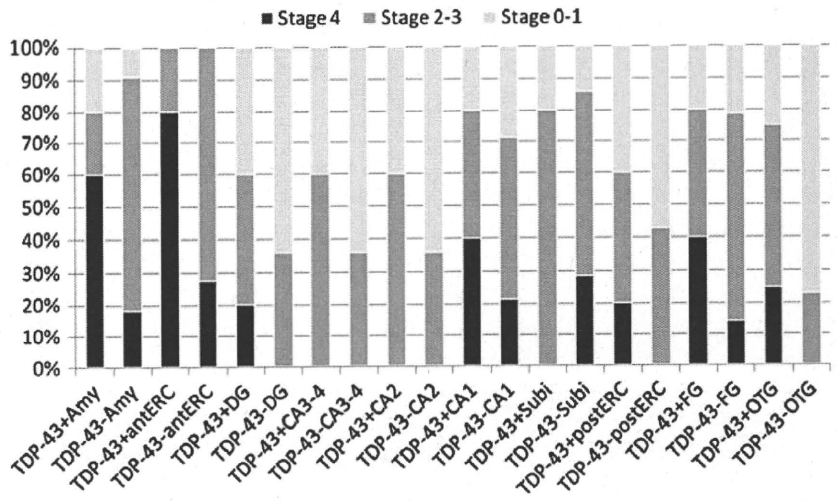

Fig. 3 Tau burden in the limbic system in PSP cases with and without TDP-43 pathology. In all regions but the subiculum, tau burden in PSP cases with TDP-43 pathology is more severe than that in PSP cases without TDP-43 pathology. Stage 0–1, no to mild tau deposition; stages 2–3, moderate to severe tau deposition; stage 4, very severe tau deposition (see detailed definition in the text). *TDP-43+* TDP-43-positive, *TDP-43−* TDP-43-negative, *Amy* amygdala, *antERC* the anterior portion of the entorhinal cortex, *DG* hippocampal dentate gyrus, *Subi* subiculum, *postERC* the posterior portion of the entorhinal cortex, *FG* fusiform gyrus, *OTG* occipitotemporal gyrus

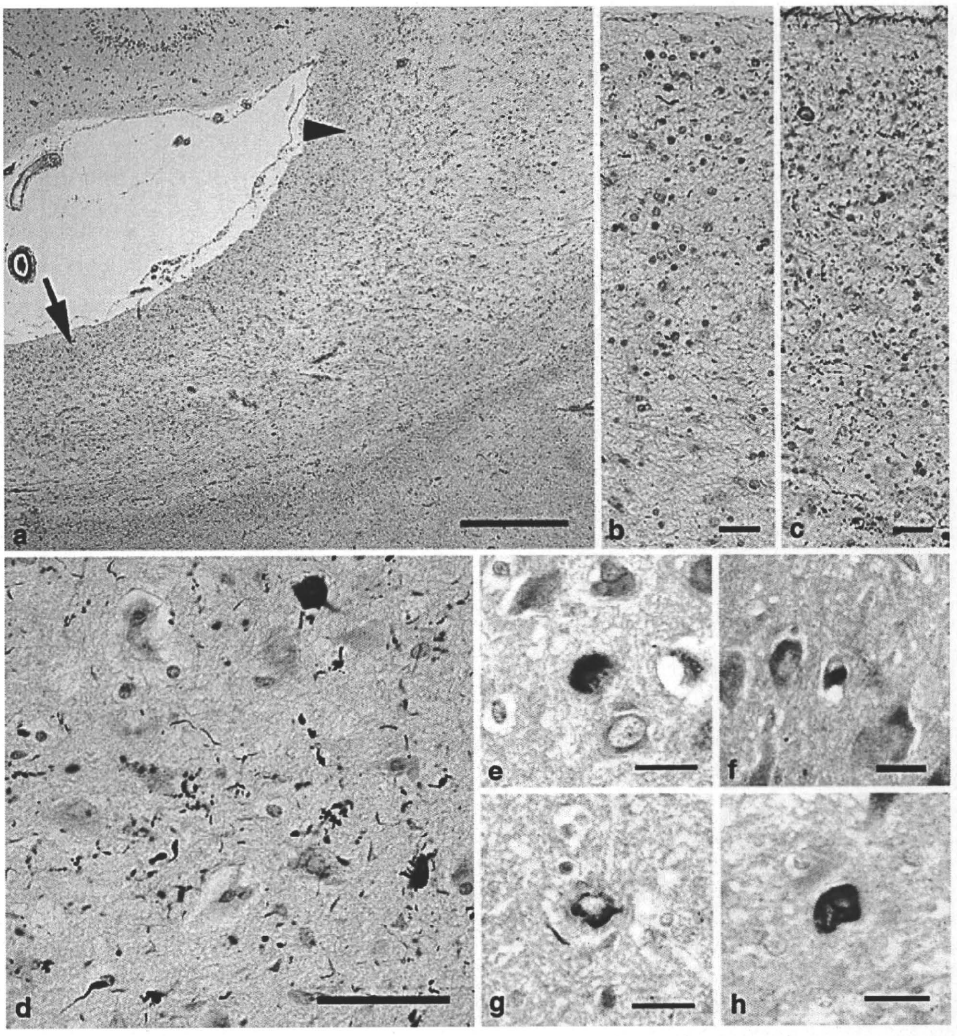

Fig. 4 Pathological features in the hippocampus in a PSP case with TDP-43, HS, and argyrophilic grains (PSP5). **a** A low power view of the hippocampal CA1 to subiculum. Severe reduction of the width with tissue rarefaction is noted in the subiculum (*arrow*) and to a lesser degree in the adjacent CA1 region (*arrowhead*). **b** A moderate power view of the subiculum on the same section as that shown in **a**. Severe neuronal loss associated with gliosis is evident. Argyrophilic threads and grains are scattered, but tangles are rare. **c** The subiculum on an adjacent section of **b**. A moderate number of tau-positive threads and grains, but only a few tangles, are seen. **d** Argyrophilic grains in CA1 region. **e**, **f** TDP-43-positive cytoplasmic inclusions in CA1 region. **g** An irregular shaped TDP-43 accumulation in the subiculum. **h** A coiled body-like TDP-43-positive inclusion in the subiculum. **a**, **b**, **d** Gallyas-Braak hematoxylin-eosin stain. **c** AT-8 immunohistochemistry. **e–h** pAb pS409/410 immunohistochemistry. *Scale bars* **a** 400 μm, **b**, **c** 25 μm, **d** 50 μm, **e–h** 20 μm

pathology in the limbic system: one case had both TDP-43 pathology and argyrophilic grains (Table 2). Two of the three PSP cases with HS had a few AT8-positive pretangles and argyrophilic grains in the CA1 and subiculum (Fig. 4b–d). Neurofibrillary tangles were rare in these regions in all PSP cases with HS (Fig. 4b). No significant ischemic changes in the hippocampal pyramidal neurons, or neuronal loss in the end plate, suggestive of a past history of severe epilepsy was noted in any of the PSP cases with HS. The frequency of HS in the TDP-43-positive PSP cases was significantly higher than that in TDP-43-negative PSP cases (60 vs. 0%, $p = 0.021$). Dementia was present in all of the 3 TDP-43-positive PSP cases with HS (100%), 4 of the 5 TDP-43-positive PSP cases with and without HS (80%), 1 of 2 TDP-43-positive PSP cases without HS (50%), and 7 of 14 PSP cases lacking both (50%). The frequency of dementia was not significantly different between PSP cases with and without HS ($p = 0.170$).

Concomitant argyrophilic grains were observed in four PSP (21%) and three CBD cases (25%) (Fig. 4d). Among these cases, one PSP and one CBD case had TDP-43 pathology (Table 2, Fig. 4e–h). There was no significant difference in the frequency of argyrophilic grains between TDP-43-positive and TDP-43-negative PSP cases, or between CBD cases with and without TDP-43 pathology, respectively. However, in the TDP-43-positive PSP and CBD cases, argyrophilic grains were found in those cases with the most severe TDP-43 pathology (Table 2).

Double immunofluorescence labeling in PSP cases

In the PSP cases examined, TDP-43 and tau pathologies were independently present in the perikarya of granular cells in the hippocampal dentate gyrus with no coexistence of these proteins (Fig. 5a–f). In contrast, in the amygdala, TDP-43 accumulation was often intermingled with tau accumulation in NCIs and dystrophic neurites, and colocalization was frequent (Fig. 5g–o). In the entorhinal cortex and parahippocampal gyrus in one PSP case with argyrophilic grains, many tau-positive grain-like structures

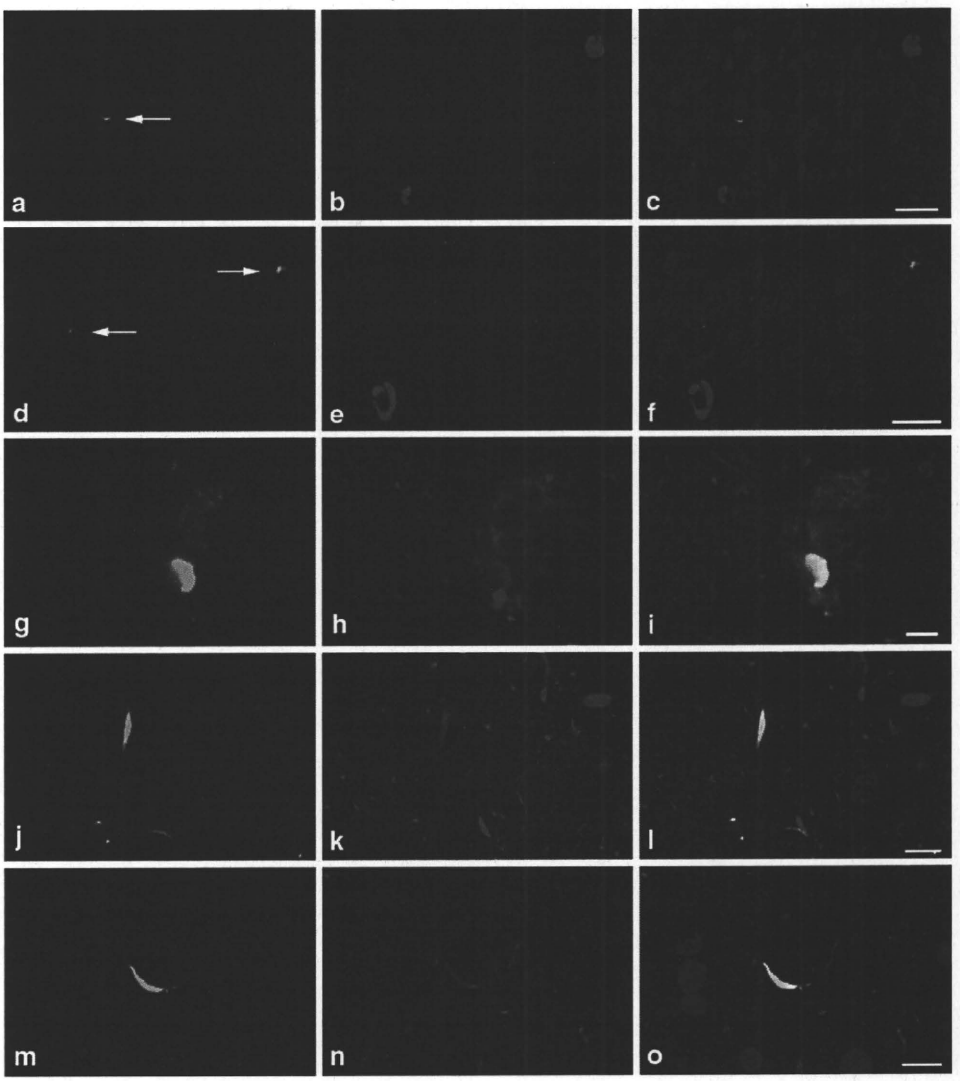

Fig. 5 Confocal double-imunofluorescence of TDP-43 (**a**, **d**, **g**, **j**, **m**) and tau (**b**, **e**, **h**, **k**, **n**) in PSP cases. *Merged images* are shown in **c**, **f**, **i**, **l**, and **o**. Blue fluorescence in merged images are nuclei. **a–f** In the hippocampal dentate gyrus, TDP-43 accumulation (*arrows*) is not colocalized with tau labeling. **g–i** In the amygdala, TDP-43 accumulation is often intermingled and colocalized with neuronal tau accumulation. **j–o** TDP-43-positive neurites (**j**, **m**) and many tau-positive neurites and granules (**k**, **n**) are seen in the amygdala. Coexistence of TDP-43 and tau is noted in some neurites (**l**, **o**). AT8 and pAb pS409/410 double immunofluorescence. *Scale bars* **a–c** 25 μm, **d–f** 25 μm, **g–i** 2.5 μm, **j–l** 7.5 μm, **m–o** 7.5 μm

were demonstrated, and TDP-43 was colocalized with tau in some of these structures (data not shown).

Biochemical analyses of TDP-43 in PSP cases

Immunoblot analysis of the sarkosyl-insoluble, urea-soluble fraction with mAb pS409/410 demonstrated distinct bands at (approximately) 45 and 25 kDa, as well as high molecular weight smears in the amygdala of a PSP case having TDP-43 pathology (Fig. 6, lane 6) and in the frontal cortex of a FTLD-TDP case (lane 5). Weak 25 and 45 kDa bands were also observed in the hippocampus in a PSP case, which had very mild TDP-43 pathology at this site (lane 7). Pathological TDP-43 bands and smear were not demonstrated in any of the other cases lacking TDP-43 pathology, including those with PSP (lanes 1 and 2) or Lewy body disease (lane 4), or in normal control cases (lane 3).

Discussion

This is the first study demonstrating abnormal accumulations of phosphorylated TDP-43 in the limbic system in a significant proportion (26%) of patients with PSP. Immunoblot analysis also demonstrated biochemical alterations in TDP-43 in tissue samples from a PSP case with TDP-43 pathology, similar to those in FTLD-TDP and ALS. Regional tau burden in PSP cases with TDP-43 pathology was higher than that in PSP cases without it, and TDP-43 burden was significantly correlated with that of tau in the occipitotemporal cortex. The frequency of HS in PSP cases with TDP-43 pathology was significantly higher than that in PSP cases without it. Collectively, these findings suggest that (1) PSP is one of the tauopathies in which pathological TDP-43 accumulation can occur in the limbic system, and (2) TDP-43 pathology may be associated with the occurrence of HS in PSP cases.

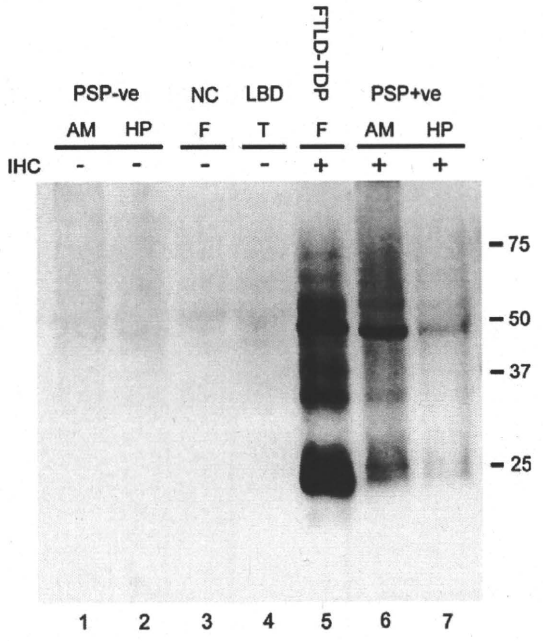

Fig. 6 Immunoblot analysis of the sarkosyl-insoluble fraction in representative PSP cases with phosphorylation-dependent monoclonal anti-TDP-43 antibody (mAb pS409/410). The 45 kDa full length TDP-43, 25 kDa fragments, and high molecular weight smear are strongly labeled in the amygdala of a PSP case with TDP-43 pathology (*lane 6*) and in the frontal cortex of a FTLD-TDP case (*lane 5*). Weakly stained 45 and 25 kDa bands are noted in the hippocampus of a PSP case (*lane 7*), in which TDP-43 pathology was mild. Similar 45 and 25 kDa bands and smears were not immunolabeled in any of the other cases without detectable TDP-43 pathology by immunohistochemistry (*lanes 1–4*). Normal 43 kDa TDP-43 is not stained by this phophorylation-dependent antibody in any case. *PSP* progressive supranuclear palsy, *LBD* Lewy body disease, *NC* normal control, *AM* amygdala, *HP* hippocampus, *F* frontal cortex, *T* temporal cortex, *IHC* pAb pS409/410 immunohistochemistry

Previous studies have demonstrated variable frequencies of concurrent TDP-43 pathology in many tauopathies: 23–56% in AD cases [2, 4, 40], 31–60% in DLB + AD cases [4, 30], 15% in CBD cases [40], and 60% in AGD cases [14]. Why no cases of PSP with TDP-43 pathology have previously been described is not clear. Our present findings show that, at least some, PSP cases may share a common pathophysiological background involving TDP-43 accumulation with other tauopathies with TDP-43 pathology. Several studies demonstrated that concurrent AD-type pathology was associated with the development of TDP-43 pathology in some neurodegenerative diseases [2, 4, 7, 14, 30]. However, it was unlikely that the development of TDP-43 pathology in our PSP series can be explained by the influence of Aβ deposits or neuritic plaques. For example, of all ten PSP cases having Aβ deposits, nine cases had only diffuse plaques, and the degree of Aβ deposition was not significantly different between TDP-43-positive and TDP-43-negative PSP cases and was not correlated with that of TDP-43 pathology in any regions. Although only one PSP case had a few neuritic plaques in the occipitotemporal gyrus, no TDP-43-positive inclusion was noted in the region.

Our findings are inconsistent with previous studies that failed to demonstrate immunohistochemical or biochemical abnormalities of TDP-43 in PSP cases [2, 3, 18, 40]. Considering that the sample size investigated in one of these previous studies [40] was far larger than that in our own study, the most plausible cause of the discrepancy may be the difference of the sensitivities of anti-TDP-43 antibodies employed: phosphorylation-dependent anti-TDP-43 antibodies do not stain normal nuclei, making true TDP-43-positive inclusions more readily identifiable [17, 37]. The distribution of TDP-43 pathology observed in our PSP cases was very similar to that reported previously in AD [2, 4, 18, 19, 40], DLB + AD [4, 30], and CBD [40], but tended to be more restricted than that in ALS/PDC of Guam [15, 16, 27]. Most frequently affected sites in these tauopathies are the amygdala and hippocampal dentate gyrus. Given these findings, it is plausible that the frequent TDP-43 accumulation in these sites in tauopathies is associated with some region-specific, rather than disease-specific, mechanism. On the other hand, it remains unclear whether TDP-43 is abnormally accumulated through an identical pathophysiological mechanism in various anatomical regions. For example, it was reported that abnormal TDP-43 accumulation was significantly correlated with the severity of tau pathology in AD cases [4] and Lewy body disease including many DLB + AD cases [30]. This same statistical relationship was observed in our PSP cases. Furthermore, in our present studies, TDP-43 was often colocalized with tau in NCIs and dystrophic neurites in the amygdala, although there were also TDP-43-positive but tau-negative lesions in this site. A coexistence of TDP-43 and tau in the same neuron in the amygdala and temporal cortex was also reported in AD and DLB cases in previous studies [4, 18]. However, in contrast to the amygdala, a coexistence of TDP-43 and tau in the same neuron in the hippocampal dentate gyrus was not seen in our PSP cases. This trend regarding non-colocalization of these two proteins was also noticed in the dentate granular cells in AD [40] and AGD brains [14]. This suggests that the mechanism underlying the accumulation of TDP-43 is different at least between the amygdala and hippocampal dentate gyrus, or that there is some unknown factor that can influence the occurrence of both TDP-43 and tau pathologies. In addition, considering the potential relationship between tau and TDP-43 in PSP presented in this paper, whether TDP-43 pathology is also noted in several other regions that are often involved by tau-associated lesions (e.g., the frontal cortex and basal ganglia) needs to be investigated in the future studies.

There is little known about the relationship between PSP and HS. In our series, 3 of 19 PSP cases (16%) had evident neuronal loss in the CA1 and/or subiculum consistent with the definition of HS. Furthermore, all of the cases with HS had TDP-43 pathology, and one of the three cases also had argyrophilic grains. It has been reported that HS cases have variable underling pathologies, including the 'pure form' of HS [1, 21, 34], FTLD-TDP [23], FTLD with motor neuron disease [29], AD [2], CBD [36], DLB [13], and AGD [5, 13]. Present findings support the possibility that the development of HS, at least in some PSP cases, may occur in association with concurrent TDP-43 pathology. On the other hand, whether the development of HS in PSP cases is correlated with the severity of tau or TDP-43 pathology remains unclear. Considering the relatively small size of the samples examined in the present study, the relationship between HS and TDP-43 accumulation in PSP, as well as the frequencies of these pathological features, needs to be confirmed in a larger case series.

Although influence of concurrent TDP-43 pathology on clinical features in tauopathies is not fully understood, some previous studies in AD, have demonstrated a comorbidity such that a concomitant TDP-43 pathology was associated with a later age at onset and death [4, 24], and significantly poorer cognitive function [24]. On the other hand, a study investigating a relatively small series of AGD did not demonstrate any significant difference in the age at death or disease duration between cases with and without TDP-43 pathology [14]. It is known that patients with PSP frequently exhibit psychiatric and behavioral disturbances, and that cognitive decline in PSP is associated with the atrophy in the orbitofrontal cortex [11] and more severe tau burden in the neocortex and hippocampus [6, 8, 22]. More recently, it was also reported that clinical presentation, including the occurrence of dementia, is influenced by the distribution and severity of tau pathology [42, 43]. In our PSP series, although not statistically significantly, the frequency of dementia in PSP cases with both TDP-43 and HS (100%), and that in all PSP cases with TDP-43 pathology (80%), were higher than that in PSP cases lacking both (50%). The potential co-morbid effect of concurrent TDP-43 pathology and/or HS on cognitive impairment in patients with PSP needs to be explored by further clinicopathological studies.

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Symposium: Advances in amyotrophic lateral sclerosis research

Phosphorylated and cleaved TDP-43 in ALS, FTLD and other neurodegenerative disorders and in cellular models of TDP-43 proteinopathy

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Transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) is a major component of the tau-negative and ubiquitin-positive inclusions that characterize amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration which is now referred to as FTLD-TDP. Concurrent TDP-43 pathology has been reported in a variety of other neurodegenerative disorders such as Alzheimer's disease, forming a group of TDP-43 proteinopathy. Accumulated TDP-43 is characterized by phosphorylation and fragmentation. There is a close relationship between the pathological subtypes of FTLD-TDP and the immunoblot pattern of the C-terminal fragments of phosphorylated TDP-43. These results suggest that proteolytic processing of accumulated TDP-43 may play an important role for the pathological process. In cultured cells, transfected C-terminal fragments of TDP-43 are more prone to form aggregates than full-length TDP-43. Transfecting the C-terminal fragment of TDP-43 harboring pathogenic mutations of TDP-43 gene identified in familial and sporadic ALS cases into cells enhanced the aggregate formation. Furthermore, we found that methylene blue and dimebon inhibit aggregation of TDP-43 in these cellular models. Understanding the mechanism of phosphorylation and truncation of TDP-43 and aggregate formation may be crucial for clarifying the pathogenesis of TDP-43 proteinopathy and for developing useful therapeutics.

Key words: α-synuclein, fragment, inclusion, phosphorylation, tau.

INTRODUCTION

Transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) is a major component of the tau-negative and ubiquitin-positive inclusions that characterize amyotrophic lateral sclerosis (ALS) and the most common pathological subtype of frontotemporal lobar degeneration (FTLD-U), which is now referred to as FTLD-TDP.[1–7] Several genes and chromosomal loci, including the progranulin gene (*PGRN*),[8,9] valosin-containing protein gene (*VCP*)[10] and an unidentified gene at chromosome 9p,[11,12] have been reported to be associated with familial forms of FTLD-TDP. 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.[13–17] These disorders are now collectively referred to as TDP-43 proteinopathy.[1–4]

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TDP-43 was first isolated as a transcriptional inactivator binding to the TAR DNA element of the HIV-1 virus.[18] It appears to belong to the group of 2 RNA-binding domain (RBD)-Gly RNA-binding proteins, which include the heterogeneous nuclear ribonucleoprotein (hnRNP) family and factors involved in RNA splicing and transport.[19] Subsequent studies reported that TDP-43 functions to inhibit expression of mouse spermatid-specific SP-10 gene and of cyclin-dependent kinase 6, to regulate alternative splicing of exon 9 of cystic fibrosis transmembrane conductase regulator (*CFTR*), exon 3 of apolipoprotein A-II (*Apo AII*), and exon 7 of survival of motor neuron 2 (*SMN2*), and to stabilize human low molecular weight neurofilament (hNFL) mRNA.[20–26] The splicing inhibitory activity requires the C-terminal region of TDP-43 by interaction with other hnRNP members.[27] Furthermore, more recent studies suggest that TDP-43 may be involved in other cellular processes such as microRNA biogenesis, apoptosis, and cell division.[28]

Ubiquitin- and TDP-43-positive pathological inclusions found in FTLD-TDP include neuronal cytoplasmic inclusions (NCIs), dystrophic neurites (DNs), neuronal intranuclear inclusions (NIIs), and glial cytoplasmic inclusions (GCIs).[1,2,29–31] Based on morphological aspects, TDP-43 proteinopathies have been classified into four subtypes.[32] 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 NIIs and Type 4 has numerous NIIs and DNs with few NCIs. Type 4 is specific for familial FTLD-TDP with mutations of VCP gene. The strong relationship between other subtypes of TDP-43 pathology and clinical phenotype is indicated. Type 1 is associated with semantic dementia, Type 2 with FTLD with motor neuron disease (MND) or clinical signs of MND, and Type 3 with progressive non-fluent aphasia.[29,33,34]

In ALS, motoneuronal skein-like inclusions immunopositive for ubiquitin had been regarded as major pathological hallmarks. Recent detailed immunohistochemical studies have clarified the wide distribution of neuronal and glial TDP-43 pathology in multiple areas of the central nervous systems, including the nigro-striatal system, the neocortical and allocortical areas, and the cerebellum.[35,36] These findings suggest that ALS does not selectively affect only the motor system, but rather is a multisystem neurodegenerative TDP-43 proteinopathy affecting both neurons and glial cells.[35,36]

Biochemical analyses of the detergent-insoluble fraction extracted from brains of patients afflicted with FTLD-TDP and ALS show that TDP-43 accumulated in these pathological structures is phosphorylated and cleaved.[1,2]

In the present review, we will focus on the histological and biochemical abnormality of TDP-43 accumulated in ALS, FTLD-TDP and other neurodegenerative disorders, and on the establishment and analyses of cellular models for intracellular aggregates of TDP-43. Using antibodies specific for phosphorylated TDP-43 (pTDP-43), we identified several phosphorylation sites in the C-terminal region of the TDP-43 that accumulates in FTLD-TDP and ALS brains.[37] Furthermore, we found a close relationship between the pathological subtypes of FTLD-TDP and ALS and the immunoblot pattern of phosphorylated C-terminal fragments of TDP-43, suggesting that proteolytic processing may be crucial in the pathological process of these diseases.[37] By transfecting deletion mutants lacking nuclear localization signal or C-terminal fragments of TDP-43, we succeeded in establishing the cellular models of TDP-43 proteinopathy. By analyzing them, we found the pathogenic effect of mutations of TDP-43 gene identified in ALS cases, and the potential therapeutic agents that inhibit the aggregate formation of TDP-43.

IMMUNOHISTOCHEMICAL AND BIOCHEMICAL ANALYSIS FOR PTDP-43 IN ALS AND FTLD-TDP

In order to identify the critical phosphorylation sites of TDP-43, we raised antibodies against 39 different synthetic phosphopeptides, representing 36 out of 63 candidate phosphorylation sites.[37] Of the generated antibodies, pS379, pS403/404, pS409, pS410 and pS409/410 stained the inclusions in immunohistochemistry, and abnormal TDP-43 species on immunoblot, in FTLD-TDP and ALS cases. Since the immunoreactivity of pS409/410 was particularly robust in both immunohistochemistry and immunoblotting, we later produced a monoclonal antibody directed against phosphoserines 409 and 410 in human TDP-43.[38] The results suggest that at least five sites on TDP-43 are phosphorylated in subjects with FTLD-TDP and ALS, and that abnormal phosphorylation takes place mainly near the carboxyl (C)-terminal region of TDP-43.

In immunohistochemistry, in contrast to the commercially obtained phosphorylation-independent anti-TDP-43 antibody, which labels both abnormal structures and normal nuclei (Fig. 1A), pTDP-43-specific antibodies recognized only abnormal structures, including NCIs (Fig. 1B), NIIs (Fig. 1B, inset), DNs (Fig. 1C), round inclusions (Fig. 1D), skein-like inclusions (Fig. 1E), and GCIs (Fig. 1F). In double immunofluorescence staining for pTDP-43 (Fig. 1G, red) and a complement protein, C4d (Fig. 1H, green), pTDP-43-positive inclusions were often found in C4d-positive oligodendrocytes (Fig. 1I), indicating that most GCIs are oligodendrocytic in origin. In the frontal cortex of the ALS case with a long duration, we

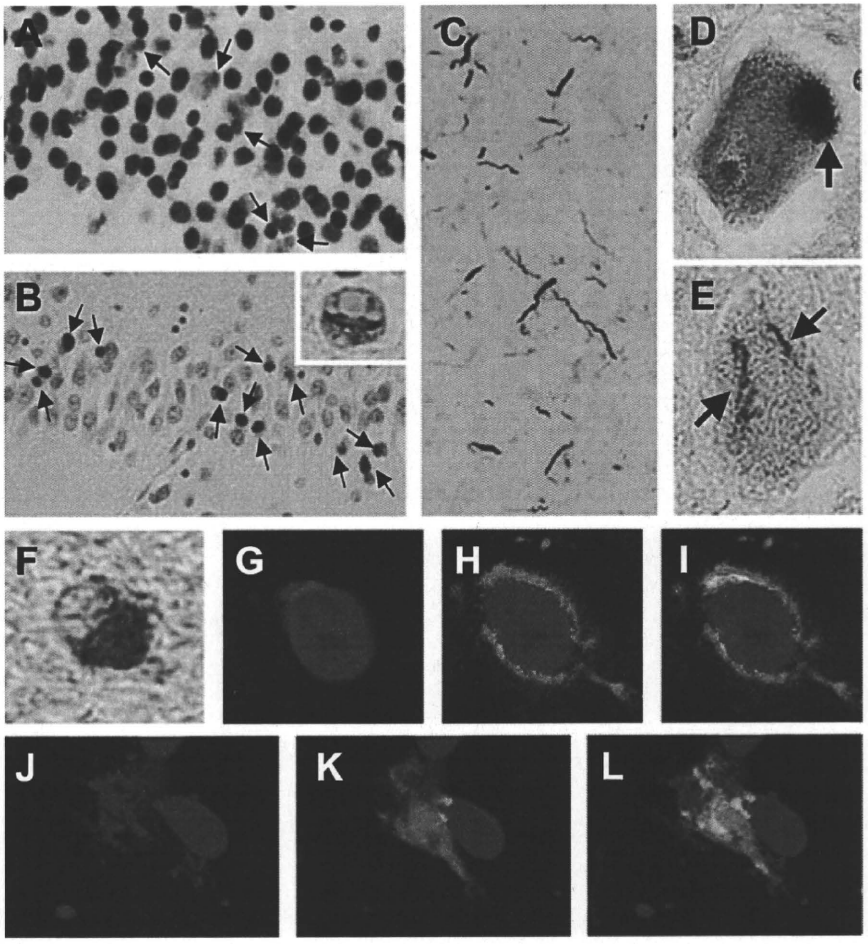

Fig. 1 Neuronal and glial inclusions immunopositive for phosphorylated transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) in frontotemporal lobar degeneration (FTLD)-TDP and ALS. A. Dentate gyrus (DG) of the hippocampus of the FTLD-TDP case stained with the commercially available phosphorylation-independent anti-TDP-43 antibody. Both neuronal cytoplasmic inclusions (NCIs) (arrows) and normal neuronal nuclei are immunopositive. B. Dentate gyrus of the FTLD-TDP case stained with the pTDP-43-specific antibody (pS409/410). NCIs are clearly stained with no nuclear staining. Inset represents neuronal intranuclear inclusions with a cat-eye shape. C. Dystrophic neurites in the temporal cortex of the FTLD-TDP positive for pS409/410. Motoneuronal round inclusion (D) and skein-like inclusion (E) of the ALS case are stained with pS409/410. F. Glial cytoplasmic inclusions in the motor system of the ALS case stained with pS409/410. In double-label immunofluorescence histochemistry using pS409/410 (red in G, I) and anti-C4d (green in H, I), the pS409/410-positive inclusion (red) is present around the nucleus of the C4d-positive oligodendrocyte (green) (I). In the frontal cortex of the ALS case with long duration, double-label immunofluorescence using pS409/410 (red in J, L) and anti-GFAP (green in K, L) shows a partial colocalization of the both proteins in the cytoplasm of the astrocyte.

found a partial colocalization of pTDP-43 and GFAP in the cytoplasm of astrocytes (Fig. 1J–L). These results suggest that all of the inclusion types previously described in FTLD-TDP and ALS contain pTDP-43.

Immunoblot analyses of sarkosyl-insoluble fractions with pTDP-43-specific antibodies revealed a single band at 45 kDa, several smaller fragments at ~25 kDa and indistinct smears in FTLD-TDP and ALS cases but not in controls (Fig. 2A). The intensity of the ~25 kDa fragments tended to be greater than that of the 45 kDa band in FTLD-TDP and in ALS. All of the immunoreactive bands were completely abolished by lambda protein phosphatase treatment, proving the specificity of the antibodies to the phosphoepitopes.

To investigate the biochemical basis of the different TDP-43 pathological subtypes (Types 1–3), we carefully compared the results of immunoblots of the sarkosyl-insoluble fractions from the cerebral cortex of cases with sporadic FTLD-TDP (Type 1), FTLD-MND (Type 2), ALS (Type 2) and familial FTLD with *PGRN* mutations (m*PGRN*) (Type 3), using pTDP-43 specific antibodies. The results showed that there is a close relationship between the pathological subtypes and the immunoblot pattern of the 18–26 kDa C-terminal fragments of pTDP-43 (Fig. 2B,C). These findings confirm and extend the previous reports[1,31] that showed C-terminal fragment composition varied between cases with Type 1 and Type 2 pathology. Furthermore, 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.[39] 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.

TDP-43-POSITIVE STRUCTURES IN A VARIETY OF NEURODEGENERATIVE DISORDERS AND THE SUBCLASSIFICATION OF TDP-43 PROTEINOPATHY

Immunohistochemical examination, using commercially available phosphorylation-independent anti-TDP-43 antibodies, had demonstrated abnormal intracellular accumulation of TDP-43 in neurodegenerative disorders other

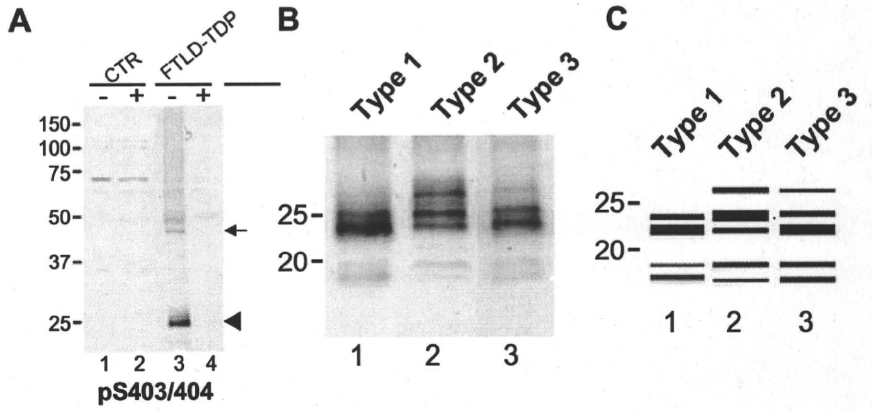

Fig. 2 Biochemical analyses using antibodies specific for phosphorylated transactivation response (TAR) DNA-binding protein of Mr 43 kDa (pTDP-43). A. Immunoblot analyses of sarkosyl-insoluble fractions from control (lanes 1, 2) and frontotemporal lobar degeneration (FTLD)-TDP (lanes 3, 4), using pS403/404, before (–) and after (+) the treatment with lambda protein phosphatase. pS403/404 specifically label the ~45 kDa band (arrow) and the ~25 kDa fragments (arrowhead) as well as a smear, only in FTLD-TDP (lane 3). These immunoreactivities are abolished after dephosphorylation. B. Representative immunoblots with the pTDP-43 specific antibody, pS409/410. The sporadic FTLD-TDP case with Type 1 pathology shows two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa (lane 1), while the FTLD-MND (motor neurone disease) case with Type 2 pathology shows three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa (lane 2). A 23 kDa band is the most intense in sporadic FTLD-TDP (lane 1), while a 24 kDa band is the most intense in FTLD-MND (lane 2). The band pattern of the case of familial FTLD with progranulin gene mutations with Type 3 pathology is not distinctive but intermediate between FTLD-TDP and FTLD-MND (lane 3). C. Schematic diagram showing the band pattern of the C-terminal fragments of pTDP-43.

than FTLD-TDP and ALS, including ALS/parkinsonism-dementia complex of Guam,[40–42] Alzheimer's disease (AD),[43–47] dementia with Lewy bodies (DLB),[44,48] Pick's disease,[2,49,50] hippocampal sclerosis,[43] and corticobasal degeneration (CBD).[47] However, the biochemical features of accumulated TDP-43, especially its phosphorylation sites and fragmentation, had been unclear in these disorders. To address these issues, we performed immunohistochemical and biochemical analyses of TDP-43 in cases of neurodegenerative disorders, using our pTDP-43-specific antibodies. As a result, we found a high frequency of pTDP-43 pathology in cases of AD (36–56%) (Fig. 3A,C), DLB (53–60%) (Fig. 3B,D), argyrophilic grain disease (AGD) (60%) (Fig. 3E), Huntington's disease (100%), and a case of familial British dementia.[51–54]

The pathological significance and mechanism of such a frequent co-occurrence of diverse protein aggregates are still unclear. A higher Braak NFT stage in the TDP-43-positive patients than in the TDP-43-negative ones was found in DLB+AD cases by Nakashima-Yasuda *et al.*[48] and in our study of AD cases.[51] We also reported parallel distribution of TDP-43-positive structures and tau-positive grains and higher AGD stages in cases with TDP-43 immunoreactivity than in those without TDP-43 immunoreactivity in AGD.[53] Double-label immunofluorescence microscopy reveals partial colocalization of tau and TDP-43 in AD, DLB, AGD, Guamanian PDC and CBD[40,41,43,44,47,48,53] or of α-synuclein and TDP-43 in DLB.[44,48,51] These findings suggest that there may be common factors or mechanisms that affect the conformation or modification of both proteins, leading to their intracellular accumulation. Nakashima-Yasuda *et al.* indicated two possibilities of the basis for those.[48] One is the direct interaction between the protein as tau and α-synuclein promote the fibrillization of each other *in vitro*.[55] The other is that the misfolding and aggregation of a disease protein disrupt normal cellular functions, leading to predisposing other proteins to aggregate.

Table 1 Subclassification of TDP-43 proteinopathy

1. pure TDP-43 proteinopathy	
Disease	Gene (locus)
A. Familial	
FTDU-17 (FTLD-TDP, Type 3)	PGRN
IBMPFD (FTLD-TDP, Type 4)	VCP
Perry syndrome	DCTN1
ALS	TARDBP (TDP-43)
FTLD-MND (FTLD-TDP, Type 2)	Chromosome 9
B. Sporadic	
FTLD-TDP (Types 1-3)	
ALS	
2. Combined TDP-43 proteinopathy	
Disease	Aggregated proteins
A. Familial	
FBD	ABri, Tau, TDP-43
HD	Huntingtin, TDP-43
MJD (SCA3)	Ataxin-3, TDP-43
B. Sporadic	
AD	Tau, TDP-43
DLB	Tau, Alpha-syn, TDP-43
CBD	Tau, TDP-43
AGD	Tau, TDP-43
C. Endemic	
Guam ALS/PDC	Tau, Alpha-syn, TDP-43
Kii ALS/PDC	Tau, Alpha-syn, TDP-43

FTDU-17, frontotemporal dementia with ubiquitinated inclusions linked to chromosome 17; PGRN, progranulin; IMBPFD, inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia; DCTN1, dynactin 1; ALS, amyotrophic lateral sclerosis; TARDBP (TDP-43), TAR DNA-binding protein of 43 kDa.

AD, Alzheimer's disease; AGD, argyrophilic grain disease; Alpha-syn, α-synuclein; CBD, corticobasal degeneration; DLB, dementia with Lewy bodies; FBD, familial British dementia; FTLD-MND, frontotemporal lobar degeneration with motor neuron disease; HD, Huntington disease; MJD, Machado-Joseph disease; PDC, parkinsonism-dementia complex; SCA, Spinocerebellar ataxia.

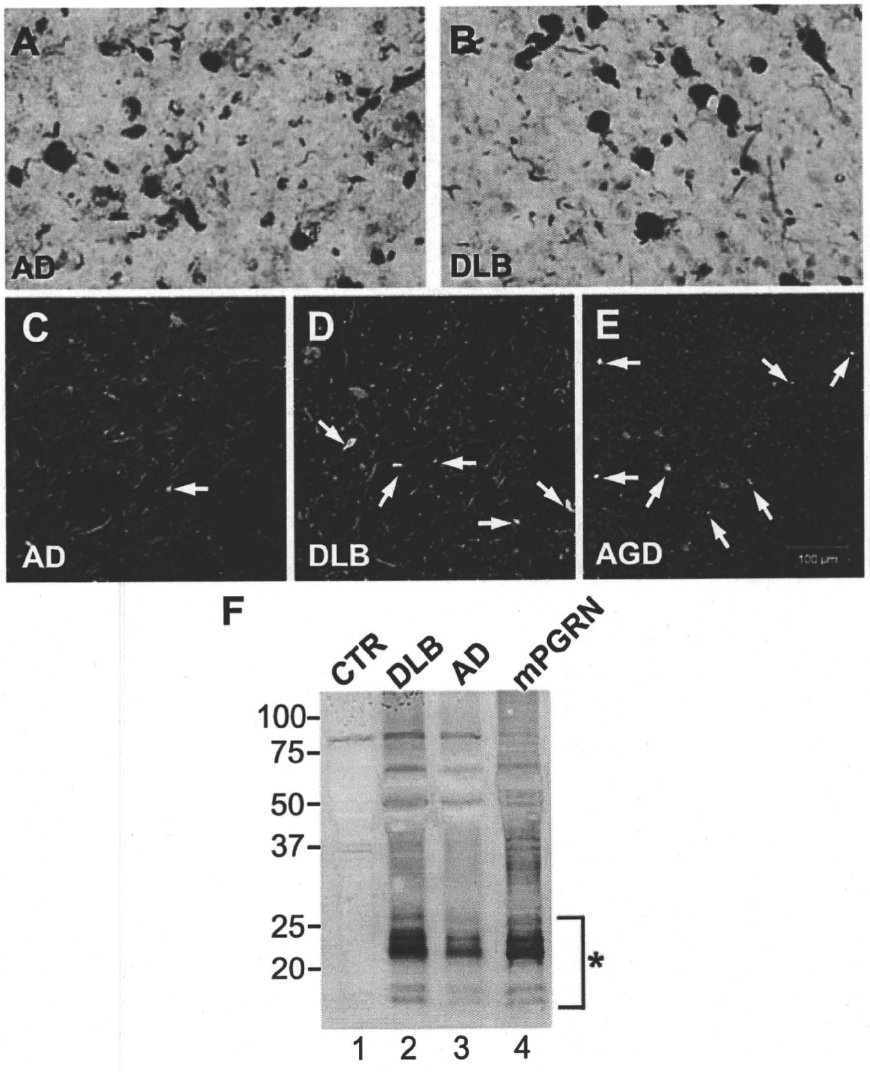

Fig. 3 Phosphorylated transactivation response (TAR) DNA-binding protein of Mr 43 kDa (pTDP-43)-positive structures in other neurodegenerative disorders. A. Neuronal cytoplasmic inclusions (NCIs) and dystrophic neurites stained with the pTDP-43-specific antibody (pS403/404) in the temporal cortex of the Alzheimer's disease (AD) case (A) and the dementia with Lewy bodies (DLB) case (B) with diffuse TDP-43 pathology. (C–E) Double-label immunofluorescence histochemistry of the temporal cortex of AD (C) and DLB (D) and of the amygdala of argyrophilic grain disease (AGD) (E). The green fluorescence reveals the immunoreactivity for phosphorylated tau (AT8) in C and E, and that for phosphorylated α-synuclein in D, while the red fluorescence represents the immunopositivity for pS403/404 in C–E. Arrows indicate the colocalization of tau and pTDP-43 in C and E, and that of α-synuclein and pTDP-43 in D. F. The band pattern of the C-terminal fragments of pTDP-43 (asterisk) in DLB (lane 2) and AD (lane 3) is similar to that in familial FTLD with progranulin gene mutation (mPGRN).

Of the TDP-43-positive cases in AD and DLB, about 20–30% showed neocortical TDP-43 pathology resembling the FTLD-TDP, Type 3[51] (Fig. 3A,B). 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-TDP, Type 3[37] (Fig. 3F). 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-TDP. Since all FTLD-TDP cases with PGRN mutations show Type 3 pathology,[56] 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.

The clinical impact of the concurrent TDP-43 pathology in other neurodegenerative disorders than FTLD-TDP and ALS is also not fully understood. Uryu *et al.* reported a lack of association between TDP-43 pathology and clinical manifestation of AD.[47] Similarly, we did not find a significant difference of clinical features between AGD cases with and without TDP-43 pathology.[53] Joseph *et al.* on the other hand, reported that AD cases with TDP-43 pathology were older at onset and death, and performed worse on the Clinical Dementia Rating Scale, Mini-Mental State Examination, and Boston Naming Test than those without TDP-43 pathology.[46] The older age at death of the AD cases with TDP-43 pathology was also observed in our study.[51] Nakashima-Yasuda *et al.* found a higher average age at death in the TDP-43 positive cases in Lewy body-related diseases with dementia.[48] Further studies using larger cohorts with more detailed clinical, radiological and pathological data are needed to elucidate the clinical impact of TDP-43 pathology in a variety of neurodegenerative disorders.