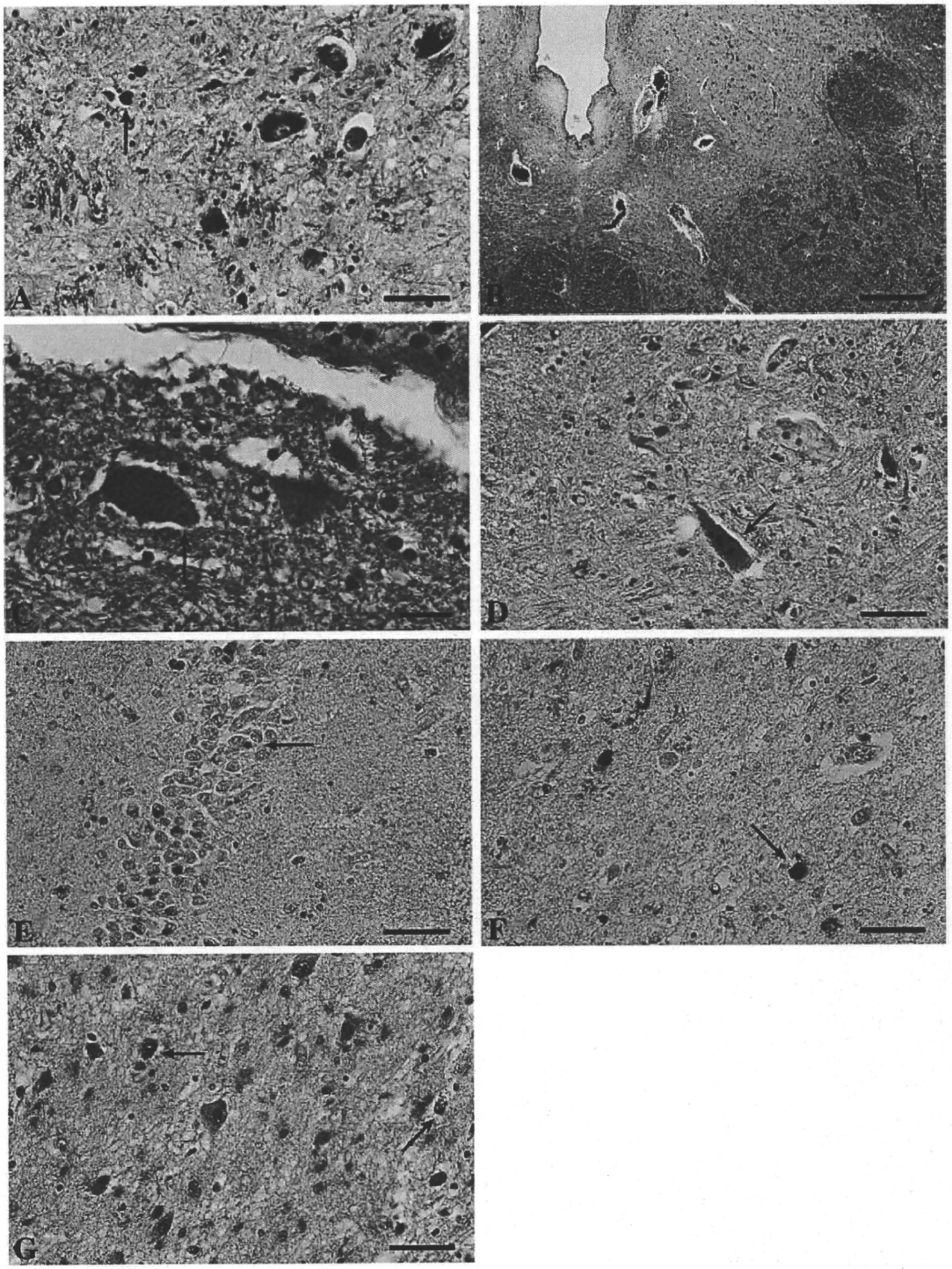

Fig. 3. A: Substantia nigra shows neuronal loss with frequent free melanins (arrow). B–D: Hypoglossal nucleus shows marked neuronal loss (B) with presence of Bunina bodies (C, D, arrow). E: Ubiquitin-positive cytoplasmic inclusions (arrow) in hippocampal dentate granule cells. F, G: Ubiquitin-positive (F) and TDP-43-positive (G) cytoplasmic inclusions (arrow) in amygdala. A, B: Klüver–Barrera staining. C: Hematoxylin and eosin staining. D: Cystatin C staining. E, F: Ubiquitin staining. G: TDP-43 staining. Bars, A: 50 μm, B: 500 μm, C: 20 μm, D–G: 50 μm.

right side, the SD symptoms including prosopagnosia were not noticed throughout the disease course. Though we could not completely rule out the possibility that the SD symptoms were difficult to be found because of progressive dysarthria, or might have been recognized afterward if the patient had lived longer, our case demonstrating type 2 TDP-43 pathology was unlikely to present with SD symptoms because most FTLD cases with SD symptoms have type 1 pathology [19]. We can assume that the etiology of FTLD–MND might be essentially different from that of FTLD with SD symptoms because both psychological symptoms and TDP-43 pathologies are mostly distinct between these two groups of FTLD. On the other hand, the severity and distribution of cerebral atrophy can be similar in a wide variety of FTLD–MND manifestations.

In conclusion, our case of FTLD–MND demonstrated a rare phenotype of severe and circumscribed atrophy of anterior temporal lobes from early clinical stage, with typical features in clinical

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symptoms and TDP-43 pathologies. Further information of similar cases will be useful for understanding the pathophysiology of FTLD and MND.

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INTERNAL MEDICINE

□ CASE REPORT □

Familial ALS with G298S Mutation in *TARDBP*: A Comparison of CSF Tau Protein Levels with those in Sporadic ALS

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Abstract

We report a 52-year-old Japanese man showing both upper and lower motor neuron signs with familial amyotrophic lateral sclerosis (ALS). Analysis of the TAR DNA-binding protein of 43 kDa (TDP-43) gene (*TARDBP*) revealed a glycine-to-serine substitution at position 298 (G298S). Cerebrospinal fluid (CSF) level of total tau protein (CSF-tau) of our patient was found to be highly elevated compared with those of sporadic ALS cases and controls. The elevated CSF-tau level might be related to the damage of neurons exhibiting a large number of TDP-43 inclusions in familial ALS with this mutation.

Key words: familial amyotrophic lateral sclerosis, TAR DNA binding protein gene, tau, cerebrospinal fluid

(Inter Med 49: 1209-1212, 2010)
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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the degeneration of motor neurons in the brain and spinal cord. In ALS, lower motor neurons exhibit ubiquitinated neuronal inclusions (UNIs), which are also detected in the brain in cases of frontotemporal lobar degeneration (FTLD) (1). TAR DNA-binding protein of 43 kDa (TDP-43) has been identified as the major component of UNIs, and both sporadic ALS (SALS) and FTLD are considered to involve a common pathological mechanism (1). This group of neurological diseases that are associated with TDP-43 accumulation are referred to as TDP-43 proteinopathies (1).

Familial ALS (FALS) is observed in 5 to 10% of all ALS cases, and exhibits an autosomal dominant inheritance (2). The most common cause of FALS was reported to be mutations in the Cu/Zn superoxide dismutase gene (*SOD-1*); however, *SOD-1* mutations have been found in about 20% of FALS cases (2). Recently, FALS cases with mutations in the TAR DNA-binding protein gene (*TARDBP*) which encodes TDP-43 have been reported (3-10). We report a Japanese FALS patient with a mutation in *TARDBP*; we examined the levels of cerebrospinal fluid (CSF)-amyloid β protein 1-42 (CSF-$A\beta_{42}$), CSF-total tau protein (CSF-tau), and CSF-phosphorylated tau protein (CSF-ptau) in our patient compared with SALS patients and controls.

Case Report

A 52-year-old Japanese man first noticed difficulty moving his right thumb, and fasciculation in all limbs and trunk developed within one month. Over the following month, the

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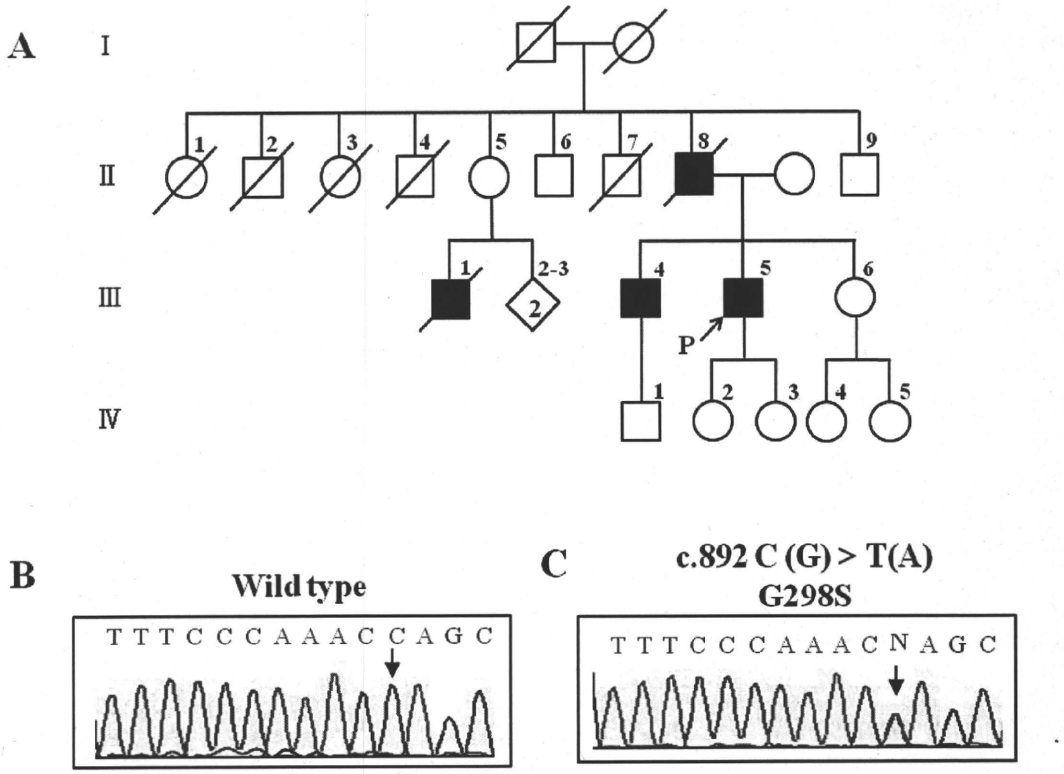

Figure 1. (A) The pedigree of our patient's family. The proband (our patient III-5) is marked with an arrow. Roman numerals indicate generations, and Arabic numerals at the upper right of the symbols indicate individuals. The symbols are as follows: squares, males; circles, females; diamond shape, individuals for whom gender was not disclosed. Multiple siblings are indicated with a number inside the symbols. Shading of the symbols indicates a diagnosis of ALS (our patient's father II-8, our patient's brother III-4 and our patient's cousin III-1). The *TARDBP* was only analyzed in our patient (III-5). (B and C) Sequencing chromatogram of a part of *TARDBP* in a sample from a control (B) and our patient (C). The chromatogram shows the heterozygous sequence trace of C (G) to T (A) at complementary DNA position c.892 for genotyping by reverse primer. The nucleotide position of the substitution is indicated by an arrow.

weakness of his right hand progressed, and cramp of the lower limbs developed. Neurological examination performed three months after the onset revealed mild weakness of the right abductor pollicis brevis, opponens pollicis, and extensor hallucis brevis; hyperreflexia in all extremities with positive right Babinski and Chaddock signs; and fasciculation in all extremities and the back. The patient's sensory perception and coordination were unremarkable. A laboratory test showed a mild elevation of serum creatinine phosphate kinase level (450 IU/L; normal, 45-163 IU/L). CSF examination showed a normal cell count and protein level. Magnetic resonance images of brain and spinal cord disclosed no remarkable findings. Electromyography revealed active denervation and renervation discharges in muscles of all limbs and the tongue. Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) indicated a full scale Intelligence Quotient (IQ) 109, verbal IQ 94, and performance IQ 106. The results of a four-factor model were as follows: verbal comprehension, 93; perceptual organization, 108; working memory, 102; and processing speed, 102. We diagnosed definite ALS according to the revised El Escorial criteria (11).

The patient was treated with 100 mg/day of riluzole; however, during the year following disease onset, weakness rapidly progressed, and the patient's gait became disturbed. The patient died due to respiratory failure 15 months after disease onset.

The patient's father (II-8), brother (III-4), and cousin (III-1) had suffered from ALS (Fig. 1A). The father (II-8) developed weakness of the right hand at age 45, which spread to his right leg, and lead to a bed-ridden state for a half-year period. The father died two years after disease onset. The brother (III-4) developed weakness of the left leg at age 54, and, 8 months after the diagnosis, he required wheelchair and noninvasive positive pressure ventilation. We could not obtain detailed information of the cousin (III-1) who had been diagnosed with ALS. There was little information of the cousin's mother (II-5) who had not developed ALS when the patient was admitted to our hospital. The family history suggests a diagnosis of FALS with autosomal dominant inheritance.

Genomic DNA was purified from whole blood. All exons and exon-intron boundaries of *SOD-1* (12) and *TARDBP* were analyzed with PCR and direct sequencing. Sequencing of *TARDBP* was performed for our patient and for 96

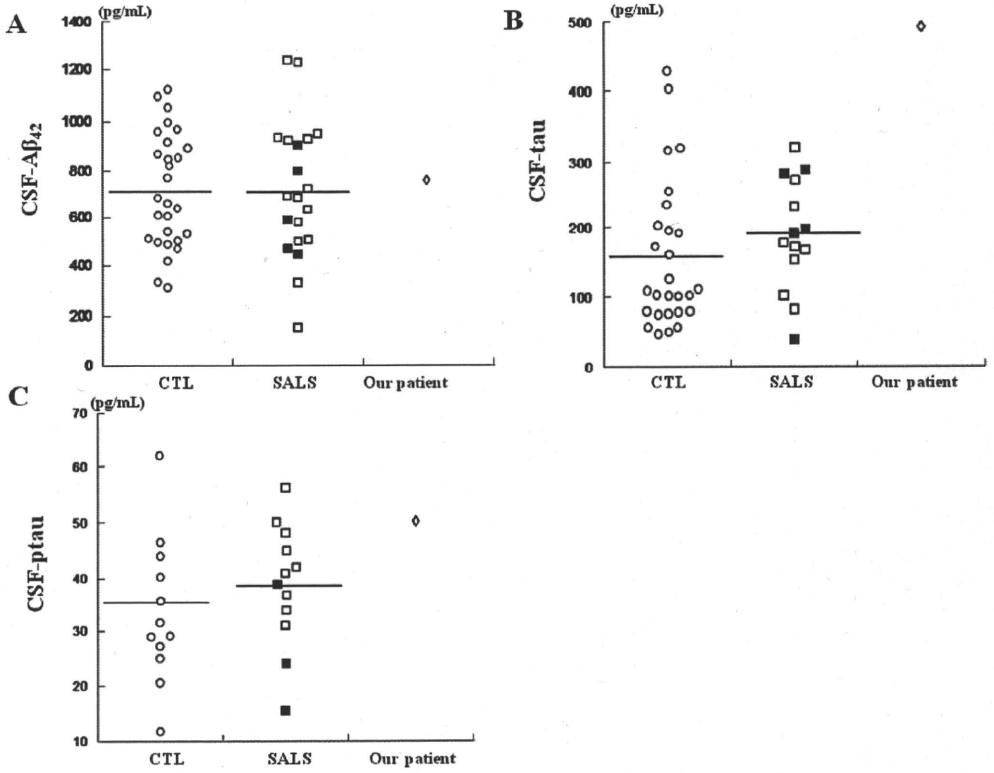

Figure 2. (A, B, and C) Comparison of cerebrospinal fluid (CSF)-amyloid β protein 1-42 (CSF-$A\beta_{42}$) (A), total tau protein (CSF-tau) (B), and phosphorylated tau protein (CSF-ptau) (C) among controls (CTL), sporadic amyotrophic lateral sclerosis (SALS) cases, and our patient. In addition to our patient, 20 patients with SALS [age, 60.8±11.0 years; clinical duration (from disease onset to admission), 23.3±21.7 months] and 27 age-matched CTL without disorders of the central nervous system (age, 53.8±17.4 years) were included in the study. Bars show the mean value. Five SALS patients with relatively rapid progression (duration of clinical course <10 months) are indicated by solid squares (see text).

healthy Japanese subjects. The genomic structure of *TARDBP* (RefGene NM_007375) was confirmed from the University of California, Santa Cruz database (http://www.genome.ucsc.edu/) and the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/>). All participants provided informed consent. The present study was approved by the ethics committees of all participating institutions.

The levels of CSF-$A\beta_{42}$, CSF-tau, and CSF-ptau in which tau protein is phosphorylated at Thr181, were measured in the following groups as previously reported (13): our patient; 20 patients with SALS; and 27 age-matched controls without disorders of the central nervous system.

Sequencing of *SOD-1* revealed no mutations. Sequencing of the coding regions of *TARDBP* revealed a heterozygous G-to-A transition at complementary DNA position 892 (c.892 G>A), which leads to the substitution of glycine by serine at position 298 (G298S) in a highly conserved region within exon 6 (Figs. 1B, 1C). We also searched for this mutation in 96 healthy controls, and none demonstrated this mutation.

When CSF-$A\beta_{42}$, CSF-tau, and CSF-ptau levels were compared between SALS cases and controls, the levels of CSF-$A\beta_{42}$ [n=20, 712.0±280.9 (mean ± standard deviation) (range, 159-1,243) pg/mL], CSF-tau [n=14, 190.6±82.7 (39-321) pg/mL], and CSF-ptau [n=12, 38.6±11.3 (15.6-56.0) pg/mL] in the SALS cases were not significantly different from those in the controls [CSF-$A\beta_{42}$, n=27, 711.7±241.5 (337-1,126) pg/mL; CSF-tau, n=27, 156.3±106.9 (53-428) pg/mL; and CSF-ptau, n=13, 35.3±14.2 (12.0-62.1) pg/mL] (Fig. 2A-C). In our patient, the concentrations of CSF-$A\beta_{42}$ (764 pg/mL) and CSF-ptau (50 pg/mL) were similar to those of the SALS cases and the controls (Figs. 2A, 2C); however, the concentration of CSF-tau (491 pg/mL) was elevated by comparison (+3SD compared with the mean value of those in the controls) (Fig. 2B). In five patients with SALS who showed relatively rapid progression (duration from onset to CSF examination <10 months), the concentration of CSF-tau was 199.6±100.8 (range, 39-282) pg/mL, which was not significantly different from those in the other nine SALS patients (Fig. 2B).

Discussion

More than 10 mutations in *TARDBP* have been identified in FALS and SALS cases (3-10). We have described a Japanese FALS patient without dementia with a G298S missense mutation of *TARDBP*. To date, this mutation has only been

reported in a Chinese FALS family (3). The family with this mutation was reported to exhibit disease onset at between 41-60 years of age with subsequent rapid progression (mean duration, 2 years) (4). The present patient and affected family members demonstrated similar ages at disease onset, and similar clinical durations (<2 years) to the reported cases (3). The aunt (II-5) [i.e. the mother of affected cousin (III-1)] was a healthy carrier, and this incomplete penetrance was compatible with the previous report (3).

CSF-tau is considered to reflect neuronal damage, but it is controversial whether CSF-tau concentration is elevated in SALS cases compared with controls (14-17). In our study, the CSF-tau levels did not differ between SALS and controls; however, the CSF-tau level of our patient was found to be elevated compared with those of SALS cases and controls. The CSF-tau levels were not associated with severity of disease progression in the SALS patients as reported in a previously report (17), and the duration of the clinical course from onset to CSF examination in our patient (three months) was similar to those in the five SALS patients with relatively rapid progression (5.6±0.9 months; range, 4-6 months). Thus, the elevated CSF-tau level cannot be simply attributed to rapid disease progression of FALS with the G298S mutation of *TARDBP*. Although there was no quantitative pathological analysis of TDP-43 positive inclusions and pre-inclusions, the reported FALS patients with the G298S mutation of *TARDBP* were reported to have more TDP-43 positive pre-inclusions in many areas of the central nervous system compared with SALS patients (3). We speculate that tau protein may be easily released from damaged motor neurons with TDP-43 positive inclusions to extracellular space. In addition, the CSF-ptau levels were not changed in this FALS patient as well as in SALS patients, suggesting the absence of hyperphosphorylated tau pathology in ALS. The CSF-$A\beta_{42}$ level has previously been reported to be significantly decreased in ALS cases compared with controls (16); in our study, however, CSF-$A\beta_{42}$ levels in our patient and in SALS patients were not significantly different from those of controls. The previous reports on FALS or SALS with *TARDBP* mutations including the G298S mutation presented no data about CSF-tau, CSF-$A\beta_{42}$, or CSF-ptau (3).

Finally, the G298S mutation of *TARDBP* has only been identified in FALS patients with an Asian ethnic background (3). It remains to be determined whether this mutation is also present in populations with other ethnic backgrounds.

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INTERNAL MEDICINE

□ CASE REPORT □

TDP-43 M337V Mutation in Familial Amyotrophic Lateral Sclerosis in Japan

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Abstract

The clinical features of a Japanese family with autosomal dominant adult-onset amyotrophic lateral sclerosis (ALS) are reported. Weakness initially affected the bulbar musculature, with later involvement of the extremities. Genetic studies failed to detect any mutations of the Cu/Zn superoxide dismutase-1 (SOD1) and Dynactin1 (DCTN1) genes, but revealed a single base pair change from wild-type adenine to guanine at position 1009 in TAR-DNA-binding protein (TDP-43), resulting in a methionine-to-valine substitution at position 337. The immunohistochemical study on autopsied brain of the proband's aunt showed TDP-43-positive cytoplasmic inclusions in the anterior horn cells of the spinal cord and in the hypoglossal nucleus, as well as glial cytoplasmic inclusions in the precentral gyrus, suggesting that a neuroglial proteinopathy was related to TDP-43. In conclusion, a characteristic clinical phenotype of familial ALS with initial bulbar symptoms occurred in this family with TDP-43 M337V substitution, the pathomechanism of which should be elucidated.

Key words: Amyotrophic lateral sclerosis (ALS), TAR-DNA-binding protein 43 (TDP-43)

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder that is characterized pathologically by the degeneration of motor neurons in the brain and spinal cord, and clinically by progressive weakness and death within a few years of onset. Recently, TAR DNA-binding protein 43 (TDP-43) was identified as the major pathological protein in the motor neuron inclusions found in sporadic ALS and superoxide dismutase 1 (SOD1)-negative familial ALS, as well as in frontotemporal lobar degeneration with ubiquitin-immunoreactive, tau-negative inclusions (FTLD-U). Although the role of TDP-43 in the pathogenesis of these neurodegenerative disorders remains to be elucidated, several mutations of TDP-43 have been identified in individuals with sporadic and familial ALS, suggesting that TDP-43 may be a causative protein for these disorders (1-6). Here we first report the detailed clinical features of affected members of a Japanese family who suffered from ALS linked to TDP-43 M337V mutation.

Case Report

The proband (III-2 in Fig. 1-1) was a Japanese woman aged 61 years. She developed dysarthria at the age of 55 years, which became progressively worse. One year later, she also noted dysphagia. Neurological examination at the age of 56 revealed minimal atrophy of the facial muscles and tongue, markedly diminished reflexes of the palatal and pharyngeal muscles, and slow movements and minimal fasciculation of the tongue. Her deep tendon reflexes, including the jaw jerk, were highly exaggerated. At the age of 57, her dysphagia worsened, and atrophy and fasciculation of the

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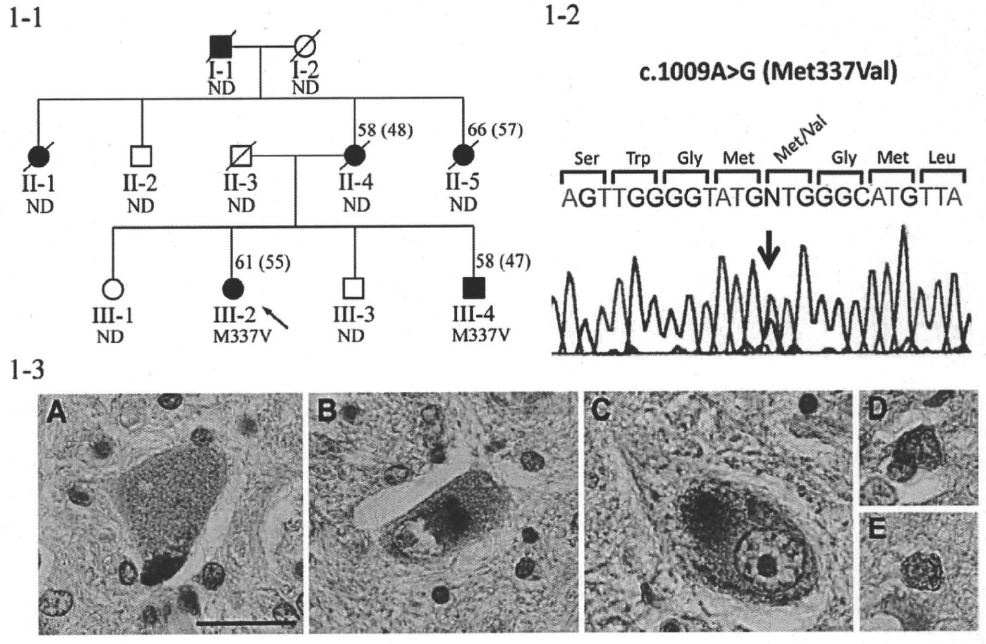

Figure 1. 1-1. Pedigree of the present family. Circles represent women and squares represent men. The slashed symbols indicate deceased subjects. Known affected persons are shown as filled symbols. The arrow represents the proband. Age at death or current age and age at disease onset in parenthesis are indicated. ND=not determined. 1-2. Chromatogram of Patient III-2 (the proband). Chromatogram shows the heterozygous sequence trace of A to G for genotyping by the reverse primer. The nucleotide position of substitution is indicated by arrow. 1-3. Immunocytochemical findings in Patient II-5. TDP-43 positive cytoplasmic inclusions in the anterior horn of the spinal cord (A, B) and in the hypoglossal nucleus (C). Glial cytoplasmic inclusions in the precentral gyrus (D, E). (A, C) Phosphorylation-independent anti-TDP-43 antibody; (B, D, E) phosphorylation-dependent anti-TDP-43 antibody (pS409/410). The sections were counterstained with hematoxylin to reveal nuclei. Bar in A=25 μm.

tongue became more prominent. Muscle weakness of the lower extremities showed slow progression, predominantly in the distal regions. At the age of 58, she was almost unable to protrude her tongue. At the age of 61, she also noted mild weakness of the upper extremities. Needle EMG showed marked neurogenic changes of the biceps, abducens pollicis brevis, vastus lateralis and tibialis anterior muscles of the right side, as well as a mild neurogenic pattern in her right masseter.

The aunt of the proband (II-5 in Fig. 1-1), a Japanese woman, developed dysarthria at the age of 57 years, followed by dysphagia, weakness of the upper extremities, and difficulty with breathing. She could walk without support until her death at the age of 66. The results of the neuropathological examination were reported in detail (7).

The younger brother of the proband (III-4 in Fig. 1-1), a Japanese man, developed dysarthiria at the age of 44 years. Neurological examination at the age of 47 showed slight dysarthria, poor movement of the soft palate, exaggerated pharyngeal reflexes and jaw jerk, slow movements, slight atrophy and fasciculation of the tongue. These findings were mainly related to pseudobulbar palsy. He also showed hyperreflexia in the upper and lower extremities (predominantly in the latter) without any pathological reflexes. Needle EMG revealed neurogenic changes of the masseter and orbicularis oris muscles, while there was a normal pattern in the tongue and extremities. He had no dysphagia, muscle weakness, or atrophy of the upper and lower extremities, as well as no sensory disturbance or vesicorectal disturbance. He could stand and walk unaided. His condition deteriorated slowly and progressively over the next 10 years. At present, he is 58 years old and virtually bed-ridden with a gastrostomy and minimal communication. Patient II-4, Patient II-1 and Patient I-1 all suffered from dysarthria until death, the details of which were unknown.

The present family demonstrated autosomal dominant inheretance of ALS and both sexes were affected. Six family members (patients I-1, II-1, II-4, II-5, III-2 and III-4) were suspected to have ALS, among whom three (II-5, III-2 and III4) had definite ALS according to the El-Escorial criteria. All six patients (2 men and 4 women) with familial ALS in this family showed dysarthria at the onset, so their clinical courses were indistinguishable from bulbar-onset ALS. There was no history of dementia and no atypical features in the kindred. Based on the information of the patients with good clinical records (patients II-4, II-5, III-2 and III-4), the mean age of symptom onset was 52.5 years (range 44-61 years) and the mean disease duration was 9.5 years (range

9-10 years) from symptom onset to death based on the outcome in patients II-4 and II-5.

After approval by the Ethics Committees of all participating institutions, sequencing of the coding regions of the TDP-43 gene in the patients (III-2 and III-4) was performed, which showed a heterozygous A-to-G transition at cDNA position 1009 (c.1009A>G) resulting in a methione-to-valine substitution at position 337 (M337V) in a highly conserved region of exon 6 (Fig. 1-2). None of the control 1,621 healthy subjects providing informed consent had this missense mutation.

Immunohistochemistry analysis of the brain of patient II-5 using both a phosphorylation-independent anti-TDP-43 antibody (10782-2-AP) and a phosphorylation-dependent anti-TDP-43 antibody (pS409/410) (8) showed neuronal cytoplasmic inclusions in the anterior horn of the spinal cord (Fig. 1-3A, B) and the hypoglossal nucleus (Fig. 1-3C), as well as glial cytoplasmic inclusions in the precentral gyrus (Fig. 1-3D, E).

Discussion

In the present study, we detected the M337V substitution in TDP-43 in a Japanese family with ALS, including one case confirmed at autopsy (patient II-5). We consider that this M337V substitution was associated with the disease, since M337V was present in two affected individuals from one generation and never in the control subjects, in addition to the fact that M337V substitution of TDP-43 has already been reported to segregate with ALS within two probably unrelated kindreds (2, 6). In a UK autosomal dominant ALS family carrying M337V substitution of TDP-43 reported by Sreedharan et al (2), three had limb-onset ALS and two had bulbar-onset ALS. The mean age of symptom onset was 47 years (range 44 to 52). Mean disease duration was 5.5 years (range 4 to 7) from symptom onset to death. The M337V mutation carrier in a US family with a strong family history of ALS reported by Rutherford et al (6) showed upper limb-onset ALS at 38 years of age, 6 years younger than the earliest onset age reported in the British M337V family (2). In the present paper, we show the first Japanese family with ALS carrying M337V substition of TDP-43, in which virtually all patients showed dysarthria at the onset, suggesting that their clinical courses were indistinguishable from bulbar-onset ALS. Among these UK, US and Japanese families carrying TDP-43 M337V mutation, the common features include no signs of dementia or other atypical features of ALS and past middle age onset of the disease. However, the signs at onset were different among these three families, and mean disease duration in the present Japanese family was longer than that in the UK family, indicating the phenotype of this mutation is quite variable. The identification of M337V in three genealogically unrelated ALS families further implies the pathogenicity of TDP-43 M337V mutation.

Regarding the pathogenecity of TDP-43 M337V mutation, Sreedharan et al (2) reported that mutant forms of TDP-43 (including M337V) fragmented *in vitro* more easily than wild-type TDP-43 and, *in vivo*, caused neuronal apoptosis and developmental delay in chick embryos, suggesting a pathophysiological link between TDP-43 and ALS. In addition, Rutherford et al (6) showed that biochemical analysis of TDP-43 in lymphoblastoid cell lines of carriers with TDP-43 mutations including M337V revealed a substantial increase in fragments possibly cleaved by caspase, including the ~25 kDa fragment, compared to control cell lines, supporting TDA-43 as a cause of ALS. Our immunohistochemical study showed TDP-43 positive cytoplasmic inclusions in the anterior horn cells of the spinal cord and in the hypoglossal nucleus, as well as glial cytoplasmic inclusions in the precentral gyrus, suggesting that a neuroglial proteinopathy was related to TDP-43. Further investigations including biochemical analysis using patients' fibroblasts or lymphoblastoid cells will be necessary to elucidate the mechanism by which TDP-43 contributes to ALS and to develop new drugs that block the pathological process related to TDP-43.

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ORIGINAL PAPER

Effect of topographical distribution of α-synuclein pathology on TDP-43 accumulation in Lewy body disease

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Abstract It has been reported that the development of TDP-43 pathology in cases of Lewy body disease (LBD) might be associated with the severity of tau pathology. However, the impact of α-synuclein pathology on TDP-43 accumulation in LBD remains unclear. To clarify whether α-synuclein pathology has an effect on TDP-43 accumulation, independent of tau pathology, we examined by immunohistochemistry 56 cases of LBD using a phosphorylation-dependent TDP-43 antibody. The frequency of TDP-43 pathology in all LBD cases was 18% (10/56). In 37 LBD cases with no or low tau burden (LBD-Ltau; Braak NFT stages 0-II), the frequency of TDP-43 pathology was 19% (7/37). The frequency of TDP-43 pathology in diffuse neocortical type LBD-Ltau cases was 36% (4/11), which was higher than those in limbic and brain stem-predominant types (11–14%). The amygdala and entorhinal cortex were the most frequently affected sites of TDP-43 pathology in LBD-Ltau cases. In LBD-Ltau cases, the proportion of diffuse neocortical type LBD was higher in the TDP-43-positive cases, than that in TDP-43-negative cases (57 vs. 23%). In all LBD cases, α-synuclein pathology in the temporal cortex was significantly more severe in TDP-43-positive cases, and significantly correlated with the severity of TDP-43 pathology in the amygdala. In a multivariate model, the presence of severe α-synuclein pathology was significantly associated with the development of TDP-43 pathology independent of age at death and tau pathology. In the amygdala, TDP-43 was often colocalized with α-synuclein or tau. Given these findings, we suggest that α-synuclein pathology is associated with TDP-43 accumulation in LBD cases.

Keywords α-Synuclein · DLB · Lewy body disease · Tau · TDP-43

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Introduction

The transactivation-responsive DNA-binding protein of M_r 43 kDa, TDP-43, is a major component of ubiquitin-positive and tau-negative inclusions in the frontotemporal cortex and motor neurons in frontotemporal lobar degeneration (FTLD-U) and in amyotrophic lateral sclerosis (ALS), and is considered to play an essential pathogenic

role in these diseases, now called TDP-43 proteinopathies [3, 6, 7, 29]. However, abnormal TDP-43 accumulations have been demonstrated in cases of Alzheimer's disease (AD) [1, 2, 15], ALS/parkinson–dementia complex of Guam (ALS/PDC of Guam) [9, 12], argyrophilic grain disease (AGD) [8], corticobasal degeneration (CBD) [32], and progressive supranuclear palsy (PSP) [34]. Additionally, some (but not all) studies have supported the possibility that the severity of tau pathology is associated with TDP-43 accumulation in AD [1, 2], AGD [8], and PSP [34].

A few studies have demonstrated a concurrent TDP-43 pathology in some cases with Lewy body disease (LBD), including ones with Parkinson's disease (PD), Parkinson's disease with dementia (PDD), and dementia with Lewy bodies (DLB). The reported frequencies of TDP-43 pathological changes in several LBD series ranged from 19 to 60% [2, 14, 28]. However, most LBD cases have variable degrees of AD-type pathology [11, 17, 21–23, 27, 30, 33]. Indeed, in the earliest (and largest) study that examined TDP-43 pathology in LBD, approximately 50% of 180 LBD cases had moderate to severe tau pathology, and a higher frequency of TDP-43 pathology was observed in cases with a more severe Braak NFT stage score [28]. In a recent study, about 70% of TDP-43-positive LBD cases had moderate to severe tau pathology (Braak NFT stages III–VI) [2]. Nonetheless, somewhat unexpectedly, it has never been examined whether the development of TDP-43 pathology in LBD is influenced by α-synuclein pathology, or can simply be explained by the effect of concurrent tau pathology. Higashi et al. [14] reported no significant difference in the severity of α-synuclein pathology between DLB cases with or without TDP-43 pathology, and AD cases with or without TDP-43 pathology. However, the number of subjects in the study was small (11 DLB cases including 5 TDP-43-positive cases, and 15 AD cases including 5 TDP-43-positive cases), and the influence of tau pathology was not compensated for.

The principal aim of this study was to investigate whether the presence of α-synuclein pathology is associated with TDP-43 accumulation in LBD. To address this, we revisited the frequency and distribution of TDP-43 pathology using a phosphorylation-dependent TDP-43 antibody in LBD cases with no or low tau burden (corresponding to Braak NFT stages 0–II [4]) (i.e., LBD-Ltau cases) and LBD cases with more severe tau burden (Braak NFT stages III–VI) (LBD-Htau cases). Secondly, we compared the severities of α-synuclein and tau pathologies between LBD cases with and without TDP-43 accumulation, and also examined the correlation between the severity of TDP-43 pathology and that of α-synuclein or tau pathology. Thirdly, we examined the frequencies of TDP-43 pathology in three subtypes of LBD (i.e., brain stem-predominant type, limbic type, and diffuse neocortical type [27]) concentrating especially on the LBD-Ltau cases, and asking whether the severity of α-synuclein pathology was independently associated with the development of TDP-43 pathology in all LBD cases using multivariate models. Finally, we performed double immunofluorescence labeling and biochemical examination in order to further understand the pathogenic mechanism underlying TDP-43 accumulation in LBD.

Materials and methods

Subjects

All of the available pathologically confirmed LBD cases ($n = 56$) in the UK Parkinson's Disease Society Tissue Bank, as well as pathologically normal controls ($n = 4$), were examined in this study. The clinical diagnosis in these cases was LBD (i.e., 29 cases of PD, 51.8%; 23 cases of PDD, 41.1%; and 4 cases of DLB, 7.1%). The clinical diagnosis of PDD was based on motor impairment preceding cognitive impairment by at least 1 year [27]. The most frequent pathological subtype of LBD in our series was limbic type (51.8%), followed by the diffuse neocortical type (30.4%) and lastly the brainstem-predominant type (17.9%). No cases having other degenerative diseases, such as PSP, CBD, and multiple system atrophy, were included in this study. The proportion of LBD cases with severe tau pathology in this series was low: 37 cases (66% of all LBD cases) had no or low tau burden corresponding to Braak NFT stages 0–II (i.e., were LBD-Ltau cases): 30 cases (53.6%) corresponded to Braak NFT stage II, six cases (10.7%) were Braak NFT stage I, and only one case completely lacked tau pathology (1.8%). The other 19 cases (34%) had a higher tau burden corresponding to Braak NFT stages III–VI (i.e., LBD-Htau cases): ten cases (17.9%) corresponded to Braak NFT stage III, four cases (7.1%) had stage IV, three cases (5.4%) had stage V, and two cases (3.6%) had stage VI. Thirty-eight cases (68% of all LBD cases) had various degrees of Aβ deposits in the hippocampus and/or temporal cortex. Argyrophilic grains were found in two LBD cases. All brains had been collected with Local Research Ethical Committee approval. Relevant clinical and pathological features for all 56 LBD cases are shown in Table 1.

Immunohistochemistry

Paraffin sections were cut at 6 μm thickness, to include the amygdala, entorhinal cortex, hippocampus, and occipitotemporal cortex, from all LBD cases and immunostained with antibodies against phosphorylated TDP-43 (pAb

Table 1 Clinical and pathological features in LBD cases with and without TDP-43 pathology

	All	TDP-43-positive	TDP-43-negative	*P* value[a]
N (%)	56 (100.0)	10 (17.9)	46 (82.1)	–
Male [*N* (%)]	42 (75.0)	8 (80.0)	34 (73.9)	1.000
Age at onset [mean (SD)]	62.8 (13.2)	65.2 (11.0)	62.3 (13.8)	0.561
Age at death [mean (SD)]	76.9 (7.2)	77.4 (6.7)	76.8 (7.3)	0.899
Duration [mean (SD)]	13.6 (7.6)	12.9 (7.4)	13.7 (7.7)	0.740
Dementia [*N* (%)]	33 (58.9)	5 (50.0)	28 (60.9)	0.725
Brain weight [g, mean (SD)]	1,303 (113)	1,343 (135)	1,297 (109)	0.540
Argyrophilic grain [*N* (%)]	2 (3.6)	0 (0.0)	2 (4.3)	1.000
Hippocampal sclerosis [*N* (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1.000
Clinical diagnosis				
Parkinson's disease	29 (51.8)	7 (70.0)	22 (47.8)	0.299
Parkinson's disease with dementia[b]	23 (41.1)	2 (20.0)	21(45.7)	0.172
Dementia with Lewy bodies	4 (7.1)	1 (10.0)	3 (6.5)	1.000
Lewy body type pathology [27]				
Brain stem type	10 (17.9)	2 (20.0)	8 (17.4)	1.000
Limbic type	29 (51.8)	2 (20.0)	27 (58.7)	0.038[c]
Diffuse neocortical type	17 (30.4)	6 (60.0)	11 (23.9)	0.052[c]
Braak NFT stage [4]				
Stages 0–II	37 (66.1)	7 (70.0)	30 (65.2)	1.000
Stages III–IV	14 (25.0)	2 (20.0)	13 (28.3)	0.713
Stages V–VI	5 (8.9)	1 (10.0)	3 (6.5)	1.000
DLB likelihood [27][d]				
Low	10 (17.9)	2 (20.0)	8 (17.4)	1.000
Intermediate	15 (26.8)	2 (20.0)	13 (28.3)	0.713
High	31 (55.4)	6 (60.0)	25 (54.3)	1.000

[a] TDP-43-positive LBD cases versus TDP-43-negative LBD cases

[b] The clinical diagnosis of Parkinson's disease with dementia was based on motor impairment preceded by at least 1 year [27]

[c] Although not significant, the frequency of limbic type of LBD pathology was higher in TDP-43-negative cases, while the frequency of diffuse neocortical type LBD was higher in TDP-43-positive cases

[d] The likelihood that Lewy body related pathology is associated with a DLB clinical syndrome

pS409/410, rabbit, polyclonal, 1:1,000 [13]), phosphorylated tau (AT8, mouse, monoclonal, 1:3,000, Innogenetics, Ghent, Belgium), phosphorylated α-synuclein (#1175, rabbit, polyclonal, 1:1,000, [30]), and Aβ (4G8, mouse, monoclonal, 1:2,000, Covance Research Products Inc., Dedham, MA). Deparaffinized sections were incubated with 1% H_2O_2 in methanol for 20 min to eliminate endogenous peroxidase activity. When using anti-α-synuclein and anti-TDP-43 antibodies, sections were pretreated in a microwave oven for 5 min in 10 mM sodium citrate buffer, pH 6.0, at 100°C to enhance immunoreaction. For Aβ immunostaining, sections were incubated in 95% formic acid for 5 min. No pretreatment was performed for AT8 immunostaining. After blocking with 10% normal serum, sections were incubated for 1 h at room temperature with one of the primary antibodies. After three 5-min washes in 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 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, α-synuclein, 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, and occipitotemporal gyrus were semiquantitatively evaluated using the following grading system blinded to any clinical or pathological information:

(a) 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. The topographic distribution of TDP-43 pathological changes was assessed using the following system, which was similar to that reported by Amador-Ortiz et al. [1]—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 in the occipitotemporal gyrus.

(b) The LBD cases were classified, irrespective of the presence or absence of dementia, into brain stem-predominant type, limbic type, and diffuse neocortical type, according to the distribution of α-synuclein pathology as recommended by the Third Consensus Guideline for DLB [27]. In addition, the severity of α-synuclein pathology in the substantia nigra, amygdala, and temporal cortex was semiquantitatively assessed at ×100 magnification using the following method, again fundamentally consistent with protocols of the Third Consensus Guideline for DLB [27]: grade 1, one Lewy body (LB) or Lewy neurites (LNs) per few fields; grade 2, one to three LBs and sparse LNs per one field; grade 3, four to ten LBs and scattered LNs per one field; grade 4, over 11 LBs and LNs per one field.

(c) Tau-positive neuronal inclusions were counted at ×100 magnification: 0, no tau-positive lesions; 1, one neuronal inclusion per few microscopic fields; 2, one to three inclusions in every field; 3, 4–30 inclusions in every field; 4, over 30 inclusions associated with numerous neurites in every field. The distribution of tau pathology in LBD cases was assessed according to Braak NFT stage on AT8 immunostained sections [4].

(d) Aβ deposits were counted at ×100 magnification: 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 CA2, 3, and 4 regions and absence of intracellular and extracellular NFTs, or ischemic changes that might explain neuronal loss in the CA1 and subiculum. HS was assessed on hematoxylin–eosin stained sections 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 two groups. Correlations between (a) the rating of TDP-43 pathology in the amygdala and clinical variables, (b) the rating of TDP-43 pathology and that of α-synuclein, tau, or Aβ pathology in each anatomical region, and (c) the rating of TDP-43 pathology in the amygdala and that of α-synuclein or tau pathology in each region were assessed by Spearman's rank-order correlation test. Multiple logistic regression models were used to assess the influence of predictor variables (age at death, the severities of tau and α-synuclein pathologies) on the occurrence of TDP-43 pathology. The effects were described as odds ratios and 95% confidence interval (CI). Statistical analysis was performed using Excel, Stat View version J-4.5, and SPSS 10.0J. A P value <0.05 was accepted as significant; however, in analyses of comparisons between two groups and correlations between two variables, a P value <0.01 was accepted as significant to interpret the results with caution because multiple tests have been done.

Confocal laser scanning microscopy

Double-labeling immunofluorescence was performed with the combination of (a) phosphorylation-dependent rabbit polyclonal anti-TDP-43 (pAb pS409/410, 1:1,200 [13]) and anti-tau antibodies (AT8, mouse, monoclonal, 1:500, Innogenetics, Ghent, Belgium), and (b) phosphorylation-dependent mouse monoclonal anti-TDP-43 (mAb pS409/410, 1:1,200 [16]) and phosphorylation-dependent anti-α-synuclein antibodies (#1175, rabbit, polyclonal, 1:1,000, [30]). Sections from the amygdala in LBD 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 phosphate buffered saline (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% Triton X-PBS for 30 min. Sections were coverslipped with Vectashield mounting media (Vector Laboratories Inc., Burlingame, CA). 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. To eliminate cross-talk between channels, the images were collected sequentially.

Immunoblotting

Frozen tissues from the amygdala, hippocampus, and frontal and temporal cortex from two LBD cases (one TDP-43-positive and one TDP-43-negative case), one FTLD-TDP case as a positive control, and one pathologically normal control case were prepared for western blotting according to

methods previously described by Neumann et al. [29]. Briefly, fresh frozen brain was homogenized in 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; BioRad, Hercules, CA). Proteins were transferred onto nitrocellulose membrane (Hybond ECL, GE Life Sciences, UK) and blocked overnight 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 [16]) for 1 h at room temperature followed by HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology Inc, CA). 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 in all LBD cases

Of the 56 LBD cases, 10 (17.9%) had TDP-43-positive neuronal intracytoplasmic inclusions (NCIs) (Table 1). The amygdala (all 10 TDP-43 positive cases) was most frequently affected by TDP-43 pathology, followed by the anterior portion of the entorhinal cortex (7/10 cases), hippocampal dentate gyrus (3 cases), subiculum (3 cases), and CA1, fusiform gyrus, and occipitotemporal gyrus (2 cases for each) (Table 2; Fig. 1). The distribution of TDP-43 pathology was the amygdala type in one case, the limbic type in seven cases, and the temporal type in two cases. No neuronal intranuclear inclusions were noted in any LBD case.

α-Synuclein and tau pathologies in LBD cases with and without TDP-43 pathology

Clinical and pathological features in those LBD cases with and without TDP-43 pathology are shown in Table 1. There was no statistically significant difference in the sex ratio, mean age at onset, age at death, disease duration, or frequency of dementia between these two groups. There was no significant correlation between demographic variables and the rating of TDP-43 pathology in the amygdala. None of our LBD cases, including TDP-43-positive cases, had significant neuronal loss in the hippocampal CA1 or subiculum consistent with the definition of HS.

α-Synuclein pathology in the 10 TDP-43-positive cases was more widely distributed than that in TDP-43-negative cases. The diffuse neocortical type of LBD was the most common pathological subtype in the TDP-43-positive cases (six cases), followed by limbic type and brainstem-predominant type (two cases each) (Fig. 2a). In contrast, in the TDP-43-negative LBD cases, the limbic type was most frequent (58.7%), while only 23.9% cases had diffuse neocortical type (Fig. 2a). The frequency of diffuse neocortical type cases in the TDP-43-positive cases tended to be higher than that in the TDP-43-negative cases (Mann–Whitney U test, $P = 0.052$). Consistent with these results, was the observation that the rating of α-synuclein pathology in the temporal cortex in the TDP-43-positive cases was significantly more severe than that in the TDP-43-negative cases (Mann–Whitney U test, $P = 0.003$; Fig. 2b). There was no significant difference in the rating of α-synuclein pathology, in either the substantia nigra or the amygdala, between the TDP-43-positive and TDP-43-negative cases. The Spearman rank correlation coefficient showed a moderate correlation between the ratings for α-synuclein pathology in the temporal cortex and TDP-43 pathology in the amygdala (Spearman rho = 0.398, $P < 0.01$). In any other regions, there was no significant correlation between the ratings for TDP-43 and α-synuclein pathologies, and Spearman rho ranged from −0.087 to 0.122.

The ratings for tau pathology in the hippocampal dentate gyrus in the TDP-43-positive cases tended to be higher than those in the TDP-43-negative cases (Mann–Whitney U test, $P = 0.037$). Likewise, although not significantly, Braak NFT stage in the TDP-43-positive cases also tended to be higher than that in the TDP-43-negative cases (Fig. 3). Although not significant, a moderate correlation was observed between the ratings for tau pathology in the hippocampal dentate gyrus and those for TDP-43

Table 2 Distribution of TDP-43 pathology in LBD cases

No.	TDP-43 pathology											Hippocampal sclerosis	Braak NFT stage	Argyrophilic grains	DLB pathology subtype	DLB likelihood	Clinical diagnosis
	Amygdala	ant.EC	DG	CA3/4	CA2	CA1	SB	post.EC	FG	OTG	Distribution						
LBD-Ltau cases																	
1	+	−	−	−	−	−	−	−	−	−	Amygdala	−	I	−	Brain stem	Low	PD
2	+	+	−	−	−	−	−	−	−	−	Limbic	−	I	−	Limbic	High	PD
3	+++	+++++	−	−	−	−	−	−	−	−	Limbic	−	II	−	Diffuse	High	PD
4	++	+	−	−	−	+	++	−	−	−	Limbic	−	II	−	Diffuse	High	PDD
5	+++++	+	+	−	−	−	−	+	−	−	Limbic	−	II	−	Diffuse	High	PD
6	++++	++	++	−	−	−	−	−	+	+	Temporal	−	I	−	Limbic	High	PDD
7	+++++	+++++	−	−	−	+	++	−	+	++	Temporal	−	II	−	Diffuse	High	DLB
%	100.0	85.7	28.6	0.0	0.0	28.6	28.6	14.3	28.6	28.6							
LBD-Htau cases																	
8	++	−	+	−	−	−	−	−	−	−	Limbic	−	III	−	Brain stem	Low	PD
9	+++	+	−	−	−	−	−	−	−	−	Limbic	−	V	−	Diffuse	Intermediate	PD
10	++++	−	−	−	−	−	+++	−	−	−	Limbic	−	VI	−	Diffuse	Intermediate	PD
%	100	33.3	33.3	0.0	0.0	0.0	33.3	0.0	0.0	0.0							

LBD-Ltau LBD with no or low tau burden of Braak NFT stages 0–II, *LBD-Htau* LBD with high tau burden of Braak NFT stages III–VI, *ant.EC* the anterior portion of the entorhinal cortex, *DG* hippocampal dentate gyrus, *SB* subiculum, *post.EC* posterior portion of the entorhinal cortex, *FG* fusiform gyrus, *OTG* occipitotemporal gyrus, *PD* Parkinson's disease, *PDD* Parkinson's disease with dementia, *DLB* dementia with Lewy bodies

DLB pathology subtype [27]: *brain stem* brain stem-predominant type, *limbic* limbic type, *diffuse* diffuse neocortical type

The stages of TDP-43 pathology: −, no lesion in the anatomical region; +, one inclusion in the anatomical region; ++, two to three inclusions in the anatomical region; +++, four to five inclusion in the anatomical region; ++++, 6–10 inclusions in the anatomical region; +++++, 11 or over inclusions in the anatomical region. The distribution of TDP-43 pathology, amygdala, amygdala type, limbic, limbic type, temporal, temporal type

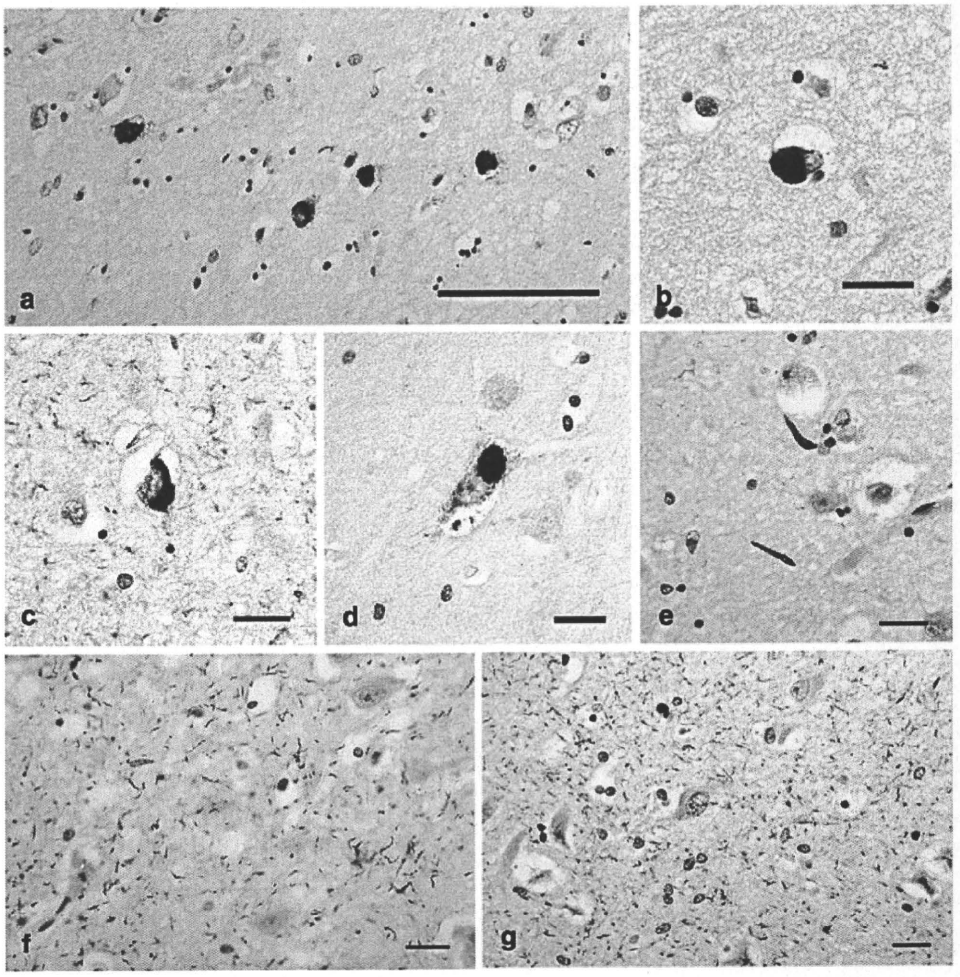

Fig. 1 Phosphorylated TDP-43 pathology in LBD-Ltau cases. TDP-43-positive NCIs in the entorhinal cortex (**a**), amygdala (**b**, **c**), and CA1 (**d**). TDP-43-positive dystrophic neurites are also scattered in the amygdala (**e**). In some cases, abundant fine and short threads-like structures are also seen in the CA1 to subiculum (**f**, **g**). pAb pS409/410 immunohistochemistry. **a**, **c**, **d**, **g** Diffuse neocortical type LBD-Ltau cases (Braak NFT stage II), **b**, **e**, **f** limbic type LBD-Ltau cases (Braak NFT stage I). *Scale bars* **a** 100 μm, **b**–**g** 20 μm

pathology in the amygdala (Spearman rho = 0.301, $P < 0.05$). In any other regions, there was no significant correlation between the ratings for TDP-43 and tau pathologies, and Spearman rho ranged from −0.092 to 0.178.

Ratings for Aβ pathology were not significantly different between the TDP-43-positive and TDP-43-negative LBD cases. Spearman correlation coefficients did not indicate any significant correlation between the severities of Aβ and TDP-43 pathologies in any region, and Spearman rho ranged from 0.103 to 0.227.

Relationship between α-synuclein and TDP-43 pathologies in LBD-Ltau and LBD-Htau cases

The relationship between α-synuclein and TDP-43 pathologies in LBD-Ltau (Braak NFT stages 0–II) and LBD-Htau cases (Braak NFT stages III–VI) was examined separately. The sex ratio, mean age at onset, age at death, disease duration, frequency of dementia were not significantly different between the TDP-43-positive and TDP-43-negative cases in the LBD-Ltau cases, as well as in the LBD-Htau cases (Table 3).

In the LBD-Ltau cases with TDP-43 pathology, the most frequent LBD subtype was the diffuse neocortical type (57.1%), followed by limbic (28.6%) and brain stem-predominant (14.3%) types (Fig. 4). In contrast, in LBD-Ltau cases without TDP-43 pathology, the limbic type was most frequent (56.7%), while the diffuse neocortical type was seen in only 23.3% cases and brain stem-predominant type in 20.0%. In the LBD-Ltau group, the rating of α-synuclein pathology in the temporal cortex in the TDP-43-positive cases tended to be higher than that in the TDP-43-negative cases (Mann–Whitney U test, $P = 0.042$). Although case numbers were small, a similar trend was seen in the LBD-Htau cases: 2 of 3 TDP-43-positive LBD cases were diffuse neocortical type, while only 4 of 16 TDP-43-negative cases were this subtype (67 vs. 25%).

Both in LBD-Ltau or in LBD-Htau cases, the ratings for tau and Aβ pathologies were not significantly different between TDP-43-positive and TDP-43-negative cases, in any region.

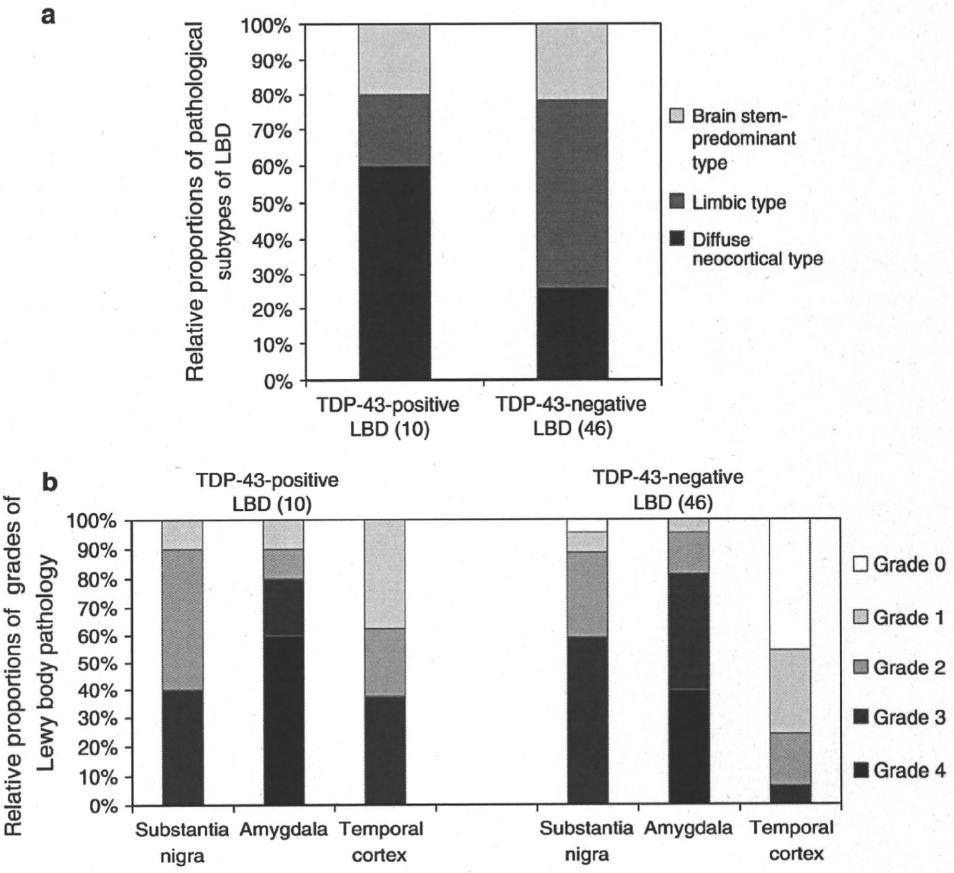

Fig. 2 α-Synuclein pathology in all LBD cases with and without TDP-43 pathology. **a** The distribution of pathological subtypes of LBD in TDP-43-positive and TDP-43-negative groups. The frequency of diffuse neocortical type in TDP-43-positive LBD cases tends to be higher than that in TDP-43-negative LBD cases ($P = 0.052$). The number of cases in each group is shown in *brackets*. **b** The rating of α-synuclein pathology in TDP-43-positive and TDP-43-negative LBD cases. α-Synuclein pathology in the temporal cortex in TDP-43-positive cases was significantly more severe than that in TDP-43-negative cases ($P = 0.003$)

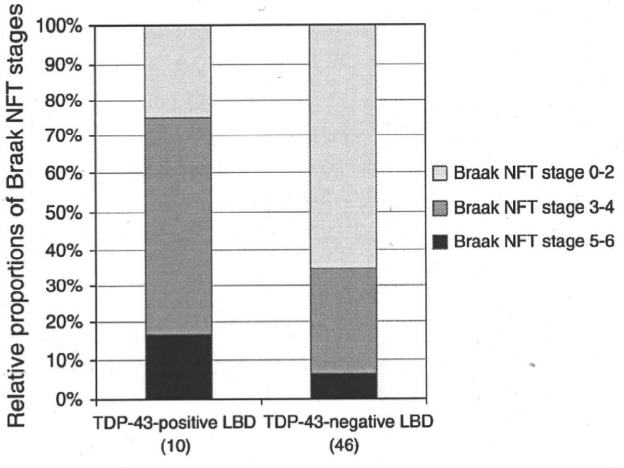

Fig. 3 Braak NFT stage in LBD cases with and without TDP-43 pathology. Tau pathology in TDP-43-positive LBD cases tended to be more severe than that in TDP-43-negative LBD cases. The number of cases in each group is shown in *brackets*

Frequency of TDP-43 pathology by clinical and pathological subtypes of LBD

There was no clear relationship between the occurrence of TDP-43 pathology and clinical phenotypes of LBD in our series: TDP-43 pathology was found in 7 of 29 PD cases (24.1%), 2 of 23 PDD cases (8.7%), and 1 of 4 DLB cases (25%). The overall frequency of TDP-43 pathology was 11.1% in LBD cases with dementia and 24.1% in LBD cases without it. However, in LBD-Ltau cases (Braak NFT stages 0–II), the duration from disease onset to the development of dementia in TDP-43-positive cases tended to be shorter than that in TDP-43-negative cases (2.0 ± 1.7 vs. 11.9 ± 7.0 years, $P = 0.046$, Mann–Whitney U test).

In contrast to clinical phenotypes, there was a trend for TDP-43 pathology to be more frequently present in cases with severe α-synuclein pathology. In LBD-Ltau cases, TDP-43 pathology was noted in 4 of 11 diffuse neocortical type LBD-Ltau cases (36.4%), whereas it was only present in 2 of 19 cases of limbic type LBD (10.5%) and in 1 of 17 brain stem-predominant type LBD (14.3%) cases. In LBD-Htau cases, 2 of the 5 diffuse neocortical type cases with severe tau pathology (Braak NFT stages V–VI) also had TDP-43 pathology. The overall frequency of TDP-43 pathology in diffuse neocortical type LBD cases was 35.3% (6 of 17 cases).

Effects of α-synuclein and tau pathologies on development of TDP-43 pathology

A multiple logistic regression model was used to evaluate whether pathological subtypes of LBD, Braak NFT stage,

Table 3 Clinical and pathological features in LBD-Ltau and LBD-Htau cases

	LBD-Ltau (Braak NFT stages 0–II)				LBD-Htau (Braak NFT stages III–VI)			
	All	TDP-43-positive	TDP-43-negative	*P* value[a]	All	TDP-43-positive	TDP-43-negative	*P* value[b]
N (%)	37 (66.1)[c]	7 (18.9)	30 (81.1)	–	19 (33.9)[c]	3 (15.8)	16 (84.2)	–
Male [*N* (%)]	29 (78.4)	6 (85.7)	23 (76.7)	0.677	13 (68.4)	2 (66.7)	11 (68.8)	1.000
Age at onset [mean (SD)]	60.9 (15.2)	65.1 (13.1)	59.9 (15.7)	0.455	66.3 (7.4)	65.3 (4.7)	66.5 (7.9)	0.958
Age at death [mean (SD)]	76.1 (7.9)	75.7 (7.2)	76.2 (8.2)	0.732	78.4 (5.2)	81.3 (3.2)	77.9 (5.4)	0.360
Duration [mean (SD)]	14.3 (7.7)	11.5 (8.4)	14.9 (7.6)	0.179	12.3 (7.5)	15.7 (4.9)	11.6 (7.8)	0.360
Dementia [*N* (%)]	23 (62.2)	5 (71.4)	18 (60.0)	0.687	10 (52.5)	0 (0.0)	10 (62.5)	0.059
Brain weight [g, mean (SD)]	1,290 (103)	1,299 (132)	1,289 (102)	1.000	1,327 (129)	1,387 (151)	1,312 (125)	0.368
Argyrophilic grain [*N* (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1.000	2 (10.5)	0 (0.0)	2 (12.5)	1.000

LBD-Ltau LBD with no or low tau burden of Braak NFT stages 0–II [4], *LBD-Htau* LBD with high tau burden of Braak NFT stages III–VI

[a] TDP-43-positive LBD-Ltau cases versus TDP-43-negative LBD-Ltau cases

[b] TDP-43-positive LBD-Htau cases versus TDP-43-negative LBD-Htau cases

[c] The proportion to all LBD cases

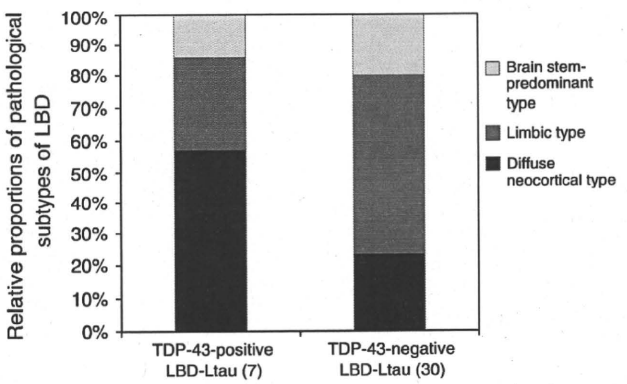

Fig. 4 Distribution of LBD subtypes in LBD-Ltau cases (Braak NFT stages 0–II). Diffuse neocortical type in TDP-43-positive cases was more frequent than that in TDP-43-negative cases. The number of cases in each group is shown in *brackets*

and age at death could be used as possible predictors for the development of TDP-43 pathology. After combining categories in which the number of cases was small (Braak NFT stages 0–II or Braak NFT stages III–VI) and pathological subtype of LBD (diffuse neocortical type or others) data were submitted as binary variables into the model. The presence of diffuse neocortical type of LBD was the only significant independent predictor of the development of TDP-43 pathology (odds ratio 7.6, 95% CI 1.46–39.1, $P = 0.016$).

Multiple logistic regression analysis was also used to examine whether the ratings of the severities of α-synuclein and tau pathologies in the amygdala and age at death were predictors of the development of TDP-43 pathology. Again, after combining categories in which the number of cases was small, the ratings of α-synuclein (grades 1–3 or grade 4) and tau pathologies (Braak NFT stages 0–II or Braak NFT stages III–IV) were submitted as binary variables into the model. However, neither of these variables predicted the development of TDP-43 pathology, although the odds ratio of severe α-synuclein pathology in the amygdala was high (odds ratio 3.5, 95% CI 0.71–17.1, $P = 0.122$).

Double-labeling confocal microscopy of phosphorylated TDP-43, tau, and α-synuclein

In the amygdala, TDP-43 accumulation was often colocalized with α-synuclein accumulation in NCIs and dystrophic neurites (Fig. 5a–i). TDP-43 was also often colocalized with tau labeling (Fig. 5j–o), but there were also some TDP-43-positive α-synuclein-negative lesions (Fig. 5d–i) and TDP-43-positive tau-negative lesions (Fig. 5j–l). In the hippocampal granular cells, TDP-43 and tau were only rarely colocalized (data not shown).

Biochemical analysis of TDP-43 in LBD cases

Immunoblot analysis of the sarkosyl-insoluble urea-soluble fraction using mAb pS409/410 in LBD cases with TDP-43 pathology demonstrated distinct bands at approximately 45 and 25 kDa, as well as high molecular weight (HMW) smears (Fig. 6, lanes 3 and 4) similar to those seen in the FTLD-TDP case (lane 6). These pathological bands and the HMW smear were not seen in the LBD cases without TDP-43 pathology (lanes 1 and 2) or in the normal control cases (lane 5).

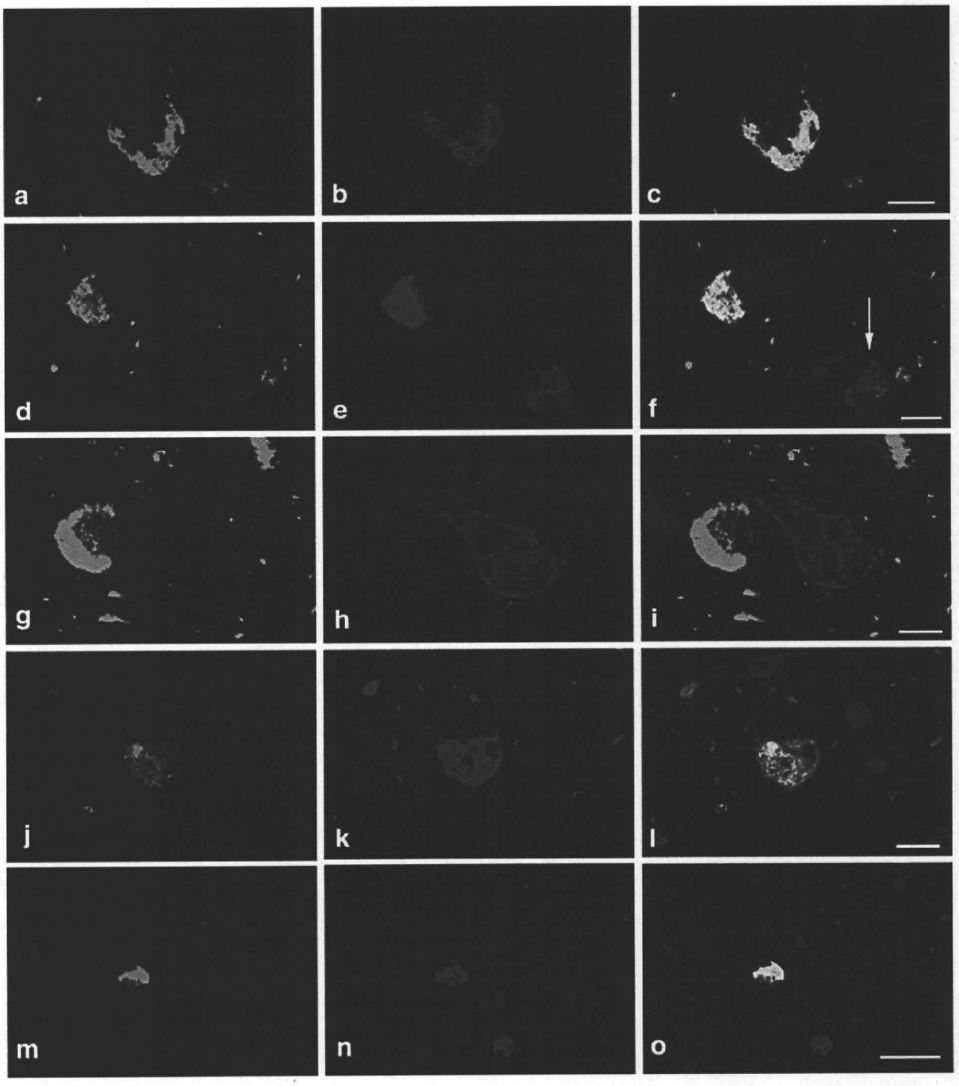

Fig. 5 Confocal double immunofluorescence of the combination of α-synuclein (**a, d, g**) and TDP-43 (**b, e, h**), and the combination of TDP-43 (**j, m**) and tau (**k, n**) in the amygdala in LBD cases. Blue fluorescence in merged images (**c, f, i, l, o**) are nuclei. **a–c** TDP-43 (*red*) is colocalized with α-synuclein (*green*) in an inclusion. **d–f** In a left inclusion, α-synuclein (*green*) is colocalized with TDP-43 (*red*). However, the right TDP-43-positive inclusion (*arrow*) shows only faint α-synuclein immunolabeling (*green*). **g–i** A left horseshoe-shaped inclusion is stained only with an α-synuclein antibody, while a right neuron shows diffuse cytoplasmic TDP-43 labeling (*red*) without α-synuclein immunoreactivity. **j–o** TDP-43 (*green*) is often colocalized with tau labeling (*red*) in cytoplasmic inclusions. There are a few TDP-43-positive tau-negative lesions (**l**, *green*). **a–i** #1175 and mAb pS409/410 double immunofluorescence in a LBD-Ltau case (Braak NFT stage II), **j–o** pAb pS409/410 and AT8 double immunofluorescence in a LBD-Htau case (Braak NFT stage VI). *Scale bars* **a–c** 7.5 μm, **d–f** 7.5 μm, **g–i** 7.5 μm, **j–l** 10 μm, **m–o** 25 μm

Discussion

This is the first study demonstrating a high frequency of TDP-43 pathology in LBD-Ltau cases (Braak NFT stages 0–II). The overall frequency of TDP-43 pathology was 19%, and the frequency of TDP-43 pathology in diffuse neocortical type LBD cases was as much as 36%, which was higher than those in other LBD subtypes (11–14%). In all LBD cases in this study, even in LBD-Ltau cases, the proportion of diffuse neocortical type LBD cases among the TDP-43-positive cases was approximately 1.5 times higher than that in the TDP-43-negative cases, and multivariate analysis demonstrated that severe α-synuclein pathology was a predictor of TDP-43 accumulation in LBD independent of age at death and tau pathology. Double immunofluorescence demonstrated that TDP-43 was often colocalized with α-synuclein or tau in the amygdala. These findings suggest that α-synuclein may play some role in the process associated with the development of TDP-43 pathology in LBD cases.

Previous data regarding TDP-43 accumulation in LBD cases are limited. In an early study by Nakashima-Yasuda et al. [28], the overall frequency of TDP-43 pathology in LBD cases was reported to be 18.9%. This frequency appears to be similar to that in all LBD cases examined in our study (17.9%), even though these authors employed a 'conventional' phosphorylation-independent TDP-43 antibody. However, these frequencies cannot be directly compared, because the pathological backgrounds between two LBD series may be different. For example, the degree of tau pathology in the Nakashima-Yasuda series tended to be more severe than that in the present series: the proportion of LBD cases having severe tau pathology (Braak NFT stages V–VI) being 21% (38 of 180 LBD cases), far higher than that in our LBD series (8.9%). Conversely, the proportion of cases of Braak NFT stages 0–II in the