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Epitope mapping of antibodies against TDP-43 and detection of protease-resistant fragments of pathological TDP-43 in amyotrophic lateral sclerosis and frontotemporal lobar degeneration

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ABSTRACT

TAR DNA-binding protein of 43 kDa (TDP-43) is the major component of the intracellular inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Here, we show that both monoclonal (60019-2-Ig) and polyclonal (10782-2-AP) anti-TDP-43 antibodies recognize amino acids 203–209 of human TDP-43. The monoclonal antibody labeled human TDP-43 by recognizing Glu204, Asp205 and Arg208, but failed to react with mouse TDP-43. The antibodies stained the abnormally phosphorylated C-terminal fragments of 24–26 kDa in addition to normal TDP-43 in ALS and FTLD brains. Immunoblot analysis after protease treatment demonstrated that the epitope of the antibodies (residues 203–209) constitutes part of the protease-resistant domain of TDP-43 aggregates which determine a common characteristic of the pathological TDP-43 in both ALS and FTLD-TDP. The antibodies and methods used in this study will be useful for the characterization of abnormal TDP-43 in human materials, as well as in vitro and animal models for TDP-43 proteinopathies.

1. Introduction

TDP-43 is a nuclear ribonucleoprotein implicated in exon splicing, gene transcription, regulation of mRNA stability, mRNA biosynthesis, and formation of nuclear bodies [1–5]. It has been identified as the major component of the ubiquitin-positive tau-negative intracytoplasmic inclusions in frontotemporal lobar degeneration (FTLD), amyotrophic lateral sclerosis (ALS) [6,7] and other neurodegenerative disorders [8–12]. Identification of mutations in familial and sporadic ALS and FTLD cases demonstrated a direct link between the genetic lesion and development of TDP-43 pathology [13–16]. Immunohistochemical studies using anti-TDP-43 antibodies revealed that TDP-43 translocates from its normal nuclear localization into the cytoplasm in these disorders. Furthermore, biochemical analysis detected abnormally phosphorylated TDP-43 of 45 kDa, high-molecular-weight smearing and C-terminal fragments of approximately 25 kDa, as well as normal TDP-43 of 43 kDa in the detergent-insoluble, urea-soluble fraction from affected brains. The antibodies generated by immunizing C-terminal phosphopeptides of TDP-43, such as pS409/410 and pS403/404, strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulations in neuropathological examination [17]. Immunoblotting of the Sarkosyl-insoluble fractions from FTLD and ALS cases using these phosphospecific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 of 45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD and ALS, and the band patterns of the C-terminal fragments of phosphorylated TDP-43 correspond to the neuropathological subtypes.

Anti-TDP-43 monoclonal antibody (mAb) (60019-2-Ig; Proteintech Group Inc., Chicago, IL) and polyclonal antibody (pAb) (10782-2-AP; Proteintech Group Inc., Chicago, IL) are widely used for the investigation of TDP-43 pathology [6,7,9,18–21]. According to the manufacturer's specifications, anti-TDP-43 mAb and pAb were generated against the N-terminal 260 amino acids (aa) of the protein, but the precise epitope has not yet been identified. Another mouse monoclonal antibody against TDP-43 (2E2-D3; Abnova Corporation, Taipei, Taiwan) is also commercially available; it recognizes residues 205–222 of human TDP-43, but does not recognize mouse or rat TDP-43 [22].

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In this study, we mapped the epitope for anti-TDP-43 mAb and pAb (Proteintech Group Inc.). We also showed that anti-TDP-43 mAb recognizes human TDP-43, but not mouse TDP-43. Using these antibodies, we investigated the abnormal forms of TDP-43 from ALS and FTLD brains, and found that the antibodies recognized the amino-terminus of the TDP-43 C-terminal fragments of 24–26 kDa. Immunoblot analysis of Sarkosyl-insoluble fractions after treatment of proteases also demonstrated that the epitope is apparently resistant to trypsin and chymotrypsin in the abnormal TDP-43, suggesting that the epitope region is important for the formation of the pathological structure of TDP-43 in ALS and FTLD.

2. Materials and methods

2.1. Construction of plasmids

GFP-tagged TDP-43 C-terminal or N-terminal fragments were constructed as described [23] by amplifying a cDNA encoding full-length TDP-43 by means of PCR and inserting the fragment into the pEGFP-C1 vector (Clontech). To investigate the specificity of TDP-43 mAb for human TDP-43, site-directed mutagenesis of GFP-tagged full-length TDP-43 was carried out to substitute Glu204 to Ala (E204A), Asp205 to Glu (D205E), Arg208 to Gln (R208Q), Glu209 to Gln (E209Q), Ser212 to Cys (S212C), Asp216 to Glu (D216E), and Met218 to Val (M218V), using a site-directed mutagenesis kit (Strategene)(Fig. 4). All constructs were verified by DNA sequencing.

2.2. Antibodies

TDP-43 polyclonal antibody, 10782-2-AP, and TDP-43 monoclonal antibody, 60019-2-Ig, were purchased from Proteintech Group Inc. Anti-GFP monoclonal antibody was purchased from MBL (Nagoya, Japan). A polyclonal antibody specific for phosphorylated TDP-43 (pS409/410) was prepared as described [17].

2.3. Cell culture and expression of plasmids

Human neuroblastoma cell line SH-SY5Y and mouse neuroblastoma cell line Neuro 2a were maintained in appropriate medium as described previously [24,25]. Cells were then transfected with expression plasmids using FuGENE6 (Roche) according to the manufacturer's instructions.

2.4. Immunoblotting

Expressed proteins in cell lysates were separated by 10% SDS–PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 3% gelatin, membranes were incubated overnight with primary antibodies (1:1000) at room temperature. After incubation with an appropriate biotinylated secondary antibody, labeling was detected using the ABC system (Vector Lab., Burlingame, CA) coupled with a diaminobenzidine (DAB) reaction intensified with nickel chloride.

2.5. Analysis of abnormal TDP-43 in ALS and FTLD-TDP brain

Brains from two cases with Alzheimer's disease (AD), two with ALS, two with FTLD-TDP (type A), two with FTLD-TDP (type B) and two with FTLD-TDP (type C) were employed in this study. The two AD cases had no TDP-43 pathology. The age, sex, brain weight, and diagnosis are given in Table 1. Sarkosyl-insoluble, urea-soluble fractions were extracted from these brains as previously described [6,9]. The samples were loaded onto 15% polyacrylamide gel and transferred onto a membrane. The membrane was cut in the center of the loaded lane, and the same samples were reacted separately with anti-TDP-43 Abs and pS409/410 as described above.

Table 1
Description of subjects.

Case No.	Diagnosis	Age (years)	Sex	BW (g)
1	AD	65	F	1165
2	AD	70	F	1126
3	ALS	62	M	1230
4	ALS	42	F	1140
5	FTLD-TDP (type A)	71	F	863
6	FTLD-TDP (type A)	66	F	1100
7	FTLD-TDP (type B)	45	M	1260
8	FTLD-TDP (type B)	67	M	1280
9	FTLD-TDP (type C)	67	M	na
10	FTLD-TDP (type C)	59	M	na

BW, brain weight; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; FTLD-TDP, frontotemporal lobar degeneration with TDP-43 pathology; na, not available.

2.6. Protease treatment of TDP-43

Sarkosyl-insoluble fractions extracted from neocortical regions of the brains were treated with trypsin (at a final concentration of 100 μg/ml, Promega, Madison, USA) or chymotrypsin (at a concentration of 10 μg/ml, Sigma–Aldrich, St. Louis, USA) at 37 °C for 30 min. The reaction was stopped by boiling for 5 min. After centrifuging at 15,000 rpm for 1 min, the samples were analyzed by immunoblotting with anti-TDP-43 pAb and mAb as described above.

3. Results

3.1. Epitope mapping of anti-TDP-43 antibody

Our previous study showed that both TDP-43 mAb and pAb reacted with GFP-tagged TDP-43 C-terminal fragment (GFP-TDP 162–414), but failed to detect GFP-TDP 218–414 [23]. To map the epitope of these antibodies, we expressed a series of GFP-tagged human TDP-43 C-terminal fragments (Fig. 1A) in SH-SY5Y cells and immunoblotted them with the antibodies. Both anti-TDP-43 pAb and mAb detected endogenous human TDP-43 of 43 kDa and exogenous GFP-tagged full-length, 171–414, 181–414, 191–414 and 201–414 TDP-43. However, both antibodies failed to detect 211–414 (Fig. 1A). These results suggest that the epitopes of these antibodies are located within residues 201–210.

To narrow down the epitope structure further, another series of GFP-tagged C-terminal fragments of TDP-43 was expressed in SH-SY5Y cells (Fig. 1B) and tested. Both antibodies reacted with GFP-TDP 203–414, but failed to recognize GFP-TDP 204–414, 205–414 and 207–414 (Fig. 1B), demonstrating that Thr203 forms the N-terminal border of the epitope for both antibodies.

To determine the C-terminus of the epitope, a series of GFP-tagged N-terminal fragments of TDP-43 was expressed and immunoblotted with these antibodies (Fig 1C). Anti-TDP-43 pAb reacted with all of the N-terminal fragments tested, although it stained the 1-212 fragment most strongly. This suggests that one of the pAb epitopes is located at the N-terminal region of TDP-43, in addition to the central epitope. Anti-TDP-43 mAb strongly stained GFP-TDP 1-212, moderately stained GFP-TDP 1-210, and barely stained GFP-TDP 1-209, while it failed to react with GFP 1-208 and 1-207 (Fig. 1C), indicating that Glu209 forms the C-terminus of the epitope for anti-TDP-43 mAb. Thus, anti-TDP-43 mAb recognizes residues 203–209 of human TDP-43.

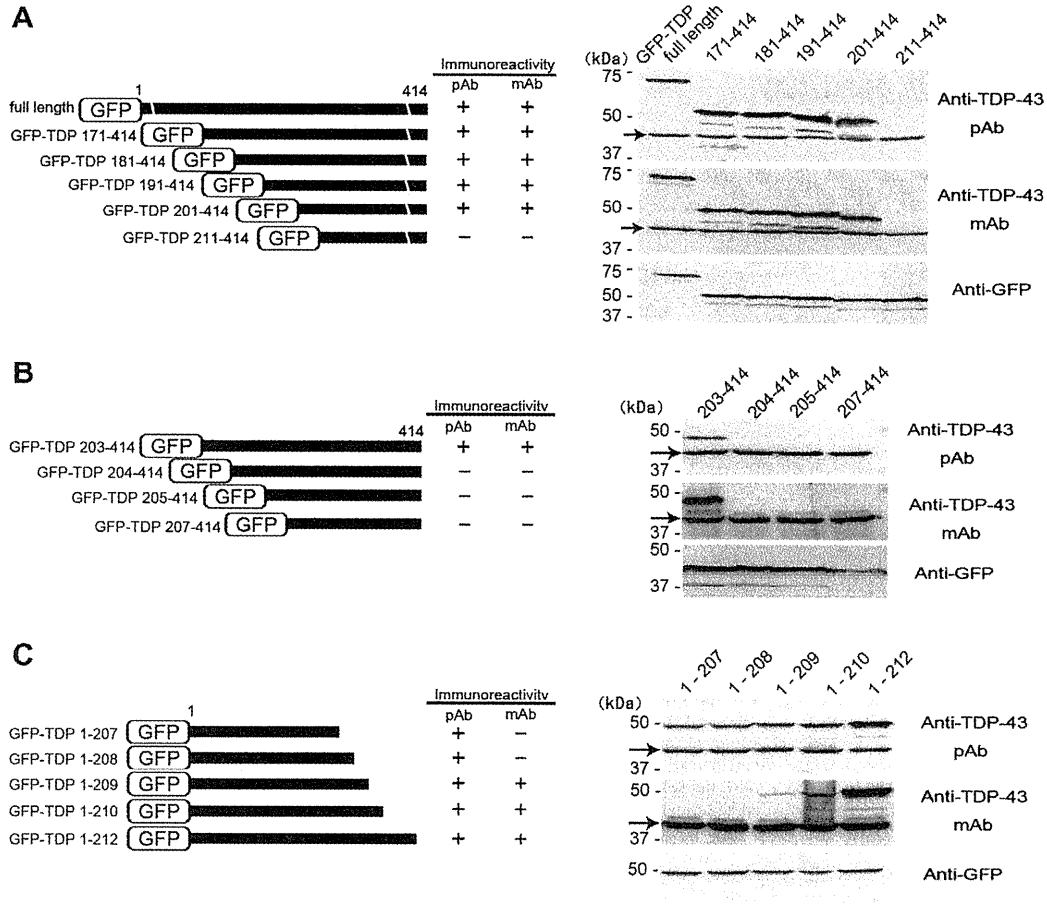

Fig. 1. Epitope mapping of anti-TDP-43 polyclonal and monoclonal antibodies. (A) Schematic diagram of GFP-tagged full-length TDP-43 (GFP-TDP) and the C-terminal fragments. Immunoblot analyses of GFP-TDP and the C-terminal fragments in SH-SY5Y cells. Both mAb and pAb reacted with GFP-TDP and the C-terminal fragments, except for 211–414. The anti-GFP antibody recognizes all the proteins expressed. (B) Further epitope mapping of anti-TDP-43 antibodies. Immunoblot analyses of the GFP tagged C-terminal fragments of TDP-43. Both mAb and pAb reacted with 203–414, but failed to recognize 204–414, 205–414, and 207–414. The anti-GFP antibody recognizes all of the fragments. (C) Epitope mapping of the C-terminus recognized by anti-TDP-43 polyclonal and monoclonal antibodies. Immunoblot analyses of GFP-TDP and N-terminal fragments in SH-SY5Y cells. Anti-TDP-43 pAb reacted with all of the N-terminal fragments, although it stained 1-212 fragment most strongly. In contrast, anti-TDP-43 mAb strongly stained GFP-TDP 1-212, moderately stained GFP-TDP 1-210, and barely stained GFP-TDP 1-209, while it failed to react with GFP 1-208 and 1-207. The anti-GFP antibody recognized all of the fragments equally. The arrows indicate endogenous TDP-43 in SH-SY5Y cells.

3.2. Amino acid sequence differences between human and mouse TDP-43

The anti-TDP-43 mAb reacted with endogenous TDP-43 of human neuroblastoma SH-SY5Y cells, but not with TDP-43 of mouse neuroblastoma Neuro2a cells (Fig. 1B, 1C, 2B). Similarly, the mAb recognized TDP-43 in human brain extract, but failed to detect TDP-43 in mouse brain extract, suggesting that the mAb does not recognize mouse TDP-43 (data not shown). The absence of reactivity with mouse TDP-43 is explained by the sequence differences around the epitope between human and mouse TDP-43 (Fig. 2A). Each different amino acid of human TDP-43 was substituted to that of mouse TDP-43. The mutated proteins were expressed in Neuro2a cells and immunoreactivity with anti-TDP-43 mAb was examined. Substitution of D216 to E and M218 to V did not affect the immunoreactivity (Fig. 2B), whereas substitutions of E204 to A, D205 to E, and R208 to Q abolished the immunoreactivity of anti-TDP-43 mAb, indicating that these residues are necessary for recognition by the mAb. Anti-TDP-43 pAb reacted with these mutants, although a marked decrease in immunoreactivity was observed in the cases of E204A, D205A, R208Q, and S212C.

3.3. Biochemical analysis of abnormal TDP-43 in ALS and FTLD brains with anti-TDP-43 mAb

On immunoblots of Sarkosyl-insoluble fractions extracted from the brain of patients with ALS and FTLD-TDP (type A), the anti-TDP-43 mAb detected phosphorylated full-length TDP-43 at 45 kDa, two bands around 25 kDa and high-molecular-weight smears, in addition to the normal TDP-43 band at 43 kDa, which can also be detected in control cases. Immunoblot analysis of the split membrane with a phosphorylation-dependent anti-TDP-43 antibody pS409/410 revealed that the two bands around 25 kDa stained with the mAb corresponded to the C-terminal fragments of 24 and 26 kDa recognized by pS409/410 (Fig. 3)[17]. These results demonstrated that these 24 and 26 kDa C-terminal fragments contain the epitope of the mAb, residues 203–209, and that the cleavage sites of these C-terminal fragments are located at the N-terminal side of Thr203.

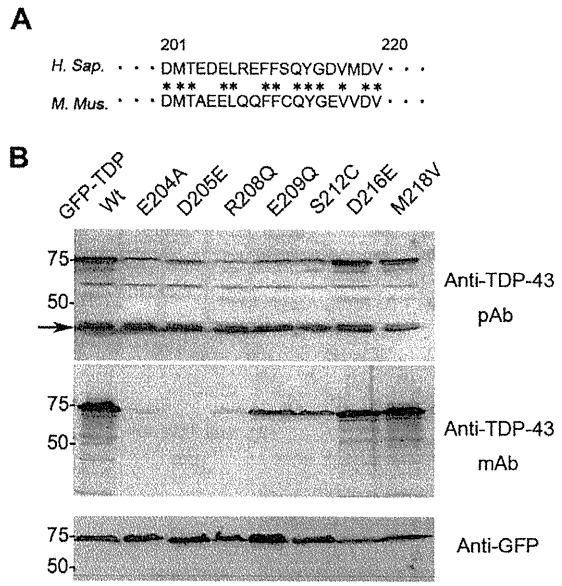

Fig. 2. Alignment of human and mouse TDP-43 (A) and immunoblot analyses of mutated TDP-43 with anti-TDP-43 antibodies. (A) The amino acid sequences of human (upper) and mouse (lower) TDP-43 around the epitope of anti-TDP-43 mAb. The asterisks show identical amino acids. (B) Immunoblot analyses of GFP-TDP wild type (Wt) and GFP-TDP mutants expressed in Neuro2a cells. Substitution of D216 to E and M218 to V did not affect the immunoreactivity, whereas substitutions of E204 to A, D205 to E, and R208 to Q, abolished the immunoreactivity of anti-TDP-43 mAb. Anti-TDP-43 pAb reacted with all these mutants, although markedly decreased immunoreactivities were observed in E204A, D205A, R208Q, and S212C. The arrows indicated endogenous TDP-43 in Neuro2A cells. Note that endogenous mouse TDP-43 in Neuro 2a cells was not recognized by anti-TDP-43 mAb.

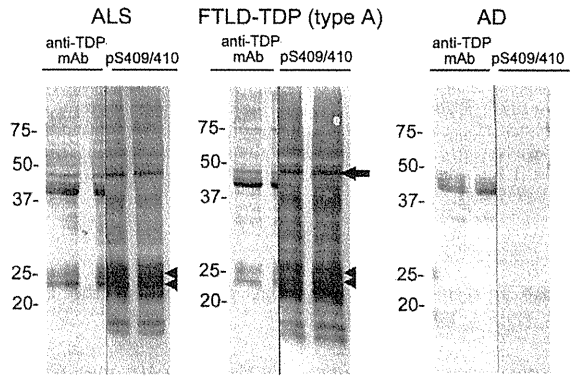

Fig. 3. Immunoblot analyses of Sarkosyl-insoluble fractions from ALS, FTLD-TDP (type A), and AD brains with anti-TDP-43 monoclonal antibody and phosphorylation-dependent anti-TDP-43 antibody, pS409/410. With pS409/410, fragments of approximately 45 kDa and 18–26 kDa, as well as smearing, were detected. The banding pattern of 18–26 kDa fragments showed three major bands at 23, 24, and 26 kDa, and 2 minor bands at 18 and 19 kDa, with the 24 kDa band being the most intense. In addition to the normal full-length TDP-43 at 43 kDa, anti-TDP-43 mAb labeled phosphorylated full-length TDP-43 at 45 kDa, high-molecular-weight smears and two bands at 26 kDa and 24 kDa (arrowheads), which were not seen in the AD case. The two bands corresponded to the major 26 and 24 kDa bands were detected with pS409/410.

3.4. The epitope of these TDP-43 antibodies constitute part of protease-resistant core domain of TDP-43 in ALS and FTLD brains

In order to characterize the epitope further, we treated the Sarkosyl-insoluble fractions extracted from brains of patients with proteases and analyzed them with these antibodies. Without protease treatment, both antibodies strongly stained normal full-length TDP-43 of 43 kDa in all cases examined including AD cases which were without TDP-43 pathology. In ALS and FTLD-TDP cases, phosphorylated full-length TDP-43 of 45 kDa (Fig 4A, arrows) and the ~25 kDa fragments (Fig 4A, arrow heads) were detected with these antibodies. After trypsin treatment, the full-length band of TDP-43 was disappeared and the protease-resistant fragments around 25 kDa (Fig 4B, white arrows) and smearing substances appeared in the ALS and FTLD-TDP cases. Similarly, after chymotrypsin treatment, protease-resistant triplet bands of 16, 20 and 25 kDa (Fig 4C, white arrow heads) and smearing substances were clearly detected in ALS and FTLD-TDP-cases with the mAb, while no such bands were seen in AD cases. On blot with the pAb, multiple bands were detected in addition to the triplet, and some of these bands were also detected in AD cases, suggesting that the pAb stained some normal fragments in addition to the abnormal TDP-43 bands. In the cases examined, apparent difference was not detected in these trypsin-resistant and chymotrypsin-resistant bands detected among the clinicopathological phenotypes of the diseases. By proteinase K treatment, immunoreactivities with these antibodies were completely abolished (data not shown), suggesting that the epitope is not entirely resistant to any proteases. However, it is obvious that the epitope of the TDP-43 deposited in the patients is fairly protease-resistant compared to the normal protein. These results indicate that the epitope of the mAb (residues 203–209 of TDP-43) constitute part of the protease-resistant domain of TDP-43 which determine a common characteristic of the abnormal TDP-43 in both ALS and FTLD-TDP.

4. Discussion

This is the first analysis of the epitopes of Proteintech's anti-TDP-43 polyclonal and monoclonal antibodies, which have often been used to research TDP-43 proteinopathies since 2006 [6,7]. We demonstrated that anti-TDP-43 mAb specifically recognizes residues 203–209 of human TDP-43, which form a part of the second RNA-recognition motif (RRM2, residues 193–257) of normal TDP-43 [26], but constitute part of the protease-resistant core domain of TDP-43 aggregates that determine the common characteristic of abnormal TDP-43 in ALS and FTLD-TDP-43.

RRM2 is a functional domain with distinct RNA/DNA binding characteristics. The anti-TDP-43 mAb recognized human TDP-43, but not mouse TDP-43. Site-directed mutagenesis and subsequent immunoblot analysis revealed that Glu204, Asp205 and Arg208 residues in human TDP-43 are important for the specific recognition by the mAb (Fig. 2). In fact, human TDP-43 shares 98.5% homology with mouse TDP-43 at the amino acid level, but the RRM2 domain has only 66% homology.

We also showed that one of the major epitopes of the pAb is located in almost the same region at that of the mAb (Fig. 1), although the pAb also recognizes the N-terminal region of TDP-43. Recently, TDP-43 transgenic mice overexpressing human TDP-43 have been produced as animal models of TDP-43 proteinopathy [27]. However, abnormal TDP-43 pathologies in these mice are very rare, so new transgenic or other animal models that develop abundant TDP-43 pathology are still required. Since the TDP-43 mAb recognizes human TDP-43, but not mouse TDP-43, it will be a useful reagent for the characterization of mouse lines transgenic for human TDP-43, together with phosphorylation-dependent antibodies.

Biochemical analyses of TDP-43 proteinopathies have demonstrated that abnormally phosphorylated full-length and C-terminal fragments of TDP-43 are the major species in the inclusions. The band patterns of the C-terminal fragments at 18–26 kDa are closely correlated with the clinicopathological subtypes of TDP-43 proteinopathies [17]. In addition, most of the pathogenic mutations

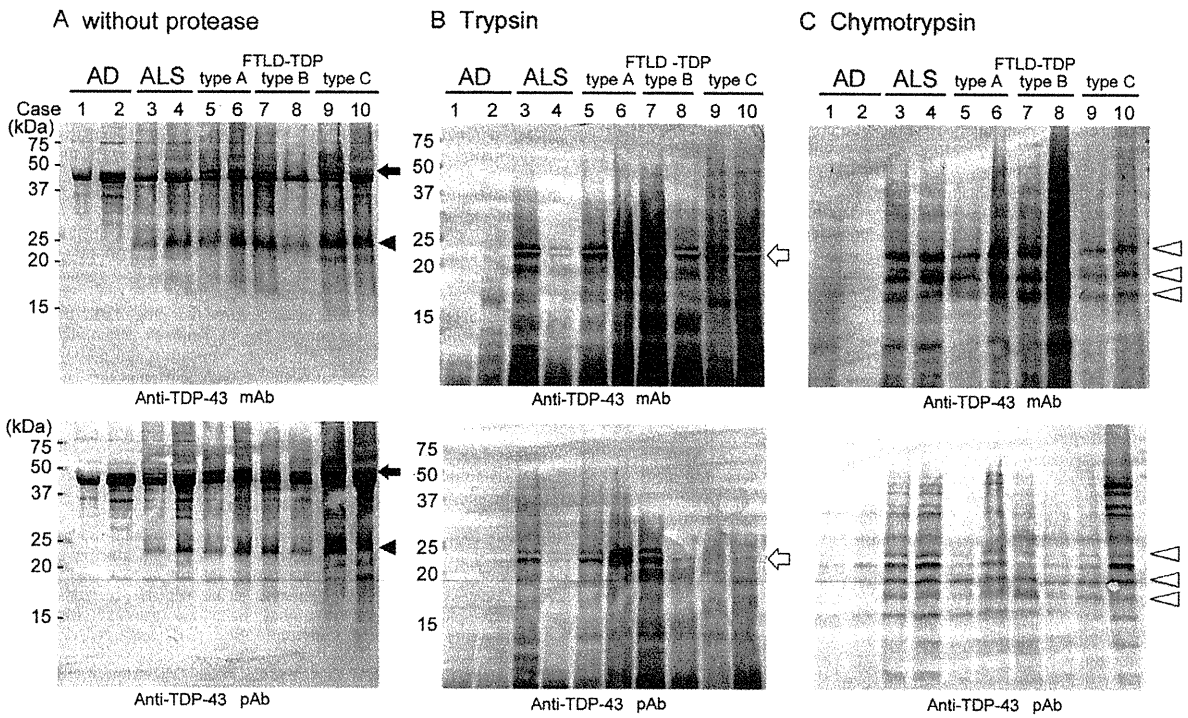

Fig. 4. Immunoblot analysis of Sarkosyl-insoluble fractions from AD and TDP-43 proteinopathies before and after protease treatment. (A) Without protease treatment, normal TDP-43 of 43 kDa was detected with these antibodies in all cases examined. In the ALS and FTLD-TDP cases, phosphorylated full-length TDP-43 of 45 kDa (arrows), high-molecular-weight smears, and the 24–26 kDa fragments (arrow heads) were detected in addition to the normal TDP-43. (B) Upon trypsin treatment, full-length TDP-43 disappeared, and the protease-resistant ~25 kDa fragments (white arrows) and smears appeared in ALS and FTLD-TDP cases, but not in AD cases. (C) After chymotrypsin treatment, triplet bands (white arrowheads) were detected in ALS and FTLD-TDP cases with the mAb and multiple bands were detected with pAb, whereas such immunoreactivities were hardly detected in AD cases.

are found in the C-terminal half of the TDP-43 [13–16]. Therefore, misfolding or structural alteration of the C-terminal half of TDP-43 seems to be the key to the pathogenesis of TDP-43 proteinopathies. By mass spectrometric analysis of the 23 kDa band in Sarkosyl-insoluble fraction from FTLD-TDP (type A), we identified the cleavage site as the N-terminus of Asp219 [23]. Another group reported cleavage at Asp208, based on N-terminal sequencing of urea extracts of FTLD-TDP brain [28]. However, the cleavage sites of the other major C-terminal fragments of 24 and 26 kDa have not been determined yet. In this study, we showed that the pathological TDP-43 C-terminal fragments of 24 and 26 kDa in ALS and FTLD-TDP type A contain the epitope of anti-TDP-43 mAb, residues 203–209, by comparing the immunoblotting results with those using pS409/410 (Fig. 3). This result suggests that the cleavage sites of pathological TDP-43 C-terminal fragments in ALS and FTLD-TDP are located at the N-terminal side of Thr203. Although the mechanisms of generation of the C-terminal fragments are still controversial, the presence of multiple cleavage sites suggests that cleavage may occur after the aggregation or assembly of TDP-43.

Structural or conformational changes in the proteins are thought to be the most important in protein aggregation in these neurodegenerative diseases. To analyze the conformational change in the epitope of TDP-43 from normal to the abnormal states further, we treated the Sarkosyl-insoluble TDP-43 with trypsin or chymotrypsin, and immunoblotted with these antibodies. The protease-resistant TDP-43 bands and smears were detected in ALS and all subtypes of FTLD-TDP with these anti-TDP-43 antibodies (Fig. 4), while no such bands were seen in AD cases. These demonstrate that the epitope is protease-resistant in the abnormal TDP-43 but not in normal TDP-43. Using an antibody pS409/410 that recognizes the C-terminal phosphorylation sites, some protease-resistant TDP-43 bands are detected, and the band patterns are slightly different between ALS and FTLD-TDP type C [29]. On immunoblots with anti-TDP-43 pAb and mAb, such difference was not observed. This is probably due to that the epitope of the mAb and pAb is located in the amino-terminus of the protease-resistant core of the TDP-43, whereas epitope of the pS409/410 located in the C-terminus. Similar protease-resistant bands have been reported in abnormal prion in prion diseases, tau in Alzheimer's disease and alpha-synuclein in Parkinson's disease and dementia with Lewy bodies. Biochemical studies in these proteinopathies suggested that the protease-resistant bands represent the core domains of the filamentous aggregates of these proteins with cross-β structures [30–32]. By analogy with these proteins we propose that these protease-resistant C-terminal fragments represent the core of the filamentous aggregates of TDP-43. Since the epitope of the mAb and pAb are determined to locate at residues 203–209, this may be important in the formation of a core region of pathological TDP-43 aggregates which is common in all TDP-43 proteinopathies. Finally, the protease treatment used in this study may be useful for detection of the abnormal TDP-43 in brains of patients, animal models, culture cells and in vitro models with these anti-TDP-43 antibodies more specifically, as used for detection of abnormal prion proteins.

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Molecular Dissection of TDP-43 Proteinopathies

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Abstract TDP-43 has been identified as a major component of ubiquitin-positive tau-negative cytoplasmic inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and in amyotrophic lateral sclerosis (ALS). We raised antibodies to phosphopeptides representing 36 out of 64 candidate phosphorylation sites of human TDP-43 and showed that the antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 labeled the inclusions, but not the nuclei. Immunoblot analyses demonstrated that the antibodies recognized TDP-43 at ~45 kDa, smearing substances and 18–26 kDa C-terminal fragments. Furthermore, the band patterns of the C-terminal fragments differed between neuropathological subtypes, but were indistinguishable between brain regions and spinal cord in each individual patient. Protease treatment of Sarkosyl-insoluble TDP-43 suggests that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between the diseases. These results suggest that molecular species of abnormal TDP-43 are different between the diseases and that they propagate from affected cells to other cells during disease progression and determine the clinicopathological phenotypes of the diseases.

Keywords Propagation · Phosphorylation · Tau · α-Synuclein · Prion · Cancer

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Introduction

TAR DNA-binding protein of M_r=43 kDa (TDP-43) is a nuclear factor that functions in regulating transcription and splicing. It is structurally characterized by two RNA recognition motifs and the C-terminal tail containing a glycine-rich region, and resembles a heterogeneous ribonucleoprotein (hnRNP) (Ayala et al. 2005). It has been shown to interact with several nuclear ribonucleoproteins (RNP), including hnRNP A and B and survival motor neuron protein, inhibiting alternative splicing (Buratti et al. 2005; Bose et al. 2008). In 2006, TDP-43 was identified as a major component of ubiquitin-positive inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) (Arai et al. 2006; Neumann et al. 2006). Subsequent immunohistochemical examination demonstrated abnormal accumulation of TDP-43 in neurodegenerative disorders other than FTLD-U and ALS, including ALS/parkinsonism–

dementia complex of Guam (Geser et al. 2007; Hasegawa et al. 2007), Alzheimer's disease (AD) (Amador-Ortiz et al. 2007; Higashi et al. 2007; Arai et al. 2009), dementia with Lewy bodies (DLB) (Higashi et al. 2007; Nakashima-Yasuda et al. 2007; Arai et al. 2009), Pick's disease (Arai et al. 2006; Freeman et al. 2008; Lin and Dickson 2008), argyrophilic grain disease (Fujishiro et al. 2009) and corticobasal degeneration (Uryu et al. 2008). These diseases with TDP-43 pathologies are now referred to as TDP-43 proteinopathies. In 2008, mutations in the TDP-43 gene (*TARDBP*) were discovered in familial and sporadic cases of ALS (Yokoseki et al. 2008; Gitcho et al. 2008; Sreedharan et al. 2008; Kabashi et al. 2008; Van Deerlin et al. 2008; Barmada and Finkbeiner 2010; Pesiridis et al. 2009), FTD-MND (Benajiba et al. 2009) and FTD (Borroni et al. 2009), clearly indicating that abnormality of TDP-43 protein causes neurodegeneration.

Identification of Abnormal Phosphorylation Sites of TDP-43

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 (Arai et al. 2006; Neumann et al. 2006). By producing antibodies against synthetic phosphopeptides containing 36 different phosphorylation sites from among the 56 serine/threonine residues of TDP-43, five abnormal phosphorylation sites were identified at serine residues in the C-terminal region (Hasegawa et al. 2008). The antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD-U, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulation in neuropathological examinations (Hasegawa et al. 2008). Immunoblotting of the Sarkosyl-insoluble fraction from control, FTLD-U and ALS cases using these phospho-specific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 at ~45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD-U and ALS (Hasegawa et al. 2008).

Cellular Models of TDP-43

To establish cellular models of TDP-43 proteinopathies, several deletion mutants of human TDP-43 in SH-SY5Y cells were expressed and the accumulation of TDP-43 was analyzed by use of the phospho-TDP-43 antibodies and ubiquitin. Wild-type (WT) full-length TDP-43 was localized to nuclei and no inclusions were observed, whereas in cells transfected with C-terminal fragments as GFP fusions, round cytoplasmic inclusions with intense GFP fluorescence were formed (Nonaka et al. 2009b). In addition, a deletion mutant lacking the nuclear localizing signal (NLS) and six amino acids similar to the NLS also formed aggregates in cells without any treatment (Nonaka et al. 2009a). These inclusions are strongly positive for antibodies to phosphorylated TDP-43 and ubiquitin. Using these cellular models, the effect of pathogenic mutations of the TDP-43 gene was analyzed. Of 14 mutants examined, seven mutants showed a significantly higher number of aggregates than the WT C-terminal fragment, strongly suggesting that these mutations of TDP-43 accelerate aggregation of the C-terminal fragments (Nonaka et al. 2009b). In addition, when GFP-tagged C-terminal fragments were co-expressed with DsRed-tagged full-length TDP-43, cytoplasmic inclusions with both GFP and DsRed signals were formed, suggesting that exogenous full-length TDP-43 is trapped in cytoplasmic inclusions formed by C-terminal fragments. This may explain why normal nuclear staining of TDP-43 is lost in neuronal cells with inclusions in diseased brains (Nonaka et al. 2009b). Furthermore, we identified two cleavage sites of TDP-43 deposited in FTLD-U by mass spectrometric analysis, and confirmed that expression of these fragments as GFP fusions also afforded cytoplasmic inclusions positive for ubiquitin and phosphorylated TDP-43 (Nonaka et al. 2009b). The cleavage sites identified in the 23-kDa C-terminal fragment of FTLD were different from that of caspase-3, suggesting that caspase is not the enzyme responsible for generating the 23-kDa fragment (Nonaka et al. 2009b). These cellular models recapitulate many of the features of TDP-43 in patients, and therefore, should be useful for screening small molecules for activity to inhibit TDP-43 aggregate formation. We tested whether or not methylene blue and dimebon have the ability to suppress formation of pathological TDP-43 inclusions. Compared to controls, a 50% reduction in the number of inclusions with 0.05 μM methylene blue, a 45% reduction with 5 μM dimebon and an 80% reduction with the combination of 0.05 μM methylene blue and 5 μM dimebon were observed (Yamashita et al. 2009). The effects were statistically significant and the results were also confirmed by Western blotting. These results suggest that these two compounds may be effective in the therapy of ALS, FTLD-U and other TDP-43 proteinopathies.

TDP-43 C-Terminal Fragments

Based on neuropathological studies, TDP-43 proteinopathies have been classified into 4 subtypes (Cairns et al.

2007). Type 1 is characterized by dystrophic neurites (DNs) with few neuronal cytoplasmic inclusions (NCIs) and no neuronal intranuclear inclusions (NIIs), Type 2 has numerous NCIs with few DNs and no NIIs, Type 3 has numerous NCIs and DNs and occasional NIIs and Type 4 has numerous NIIs and DNs with few NCIs, a pattern which is specific for familial FTLD-U with mutations of VCP gene. There appears to be a strong relationship between other subtypes of TDP-43 pathology and clinical phenotype. Type 1 is associated with semantic dementia, type 2 with FTLD with motor neuron disease (MND), ALS or clinical signs of MND, and type 3 with progressive non-fluent aphasia or FTD with mutation in the progranulin gene. Recent studies of ALS have clarified the wide distribution of neuronal and glial TDP-43 pathology in multiple areas of the central nervous systems (Geser et al. 2008; Nishihira et al. 2009), suggesting 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.

By immunoblot analyses of the Sarkosyl-insoluble fractions from FTLD-U and ALS patients, we found that the band patterns of the C-terminal fragments of phosphorylated TDP-43 corresponded to the neuropathological subtypes. Type 1 FTLD-U showed two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa, while type 2 ALS showed three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa. Type 3 FTD with mutation in the progranulin gene showed an intermediate pattern between those two. These results clearly indicate that TDP-43 proteinopathies subclassified by neuropathological differences can also be distinguished biochemically. This strong association between the neuropathology and the biochemistry is critical for understanding the molecular pathogenesis of TDP-43 proteinopathies.

Biochemical Analysis of TDP-43 in FTLD-U and ALS

The biochemical differences of TDP-43, as shown in the different band patterns of TDP-43 C-terminal fragments, are closely linked to the morphologies of inclusions. The properties of the abnormal TDP-43 may determine the neuropathological and clinical phenotypes of TDP-43 proteinopathies. Similar biochemical and neuropathological differences have been reported in tau between PSP and CBD. Both PSP and CBD are tauopathies with deposition of 4-repeat tau isoforms; however, distinct types of C-terminal fragments are detected, i.e., a 33-kDa band in PSP and ~3-kDa bands in CBD (Arai et al. 2004).

So, what do the different band patterns mean? It is clear that the fragments are produced by cleavage at multiple sites of TDP-43. The band patterns also suggest that the cleavage sites are slightly altered between the diseases. Based on these observations, it is likely that the event may occur after the assembly or aggregation of abnormal TDP-43, and represent relatively protease-resistant domains of TDP-43, which form beta-sheet structure. That is, the different band patterns in TDP-43 proteinopathies represent different conformations of abnormal TDP-43 in the diseases.

To test this idea, we performed protease treatment of the abnormal TDP-43 recovered in the Sarkosyl-insoluble pellets, and analyzed the protease-resistant bands. Proteins can be easily cleaved by proteases if they are denatured or unstructured, but domains that have rigid structures such as beta-sheet structure, or that are structurally buried or interacting with other molecules, are highly resistant to proteases. Figure 1 shows the result of immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment. Before treatment, hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. Upon trypsin or chymotrypsin treatment, the full-length 45-kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16–26 kDa (Fig. 1). As expected, the protease-resistant band pattern of ALS is different and clearly distinguishable from that of FTLD-U. Doublet bands at ~16 kDa and a band at 25 kDa were detected in ALS, but only a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment (Fig. 1). Similarly, multiple protease-resistant bands were detected at 16–25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U (Fig. 1). These results strongly support the idea that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between ALS and FTLD-U.

TDP-43 in Different Brain Regions

Similar protease-resistant bands and differences in the band patterns have been reported in prion diseases, CJD and BSE (Collinge et al. 1996). Protease-resistant prion from new-variant CJD cases showed a different characteristic pattern from that in sporadic CJD cases, and the band pattern is indistinguishable from that of mice infected with BSE prion. This is biochemical evidence that the BSE agent has been transmitted from bovine to human.

Applying this to TDP-43 in TDP-43 proteinopathies, it is possible to determine whether there is any difference between the abnormal TDP-43 accumulated in cortex and that in spinal cord by analyzing the band patterns of the C-

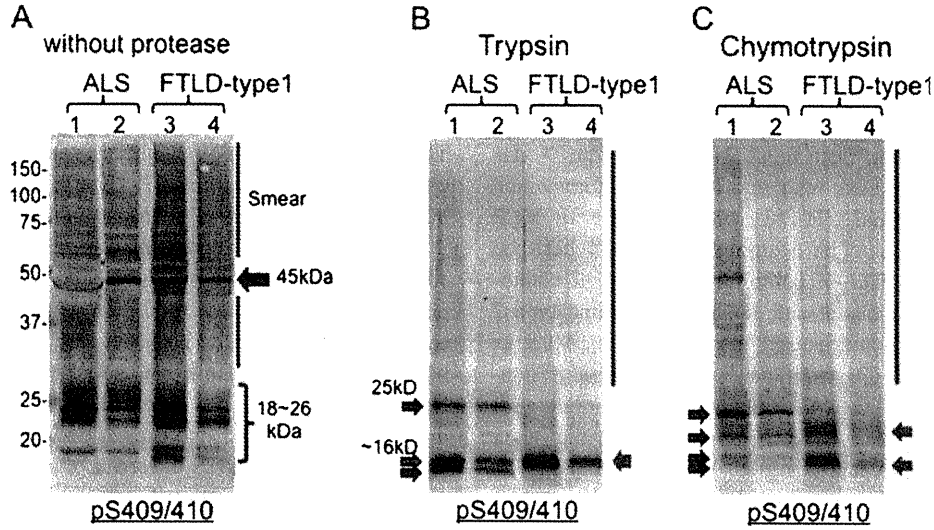

Fig. 1 Immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment with a phosphorylation dependent anti-TDP-43 rabbit polyclonal antibody (pS409/410). **a** Hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410 before treatment. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. **b** Upon trypsin treatment, the full-length 45 kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16~26 kDa. Doublet bands at ~16 kDa and a band at 25 kDa were seen in ALS, but a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment. **c** Multiple protease-resistant bands were detected at 16~25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U

terminal fragments of TDP-43. Thus, we have prepared Sarkosyl-insoluble fractions from cortex and spinal cords of three ALS cases, immunoblotted them with pS409/410 and compared the results. In all three cases, type 2 C-terminal fragments of TDP-43 were detected, and there was no significant difference between the band pattern in cortex and that in spinal cord (data not shown). This strongly suggests that the same form of abnormal TDP-43 molecule is deposited in different brain regions. Similar results were also obtained from the analysis of the C-terminal band pattern of TDP-43 in FTLD-U. It seems highly unlikely that the same conformational change would occur synchronously in different brain regions. Instead, it seems more likely that abnormal protein produced in cells is transferred to different regions and propagated. These biochemical data obtained from the brains of patients provide biochemical evidence that abnormal species of TDP-43 are transmitted from cell to cell and propagated in vivo.

Discussion

Amyloid-like protein deposition is a common neuropathological feature of many neurodegenerative diseases. Hyperphosphorylated tau in Alzheimer's disease and related tauopathies, hyperphosphorylated alpha-synuclein in Parkinson's disease and other alpha-synucleinopathies, and expanded polyglutamines in polyglutamine diseases have been identified. Importantly, the extent of the abnormal protein pathologies is closely correlated with the disease progression (Braak and Braak 1991; Braak et al. 2003; Saito et al. 2003). The proteins or protein fibrils deposited in cells in these diseases have been shown to have a common structural feature. Cross-beta structure, which is the same as in abnormal prion protein, has been demonstrated in filaments or fibrils composed of tau (Berriman et al. 2003), alpha-synuclein (Serpell et al. 2000) or expanded polyglutamines (Perutz 1999). It has not been demonstrated in TDP-43 yet, but we have shown by electron microscopy that phosphorylated TDP-43 in motor neurons of ALS patients has a fibrous structure (Hasegawa et al. 2008), suggesting that TDP-43 is also an amyloid-like protein.

For the assembly of amyloid fibrils, nucleation-dependent protein polymerization has been proposed. This comprises nucleation and elongation phases, and nucleation is the rate-limiting step. It takes a long time to form the first aggregated seed from the monomer, but once the seed is formed, the elongation step proceeds relatively quickly. More importantly, by addition of amyloid-seed, proteins are often converted to the same conformation as that of the seed. For example, WT monomeric alpha-synuclein is converted to A30P-type amyloid fibrils when it is incubated with a small amount of fibril-seeds formed with A30P mutant alpha-synuclein (Yonetani et al. 2009). Differences in the conformations of the amyloid fibrils are detected based on the differences in the protease-resistant band

patterns, as in the typing of prion proteins. There is another example of nucleation-dependent amyloid fibril formation in cultured cells. We developed a novel method for introducing amyloid seeds into cultured cells using lipofectoamine, and presented experimental evidence of seed-dependent polymerization of alpha-synuclein, leading to the formation of filamentous protein deposits and cell death (Nonaka et al. 2010). Overexpression of alpha-synuclein itself in cells does not generate abnormal inclusions, but if fibril seeds formed with alpha-synuclein are introduced into cells, abundant filamentous alpha-synuclein aggregates positive for PSer129 and ubiquitin are developed, and cells with inclusions undergo cell death. This was also clearly demonstrated in cells expressing different tau isoforms by introducing the corresponding tau fibril seeds (Nonaka et al. 2010).

The above results obtained from biochemical analyses of abnormal proteins in patients strongly suggest that intracellular amyloid-like proteins, including TDP-43, propagate from cell to cell and this propagation is the cause of disease progression, analogously to metastasis of cancer cells to multiple different tissues in cancer progression. From this point of view, we have proposed as a hypothesis that neurodegenerative diseases with amyloid-like proteins can be regarded as "protein cancers." The term prion, coined in 1982 by Stanley B. Prusiner, describes an agent transmissible among humans and a variety of mammals. On the other hand, the term "protein cancers" describes diseases that involve the spreading or propagation of abnormal proteins in tissues or individuals, even though the mechanism of propagation is basically the same as that of prions. Amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation, and the amplified amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions (Fig. 2). As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. Therefore, it is important to regulate the propagation of abnormal proteins for clinical therapy.

Conclusions

1. In ALS, FTLD-U and other TDP-43 proteinopathies, abnormally phosphorylated, ubiquitinated, and truncated TDP-43 is accumulated in a filamentous form.
2. We established cellular models which recapitulate many of the features of the abnormal TDP-43 in FTLD-U and ALS
3. ALS-related pathogenic mutations of the TDP-43 gene accelerate aggregate formation by the C-terminal fragments.
4. The band pattern of the TDP-43 C-terminal fragments is different between diseases with different clinicopathological phenotypes, and it represents different conformations of the abnormal TDP-43 between the diseases.
5. The C-terminal band patterns in several brain areas and spinal cord in each individual case of sporadic ALS are indistinguishable.
6. These and other results suggest that abnormal TDP-43, tau and alpha-synuclein are transmitted and propagated from cell to cell in different regions during disease progression. It is important to find drugs that can block the propagation of abnormal proteins for clinical therapy.

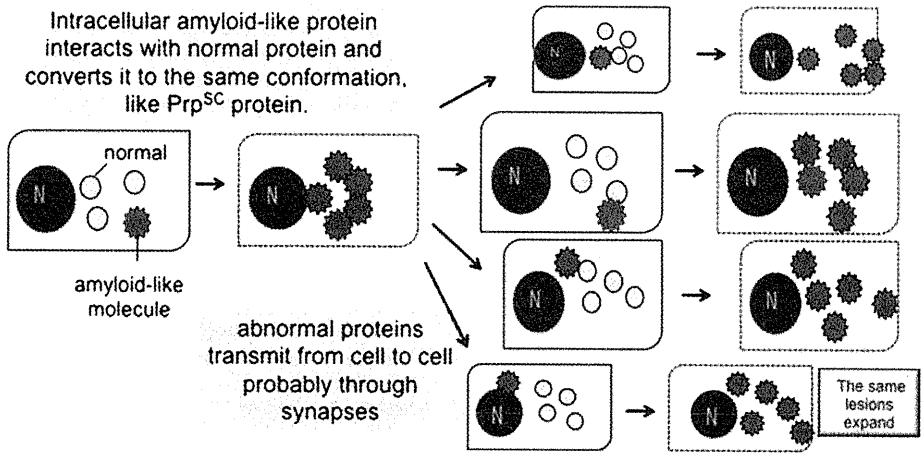

Fig. 2 Schematic representation of prion-like conversion of normal protein into amyloid-like protein and its propagation in neurodegenerative diseases. Intracellular amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation. Amplified abnormal amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions. As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. From this point of view, neurodegenerative diseases with amyloid-like proteins can be regarded as 'protein cancers"

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ARTICLE ADDENDUM

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In vitro recapitulation of aberrant protein inclusions in neurodegenerative diseases

New cellular models of neurodegenerative diseases

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Aberrant protein aggregates in affected brain cells of patients with neurodegenerative diseases are a well-known hallmark, but although the formation of these inclusions is an important pathogenic event, the mechanism involved remains unclear. We have recently established a simple method to introduce protein fibrils into cultured cells as seeds for protein aggregation, and we showed that intracellular soluble α-synuclein or tau can aggregate in cultured cells dependently upon seeds introduced in this way. Seeded aggregation of α-synuclein induced necrotic cell death, which was suppressed by the addition of various polyphenols. Our cellular models are expected to be valuable tools not only for elucidating the molecular mechanisms of onset of neurodegenerative diseases, but also for drug discovery.

Key words: alpha-synuclein, tau, intracellular aggregates formation, transduction of protein fibrils, cell death

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In patients with neurodegenerative disorders, intracellular aberrant protein inclusions are often found in the brain, including neurofibrillary tangles in Alzheimer's disease or Lewy bodies in Parkinson's disease and dementia with Lewy bodies. These aberrant protein aggregates are often observed in the most affected regions of diseased brains, suggesting they may cause neuronal cell death, leading to onset of these diseases. Tau and α-synuclein are well-known cytosolic proteins that are the main components of neurofibrillary tangles and Lewy bodies, respectively. They are soluble and natively unfolded proteins, and it remains unclear how they become aggregated in neuronal cells. Indeed, intracellular aggregate formation of these proteins does not occur when cultured cells are transfected with expression plasmids encoding these proteins. On the other hand, many in vitro studies using recombinant proteins, such as Abeta, tau, α-synuclein or poly glutamine-containing protein, have shown that these proteins are readily aggregated into fibrils in the presence of seeds for aggregation. These findings prompted us to examine whether seeds-dependent aggregation would occur in cultured cells. Thus, we aimed to introduce protein fibrils into cultured cells as seeds for aggregation.

Transfection of plasmid DNA into cultured cells is conducted routinely by the use of liposomes of polycationic and neutral lipids in water, based on the principle of cell fusion. Several commercially available reagents such as Lipofectoamine, Lipofectamine 2000 or FuGENE6 are available to efficiently transfect plasmid DNA into cultured cells. We tested whether these transfection reagents could transfect cultured SH-SY5Y cells not only with plasmid DNA, but also with protein fibrils. After much trial and error, we finally succeeded in transfecting α-synuclein fibrils into these cells using Lipofectamine reagent. We found that the introduced recombinant α-synuclein fibrils are phosphorylated at Ser129 in cultured cells, indicating that they had been introduced by Lipofectamine.[1] Interestingly, monomeric and oligomeric α-synuclein could not be introduced by the use of Lipofectamine. We applied for a patent covering the use of Lipofectamine for transduction of recombinant protein fibrils into cultured cells in 2005 (patents

pending in the United States: 12/086124, the European Union: 06834541.2 and Japan: 2007-549210). Recently, other groups have also reported introduction of fibrillar protein into cultured cells with or without specific reagents.[2-5]

Next, we examined whether intracellular α-synuclein can be aggregated dependently upon introduced seeds. When α-synuclein fibrils mixed with Lipofectamine were introduced into cells transiently expressing α-synuclein, phosphorylated and ubiquitinated α-synuclein inclusions (~10 μm in diameter) were observed by means of confocal laser microscopy, indicating that plasmid-derived soluble α-synuclein formed aggregates in the presence of exogenous α-synuclein fibrils in cells, and these inclusions resembled Lewy bodies in diseased brains. Others have also reported that α-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells.[4] On the other hand, introduced tau fibrils were also shown to act as seeds for intracellular aggregation of plasmid-derived soluble tau protein. Interestingly, we found that fibrils composed of 3-repeat tau isoform serve as seeds for intracellular aggregation of soluble 3-repeat tau, but not soluble 4-repeat tau and fibrils of 4-repeat tau seed serve as seeds for aggregation of soluble 4-repeat tau, but not soluble 3-repeat tau. Likewise, introduction of α-synuclein fibrils did not elicit intracellular tau aggregation in cells and soluble α-synuclein did not form intracellular aggregates in the presence of any tau fibrils. These results clearly indicate that intracellular protein aggregation is highly dependent on the species of protein fibril seeds. Now, we are examining whether detergent-insoluble fractions prepared from several diseased brains can be introduced into cells by Lipofectamine and can serve as seeds for intracellular aggregate formation of soluble α-synuclein, tau or TDP-43.

Does the formation of these inclusions lead to cell death or toxicity? The answer is yes. We observed non-apoptotic cell death in cells harboring these inclusions. In these cells, proteasome activity was found to be significantly reduced. This suppression may be related to the cause of cell death. Furthermore, we showed that cell death in cells with α-synuclein inclusions is effectively suppressed by the addition of various small molecules to the culture medium; polyphenols such as exifone and gossypetin were the most effective, suggesting that these compounds may be possible new drugs for the treatment of neurodegenerative diseases.

Our study strongly supports a seed-dependent mechanism for the formation of the intracellular protein aggregates. Recently, the intercellular transfer of inclusions made of tau,[3,6] α-synuclein[2,7,8] and huntingtin[9] has been reported, suggesting the existence of mechanisms reminiscent of those by which prions spread through the nervous system. It remains to be clarified whether the incorporation of amyloid seeds into neurons or glial cells, as shown in our study, also occurs in vivo, but our results strongly suggest that extracellular aggregates may be taken up into neurons by endocytosis or under certain specific conditions. Therefore, it may be crucial to inhibit not only the production of intracellular amyloid seeds, but also their spread into extracellular space and their propagation. Vaccination against α-synuclein[10] or tau may be an effective treatment, together with the inhibition of intracellular aggregates formation with small-molecular compounds, for the therapy of neurodegenerative diseases.

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Neurological CPC

左側の失行とパーキンソニズムを認め，経過6年で寝たきりとなった84歳男性

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Neurological CPC

左側の失行とパーキンソニズムを認め，経過6年で寝たきりとなった84歳男性*

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Masashi Takanashi[1)]（Case Presenter）　Sayaka Funabe[1)]（Pathology）　Shigeo Murayama[2)]（Commentator）　Masayuki Yokochi[3)]（Moderator）

第15回 Neuro CPC 第2例　2010年9月24日　於：東京慈恵会医科大学　1号館　5階講堂

症例呈示

司会（横地）　それでは高梨先生から臨床の報告をお願いします。

主治医（高梨）　症例は，死亡時年齢84歳の男性。既往歴は高血圧で内服加療。前立腺肥大があるが特に加療なし。家族歴なし。

現病歴は2001年76歳頃から，左上肢を使うときに力が入らないということを自覚。そのまま様子をみていたが，2002年9月に，風邪による発熱を契機に，急に左上肢の使いづらさが増悪したということで，A大学付属病院脳神経内科を緊急受診した。その際，診察上左上肢に明らかな麻痺，筋力低下はなかったが，動きは全体的に緩慢で，回内・回外運動や指タップがうまくできなかった。また，左手のみに固縮が軽度に認められた。歩行は，ややバランスが悪かったそうだが，明らかな失調性歩行や麻痺性歩行ではなかった。脳梗塞を疑って脳のMRIを撮ったが，明らかな急性期の脳梗塞は存在せず，左に比べてやや右側の側脳室体部が大きいという所見のみであった。

左手にパーキンソニズムと思われる症状を認めたことから，Parkinson症候群を疑い，外来通院を促したが，その後，本人より通院を中断してしまった。

その2年後の2004年3月には，左足の出が悪いという主訴で，同じ大学病院の外来を受診した。患者本人の話では2003年ぐらいから，徐々に左足の出が悪くなってきたこと，転ぶことも増えてきたことの訴えがあった。

この時の所見から，左側のパーキンソニズムと，歩行時左側の足の出が悪いことなど，左側に非常に限局した運動症状であるということと，左側に構成失行を認めることで，大脳皮質基底核変性症（corticobasal degeneration：CBD），ではないかと診断された。治療としてレボドーパを徐々に増やして750 mg/日まで増量したが，運動症状に関しては改善がなかった。

2004年3月受診時の神経学的所見は意識は清明，改訂長谷川式認知症スケール（Hasegawa Dementia Scale revised：HDS-R）は，24/30点。高次脳機能に関しては，失語は明らかでなく，失行は，この際認めたものでは構成失行，左手で立体の模写が不可でした。失認はなかった。

脳神経系は，明らかな異常はなく，眼球運動制限も特になし。構音障害，嚥下障害なし。歩行に関しては，左足がややすり足で，後方転倒傾向があった。後方へのプ

* A 84-year-old Man with Apraxia and Parkinsonism on His Left Side, who Became Bedridden for 6 Years

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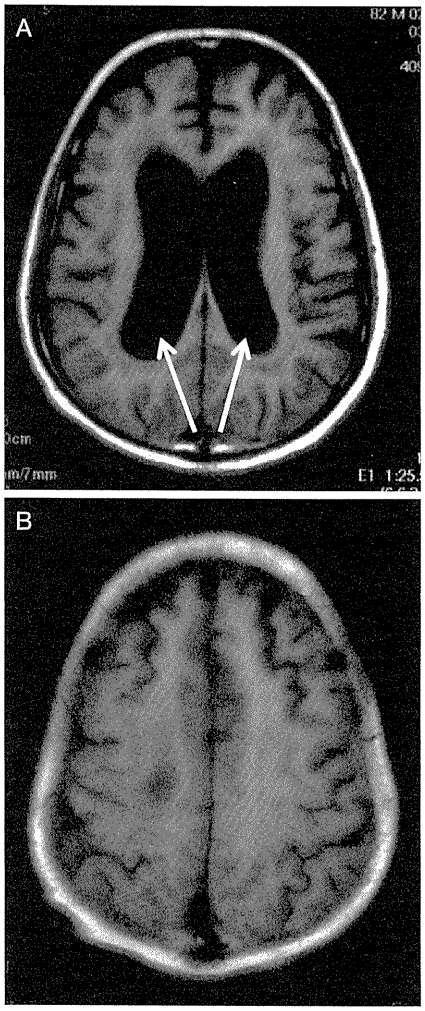

Fig. 1　発症 6 年経過時の脳 MRI
T_1 強調画像。A：側脳室の拡大（矢印），
B：前頭葉の軽度萎縮。

ルテストは自力で止まれなかった。筋力は正常で，麻痺はなし。無動症状は，全身の動作が緩徐で，回内・回外運動や，指タップ，手の開閉運動は，右は問題なかったが，左上肢で緩徐かつ拙劣であった。筋緊張は左優位で，上下肢に固縮を認めたが，右上肢にも軽度の固縮が認められた。

不随意運動は明らかなものはなく，小脳失調もなし。深部腱反射は正常範囲内，病的反射も問題なく，前頭葉徴候などもなし。感覚・自律神経も問題なかった。

2004 年の検査所見はカルテの記述では，2002 年の救急外来受診時に比べて右側脳室体部の拡大が目立っていた。同様に左の側脳室体部も，以前に比べて拡大していた。脳血流 SPECT では，右前頭頭頂葉に血流低下を認めた。

このような，左右差が非常にはっきりしているパーキンソニズムと，失行を認めることから，外来では CBD という診断でフォローされていた。

その後，当初は左上肢に，固縮・無動が，徐々に進行し，だんだん命令動作に従えなくなり，肢節運動失行も明らかになってきた。観念運動性失行も加わり，パーキンソニズムの後から徐々に失行が認められたと記載があった。

2005 年にはさらに転倒を繰り返し，たびたび外傷で救急外来を受診。この頃から，左上肢にいままで認められなかったミオクローヌス様の細かい振えが，安静時，姿勢時ともに出現するようになった。

2005 年の時点では，眼球運動が上方視では 3/5，下方視は 4/5 の制限を認めた。異常眼球運動所見は認めなかった。

2006 年になると，無動症状が進行し，転倒傾向も非常に強く，自力での歩行・立位が困難となった。また，目の前にある食物を手当たり次第に食べてしまうという脱抑制行動が，徐々に認められるようになった。

2007 年頃になると嚥下が障害され，食事でむせるようになった。また，この頃から左手指は屈曲位で拘縮し，ずっと握ったままの状態であった。足は両足伸展で拘縮して，首は後屈位であった。右の手指の開閉運動は命令に応じて可能で，拘縮するような筋緊張の亢進は認めなかった。

2008 年になると，ほとんど発語がなくなり，立位は介助しても不可能。ほぼ 1 日車椅子，もしくはベッドでじっとしているような生活になった。また，この頃から終日閉眼して，開眼指示に従うことはなかった。無理に目を開けようとすると，Gegenhalten のためか，ギュッと力強く閉眼してしまった。

このような経過で，経口摂取困難となったため，胃瘻増設目的で 2009 年 7 月 24 日に A 大学付属病院に入院したが，入院後，38°C台の発熱が持続，敗血症を合併し，敗血症性ショックで死亡。

画像所見

主治医　発症から約 6 年経過時の MRI (Fig. 1) を呈示する。当初は，側脳室の拡大が，左に比べて右に強く目立つということだったが，この頃にはどちらも拡大していると思われる。

一方，側脳室の拡大が目立つわりには，大脳弁蓋部では，前頭葉皮質の萎縮は認めるものの，そんなに強く萎縮が目立たなかったと思われる。

皮質下核周辺の変化は割と目立っており，T_1 強調画

像の axial 像（Fig. 2 A）では，第三脳室の開大がかなり大きく目立った。

Sagital 像（Fig. 2 B）では，中脳被蓋が萎縮していて，一見ハミングバード像（hummingbird sign）といえそうな中脳被蓋の萎縮を認めた。

このように，大脳皮質表面は，そんなに強い左右差，もしくは萎縮は目立たなかったが，側脳室体部の拡大および第三脳室の開大，中脳被蓋の萎縮が経過とともに目立ってきていた。

SPECT は ECD の核種で行い，eZIS で統計処理をした。

2004 年の時点では左右差があり，右前頭頭頂葉の血流低下がいわれていた。2007 年では，側頭葉の下面に左右差があるが，帯状回や前頭葉では，左右の血流低下が目立たなくなってきている。

画像は，経過を経るごとに，徐々に左右差が目立たなくなってきたということが，特徴であった。

臨床診断

・大脳皮質基底核変性症

ディスカッション 1

司会　それでは，まず臨床経過，既往経過，症状についてコメントをお願いします。

橋本（慈恵医大）　この症例の臨床経過から，みなさん CBD が頭の中にあると思いますが，認知症の合併はないのでしょうか。もしくは，本症例を cortico basal syndrome（CBS）として考えた場合，Alzheimer 型認知症などの認知症だけで本例を説明できないでしょうか。認知症の病歴がほとんどありませんが，聴取できていれば教えてください。

主治医　長い経過で外来カルテを掘り起こしてつくった病歴なのですが，当初は，HDS-R で 24 点程度と，年齢の割には認知機能は保たれています。徐々に無動症状が強くなってきたのと，脱抑制の症状が出てきて，どちらかというと前頭側頭型の症状が徐々に目立ってきている記載があります。アミロイドが関連するような認知症というよりは，タウオパチーや前頭側頭葉変性症（fronto-temporal lobar degeneration：FTLD）のような認知症がみられた経過だと思います。

司会　HDS-R を 1 回やって点数が 24 点ですが，失点の 6 点の内容はわかっていますか。

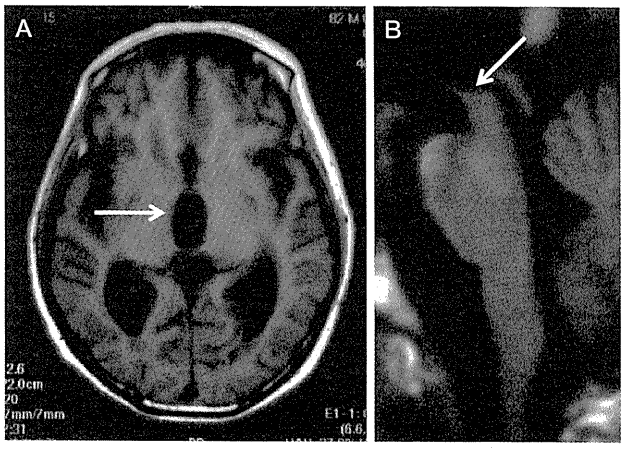

Fig. 2　発症 6 年経過時の脳 MRI

T_1 強調画像。A：Axial 像。第 3 脳室の開大が目立つ（矢印），B：Sagital 像。中脳被蓋の萎縮（矢印）。

主治医　HDS-R は，野菜の名前が 5 点満点中の 3 点，あと 5 つの物品のうちの 1 つがいえなかったことと，3 つの言葉の記憶が－1 点と，数字の逆唱が 1 回できなかったことです。

司会　その評価内容は，Alzheimer 病にみられるタイプじゃないですよね。見当識はよく保たれていますから。

河村（昭和大学）　その点を聞きたかったんです。野菜の名前は 5 点満点ですね。いくついえたんですか。

主治医　7 ついえて，失点は 2 点です。

河村　それも含めて，失行症状を伺いたいんですが，最初は構成失行だけで，それから肢節運動失行，観念運動性失行が出てくる，割に珍しい経過ですが，構成失行はどんな内容だったのでしょうか。

主治医　一応，左手の模倣ができませんでした。

河村　でも，それは観念運動性失行でも，肢節運動失行でも起こる症状ですよね。なぜ，構成失行というふうに思われたのですか？

主治医　立体の図を描かせて，それがうまく描けなかったということもあり，当時の担当医は構成失行と考えたようです。

河村　左手だけですか。

主治医　そうです。

河村　脳梁病変など特殊な場合を除いて，構成失行は，左と右と，両方の手に起こります。まずこれは構成失行でない可能性があります。その後の肢節運動失行はどうですか。先ほど観念運動性失行のところで，物品の使用があまりよくない，だから観念運動性失行というふうにおっしゃったけれど，観念運動性失行は物品の使用が比較的よいのが特徴なんです。だから，ここの考え方は，

全体的に自然ではないのではないでしょうか。

主治医　表現が拙かったことはお詫びします。

河村　この所見では，なんらかの症状があったとしても実態がよくわからない。この点を指摘しておきます。

主治医　今回カルテの記載をひも解いてプレゼンテーションしましたので，詳しいところまでは表現が行き届かなかったと思います。

司会　最初のところで，交互変換運動，それから指タップができなかったということですが，「うまくできなかった」の表現は，「impossible」という意味ですか，それとも「clumsy」という意味ですか。

主治医　「clumsy」です。

司会　そうすると，パーキンソニズムとしてもよい程度のことなんですね。

河村　失行に続いて，左手での模写が不可になったということですが，この症状はなんでしょうか。

主治医　先ほどいった，立体的な画を模写できなかったということを構成失行という表現をしましたが，先ほどのご指摘からはこの表現は誤りのようです。

河村　片側に構成失行が起こるということは，ごく稀なんですが，ないことはないんです。脳梁病変で，時々右手の構成失行というのが起こります。けれども，それ以外に片手で起こることはないので，模写できなかった，その症状の内容が，本当は知りたいです。どんなものか。ぜんぜん違った図を描いているのか，それともちょっと下手だったのか。

きっと失行，行為障害といってもいいですが，それが進んでいるんだと思います。具体的な症状を本当は知りたいですよね。

鈴木（慈恵医大青戸病院）　SPECTの画像がeZISで出ていますが，これらが診断に本当に役立つ画像情報を提供しているかどうか疑問に思います。側脳室があれだけ開大していますと，こういう統計画像を解釈するときには，非常に注意が必要です。ですから，eZISではなくて，通常のaxial画像があれば教えていただけますか。

主治医　記録に残っているのがeZIS処理後の画像のみであり，これだけが判断材料になっています。

鈴木　このeZIS画像を診断に用いるのは，疑問が残ります。

主治医　帯状回は，かなり脳室拡大の影響を受けているのではないかと思います。

鈴木　先ほどの橋本先生も，その辺りを考えて質問されていると思うのですが，やはり後部帯状回とか，楔前部の所見というのは，Alzheimer病を考えるうえで最も大事な所見なので，この画像から何かをいうのは止めたほうがいいと思います。臨床所見からみていくしかないのかなと思いました。

河村　MRI（Fig. 1）をみると，左の前頭葉がちょっと萎縮していませんか。つまり，上前頭溝はあまり左右差がないのですが，そのほかの前頭葉の溝は左優位に幅が広いですよね。一方，頭頂間溝は，あまり拡大がないですね。左優位に前頭葉萎縮があるんじゃないでしょうか。

主治医　そういう眼でみると，頭頂間溝に関しては若干左のほうが開いているようにもみえます。

河村　そうですね。しかし，頭頂間溝より上前頭溝のほうがよっぽど幅が拡大してます。脳室も大きく開いているけれども，脳室も，側脳室も，前のほうが拡大が強いんじゃないですか。

主治医　前頭葉萎縮がないといっているわけではなく，皮質に比べ皮質下の脳室拡大が軽いと思っています。

河村　そうすると，SPECT所見やeZIS所見と矛盾しませんか。前頭葉萎縮にみえます。

司会　河村先生，この症例に関しては，運動障害のほかに失行はありますか，ないですか。

河村　逆にそれを教えてほしいと思います。何も根拠になるような話がなくて，失行が「ある」ということしかいわれていません。構成失行が左手に起こったなんていうのは普通は考えられないと思います。

病理所見

病理医（舟邉）　まずはマクロの所見です。肉眼所見は，過固定になってしまい，萎縮の評価は困難です。脳重は1,210 gでした。

右側の内側面も過固定になり，萎縮などの評価は困難でした。左側は凍結に回してしまったので写真はありません。

脳幹をみると，黒質は色調がかなり落ちていて，それに比べると青斑核の色調は，比較的保たれていました。小脳は歯状核が褐色調を呈していました。右半球の冠状断前方は特に問題はありません。基底核はあまり所見が目立たないのですが，淡蒼球の内節・外節ともに萎縮していて，褐色調を呈しています。視床下核が切れてしまっていますが，こちらも萎縮しており，褐色調を呈しております。海馬は，保たれておりました。脳梁はやや薄くなっています。後方は，特に問題ありません。

次に組織学的所見です。Klüver-Barrera（KB）染色（Fig. 3 A）では黒質の神経細胞は著明に脱落しています。ヘマトキシリン・エオジン（hematoxylin-eosin：HE）染色（Fig. 3 B）では，黒質の神経細胞はかなり減ってい

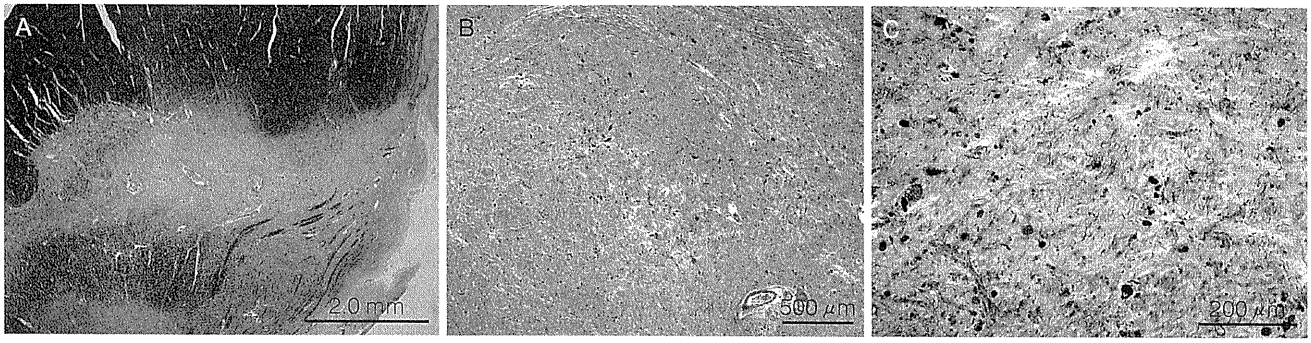

Fig. 3　中脳黒質の組織所見
A：Klüber-Barrera 染色，B：ヘマトキシリン・エオジン染色，C：Gallyas-Braak 染色。

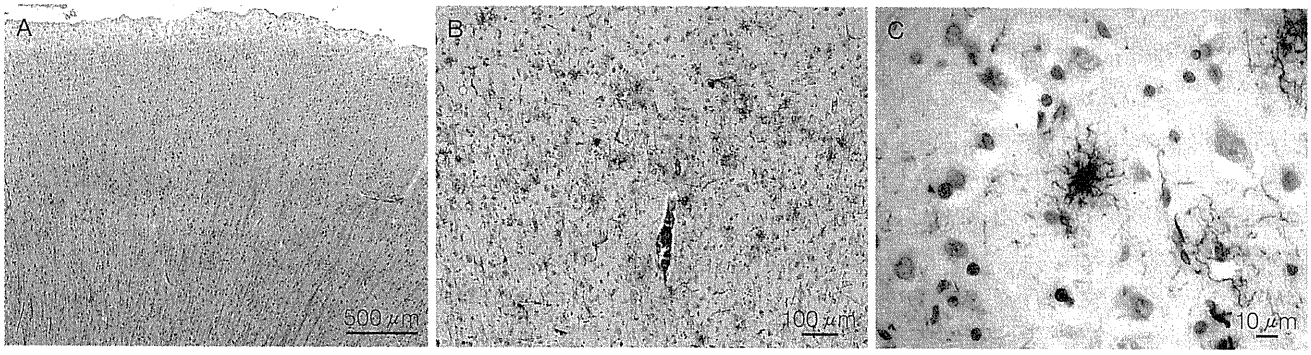

Fig. 4　上前頭回の組織所見
A：Klüber-Barrera 染色，B：Gallyas-Braak 染色，C：AT8 免疫染色。

て，メラノファジアが多数認められます。拡大像ではグリオーシスが著明でした。

中脳黒質の Gallyas-Braak 染色(Fig. 3 C)では，tufted astrocyte や coiled body，神経原線維変化（neurofibrillary tangles：NFTs），neuropil threads（NTs）が認められています。

動眼神経核にも，かなり多数の NFTs が認められました。

青斑核では KB 染色，HE 染色で神経細胞は保たれていました。Gallyas-Braak 染色で NFTs が少数みられました。

橋核の AT8 の免疫染色では，橋核神経細胞の周囲に tufted astrocyte が認められました。

延髄下オリーブ核の HE 染色では若干グリア細胞が増えている印象があり，そして NFTs がみられました。AT8 の免疫染色でも tufted astrocyte が認められました。

中脳の赤核の Gallyas-Braak 染色でも tufted astrocyte, Gallyas-Braak 染色では NFTs, NTs が多数あり，tufted astrocyte も認めました。

歯状核にもグリオーシスを認め，Bodian 染色でグルモース変性を認めます。

淡蒼球内節も，かなり著明なグリオーシスがみられ，Gallyas-Braak 染色で NFTs, NTs, tufted astrocyte が中等度にみられました。淡蒼球外節のグリオーシスも高度で，視床下核のグリオーシスも中等度認めています。

上前頭回の KB 染色（Fig. 4 A）でみると，皮質の構造は保たれ，神経細胞も保たれているのですが，Gallyas-Braak 染色（Fig. 4 B）では，かなり多数の tufted astrocyte を認めました。NFTs，NTs も多数認めました。AT8 の免疫染色（Fig. 4 C）では tufted astrocyte を皮質に多数認めておりました。

中心前回では，Betz 細胞にタウ陽性の蓄積がありました。

他に Alzheimer 型 NFTs ですが，CA1 に NFTs, NTs が少数，また嗅内野には NFTs, NTs が少数認められています。移行嗅内野にも中等度認めています。

神経病理診断は，進行性核上性麻痺（progressive supranuclear palsy：PSP）です。

本症例における NFTs や coiled body，tufted astrocyte, threads の分布の程度は，通常の進行性核上性麻痺の症例に比べると，上前頭回の NFTs，NTs，tufted astrocyte，threads，coiled body の出現がかなり目立ちました。脳幹は，比較的軽度でした。