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Symposium: Advances in amyotrophic lateral sclerosis research

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

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

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

INTRODUCTION

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

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

Ubiquitin- and TDP-43-positive pathological inclusions found in FTLD-TDP include neuronal cytoplasmic inclusions (NCIs), dystrophic neurites (DNs), neuronal intranuclear inclusions (NIIs), and glial cytoplasmic inclusions (GCIs).[1,2,29–31] Based on morphological aspects, TDP-43 proteinopathies have been classified into four subtypes.[32] Type 1 is characterized by DNs with few NCIs and no NIIs, Type 2 has numerous NCIs with few DNs and no NIIs, Type 3 has numerous NCIs and DNs and an occasional NIIs and Type 4 has numerous NIIs and DNs with few NCIs. Type 4 is specific for familial FTLD-TDP with mutations of VCP gene. The strong relationship between other subtypes of TDP-43 pathology and clinical phenotype is indicated. Type 1 is associated with semantic dementia, Type 2 with FTLD with motor neuron disease (MND) or clinical signs of MND, and Type 3 with progressive non-fluent aphasia.[29,33,34]

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

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

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

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

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

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

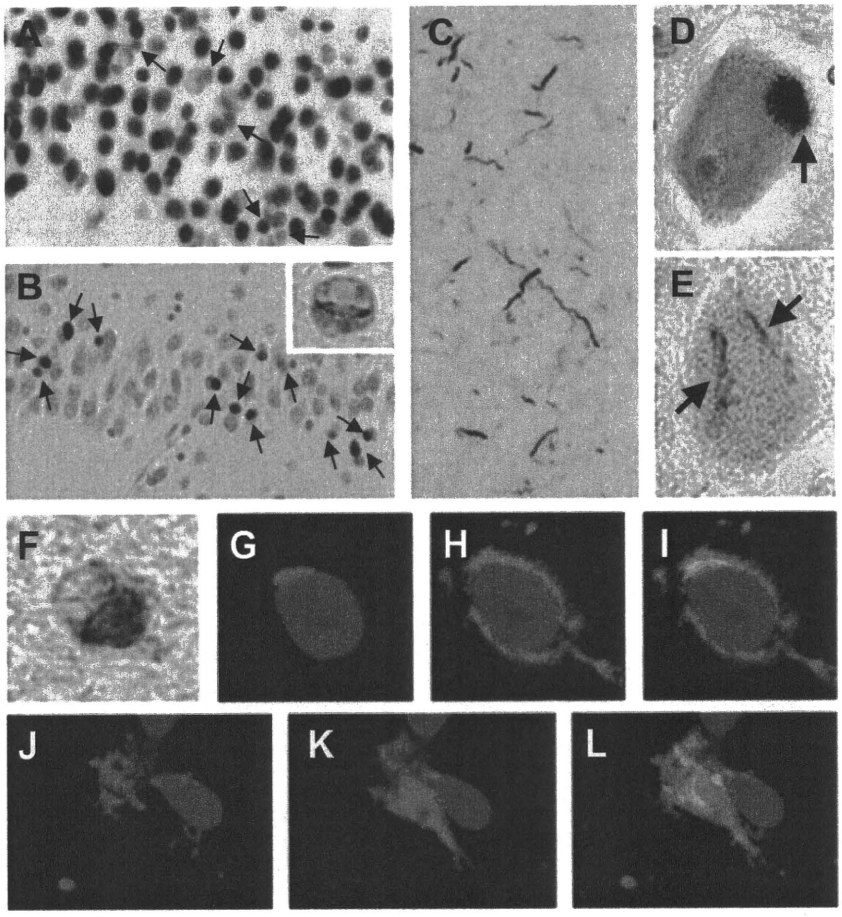

Fig. 1 Neuronal and glial inclusions immunopositive for phosphorylated transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) in frontotemporal lobar degeneration (FTLD)-TDP and ALS. A. Dentate gyrus (DG) of the hippocampus of the FTLD-TDP case stained with the commercially available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

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

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

To investigate the biochemical basis of the different TDP-43 pathological subtypes (Types 1–3), we carefully compared the results of immunoblots of the sarkosyl-insoluble fractions from the cerebral cortex of cases with sporadic FTLD-TDP (Type 1), FTLD-MND (Type 2), ALS (Type 2) and familial FTLD with *PGRN* mutations (m*PGRN*) (Type 3), using pTDP-43 specific antibodies. The results showed that there is a close relationship between the pathological subtypes and the immunoblot pattern of the 18–26 kDa C-terminal fragments of pTDP-43 (Fig. 2B,C). These findings confirm and extend the previous reports[1,31] that showed C-terminal fragment composition varied between cases with Type 1 and Type 2 pathology. Furthermore, these results parallel our earlier findings of differing C-terminal tau fragments in progressive supranuclear palsy and corticobasal degeneration, despite identical composition of tau isoforms.[39] Taken together, these results suggest that elucidating the mechanism of C-terminal fragment origination may shed light on the pathogenesis of several neurodegenerative disorders involving TDP-43 proteinopathy and tauopathy.

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

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

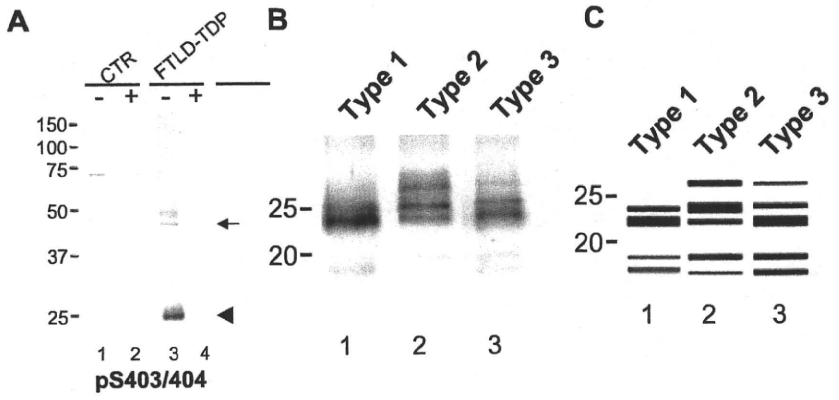

Fig. 2 Biochemical analyses using antibodies specific for phosphorylated transactivation response (TAR) DNA-binding protein of Mr 43 kDa (pTDP-43). A. Immunoblot analyses of sarkosyl-insoluble fractions from control (lanes 1, 2) and frontotemporal lobar degeneration (FTLD)-TDP (lanes 3, 4), using pS403/404,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

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

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

Table 1 Subclassification of TDP-43 proteinopathy

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

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

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

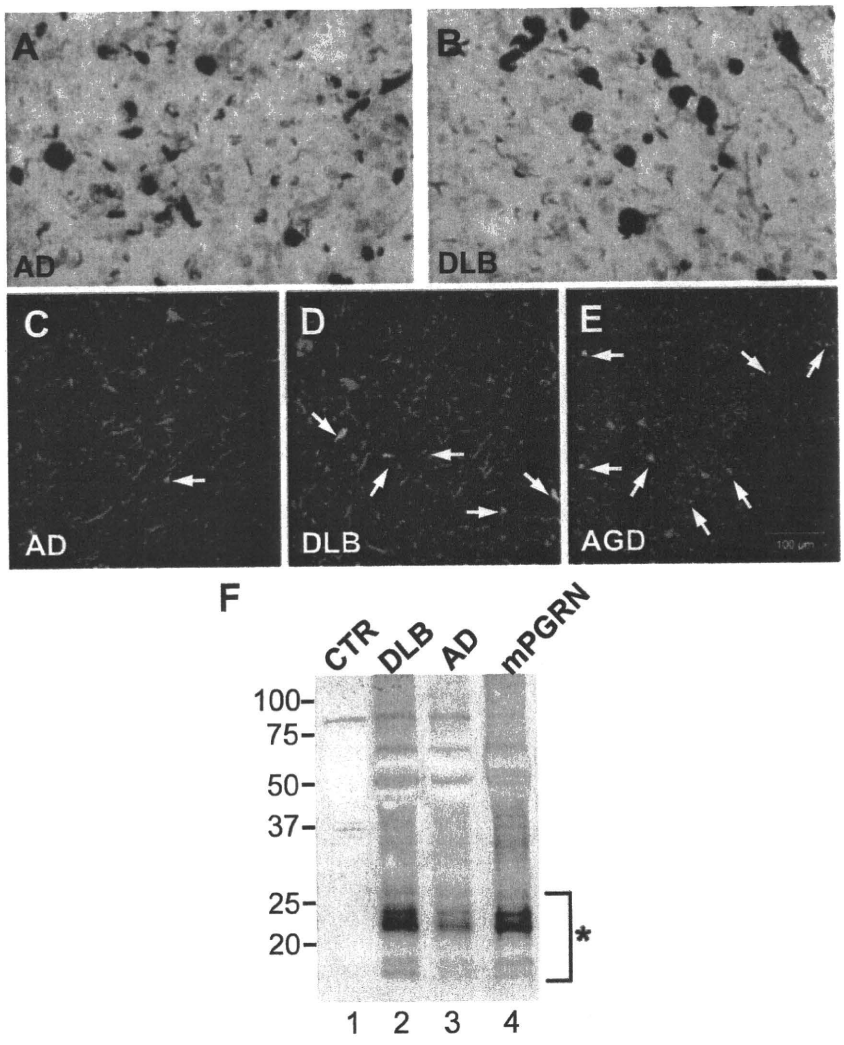

Fig. 3 Phosphorylated transactivation response (TAR) DNA-binding protein of Mr 43 kDa (pTDP-43)-positive structures in other neurodegenerative disorders. A. Neuronal cytoplasmic inclusions (NCIs) and dystrophic neurites stained with the pTDP-43-specific antibody (pS403/404) in the temporal cortex of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

Of the TDP-43-positive cases in AD and DLB, about 20–30% showed neocortical TDP-43 pathology resembling the FTLD-TDP, Type 3[51] (Fig. 3A,B). Immunoblot analyses of the sarkosyl-insoluble fraction from cases with neocortical TDP-43 pathology showed intense staining of several low-molecular-weight bands, corresponding to C-terminal fragments of TDP-43. Interestingly, the band pattern of these C-terminal fragments in AD and DLB also corresponds to that previously observed in the FTLD-TDP, Type 3[37] (Fig. 3F). These results suggest that the morphological and biochemical features of TDP-43 pathology are common between AD or DLB and a specific subtype of FTLD-TDP. Since all FTLD-TDP cases with PGRN mutations show Type 3 pathology,[56] there may be genetic factors, such as mutations or genetic variants of *PGRN* underlying the co-occurrence of abnormal deposition of TDP-43, tau and α-synuclein.

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

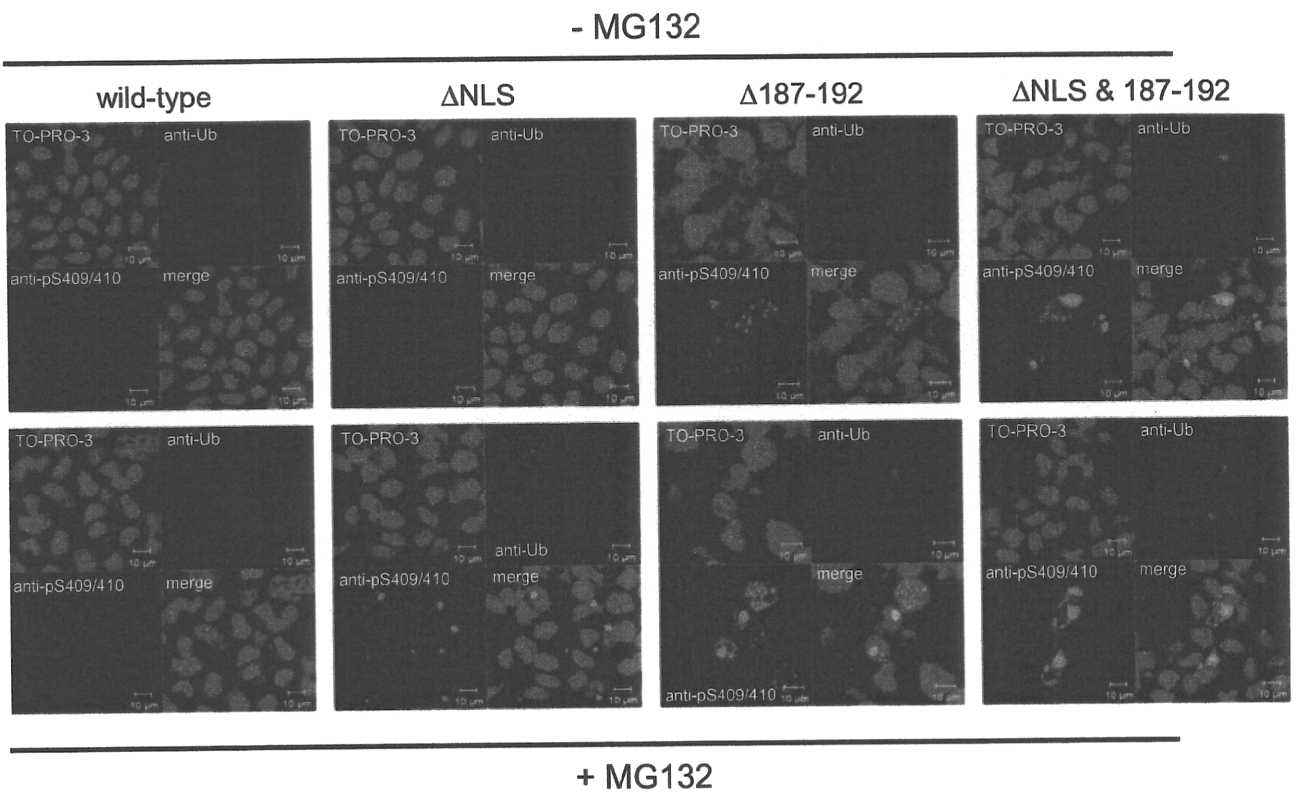

Fig. 4 The formation of inclusion-like structures in cells transfected with deletion mutants of transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43). When pcDNA3-TDP-43 wild-type was expressed in SH-SY5Y cells, no staining was observed by the phosphorylation-specific anti-TDP-43 antibody (pS409/410), indicating that transfected wild-type TDP-43 and endogenous TDP-43 are not phosphorylated at Ser409/410. The deletion mutant lacking nuclear localization signal (ΔNLS: 78–84 residues) was not recognized by pS409/410 without MG132 treatment, while round cytoplasmic inclusion-like structures were stained by both pS409/410 and anti-ubiquitin antibodies in those cells treated with MG132. In cells expressing another deletion mutant lacking 187–192 residues (Δ187–192), pS409/410-positive but ubiquitin-negative intranuclear dot-like structures were observed without treatment. With MG132, round intranuclear inclusions positive for pS409/410 and ubiquitin were formed. In cells expressing the double-deletion mutant (ΔNLS and 187–192), cytoplasmic inclusions positive for pS409/410 and ubiquitin were formed even in the absence of MG132.

Based on these findings so far, we would like to propose that TDP-43 proteinopathy can be divided into two groups (Table 1). One is "pure" TDP-43 proteinopathy, in which only TDP-43 accumulates in brains as a pathological protein. The other is "combined" TDP-43 proteinopathy, which shows multiple protein aggregates. TDP-43 pathology is always found in all cases of pure TDP-43 proteinopathy and familial and endemic cases of combined TDP-43 proteinopathy, while it is found in a subpopulation of cases with sporadic combined TDP-43 proteinopathy.

ESTABLISHMENT AND ANALYSES OF CELLULAR MODELS OF TDP-43 PROTEINOPATHY

To establish the cellular models for intracellular aggregates of TDP-43, we first examined two candidate sequences for the nuclear localization signal (NLS) (Fig. 4).[57] Deletion of residues 78–84 resulted in cytoplasmic localization of TDP-43 in SH-SY5Y cells, proving that this sequence indeed functions as NLS. This result is largely consistent with the previous report by Winton *et al.* which showed that residues 82–98 were required for TDP-43 entry into the nucleus.[58] On the other hand, the mutant lacking residues 187–192 localized in nuclei, forming unique dot-like structures. Proteasome inhibition caused these to assemble into aggregates. Furthermore, double-deletion mutant of these sequences caused cytoplasmic inclusion formation without proteasomal inhibition. Immunohistochemical and immunoblot analyses showed that these inclusions consisted of phosphorylated and ubiquitinated TDP-43, suggesting that these cellular models recapitulate the phenotypes of TDP-43 proteinopathies both pathologically and biochemically.

Then, we tried to generate and analyze the cellular models by expressing C-terminal fragments of TDP-43 in

SH-SY5Y cells, since 18–26 kDa C-terminal fragments of TDP-43 are major constituents of inclusions in FTLD-TDP and ALS brains.[37] The results showed that expression of several TDP-43 C-terminal fragments as green fluorescent protein (GFP), including 162–414, 218–414, 219–414 and 247–414, led to the formation of cytoplasmic inclusions positive for pTDP-43 and ubiquitin (Fig. 5).[59] The N-termini of the latter two peptides, 219–414 and 247–414, correspond to the cleavage sites of TDP-43 C-terminal fragments accumulated in FTLD-TDP brains identified by our mass spectra analyses. Igaz *et al.* reported another cleavage site at Arg 208 in a pathological TDP-43 C-terminal fragment from FTLD-TDP brains and inclusion formation in cultured cells expressing resultant C-terminal fragment (residues 208–414).[60] Our immunoblot analysis showed that these aggregated pTDP-43 C-terminal fragments were recovered in sarkosyl-insoluble fraction as those in brains of FTLD-TDP and ALS.

Several groups have recently reported increased accumulation of TDP-43 fragments in the brain homogenates[13] and cultured cells[15,16] in some of the pathogenic mutations of TARDBP identified in ALS. However, in our cellular models, immunoblot analyses failed to show any significant differences in the generation of fragments of TDP-43 with or without various mutations. Alternatively, pathogenic mutations consistently enhanced aggregation of TDP-43 if they are present in the C-terminal fragment, GFP-TDP 162–414 (Fig. 6). These results suggest that pathogenic mutations and N-terminal truncation synergistically promote abnormal accumulation of TDP-43.

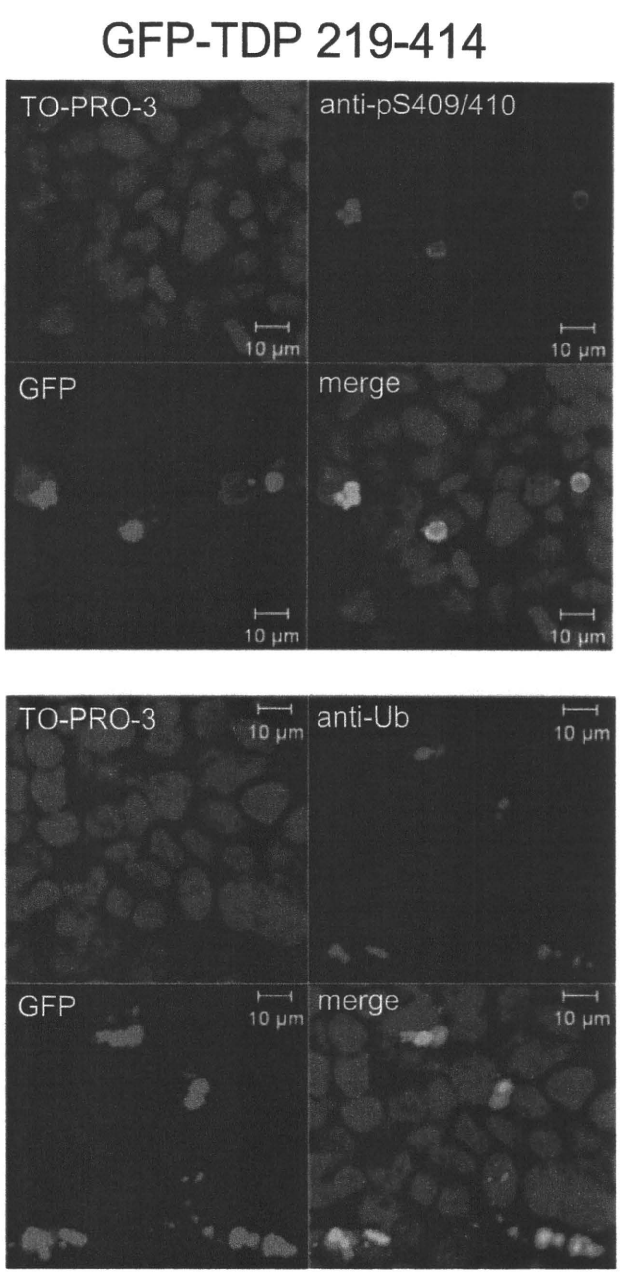

Fig. 5 Transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) C-terminal fragments identified in diseased brains form cytoplasmic inclusions in cells. Round cytoplasmic inclusions with strong green fluorescent protein (GFP) intensities were observed in SH-SY5Y cells expressing GFP-TDP 219–414. These were positive for pS409/410 and ubiquitin Ub).

METHYLENE BLUE AND DIMEBON INHIBIT AGGREGATION OF TDP-43 IN CELLULAR MODELS

Inhibition of the aggregation of TDP-43 and promotion of its clearance are considered to be major therapeutic avenues for ALS and FTLD-TDP. As for other neurodegenerative diseases, current tools include antibodies, synthetic peptides, molecular chaperones and chemical compounds. Of the latter, methylene blue (MB) and dimebon have recently been reported to have significant beneficial effects in phase II clinical trials of AD.[61,62] MB is a phenothiazine compound that has been used for treating methemoglobinemia,[63,64] inhibiting nitric oxide synthase,[65] reducing nGMP,[66] enhancing β-oxidation in mitochondria,[67] inhibiting of noradrenalin re-uptake[68] and enhancing brain mitochondrial cytochrome oxidase activity.[69,70] It has also been shown to inhibit AD-like Aβ and tau aggregation *in vitro*.[71,72] Dimebon is a non-selective antihistaminergic compound that was in clinical use for many years before more selective agents became available.[73] It has been reported to inhibit butyrylcholinesterase, acetylcholinesterase, NMDA receptors, voltage-gated calcium channels, adrenergic receptors, histamine H1 receptors, histamine H2 receptors and serotonin receptors, as well as to stabilize glutamate-induced Ca2+ signals.[74–76] The effects of dimebon on pathological protein aggregation have not been studied in detail.

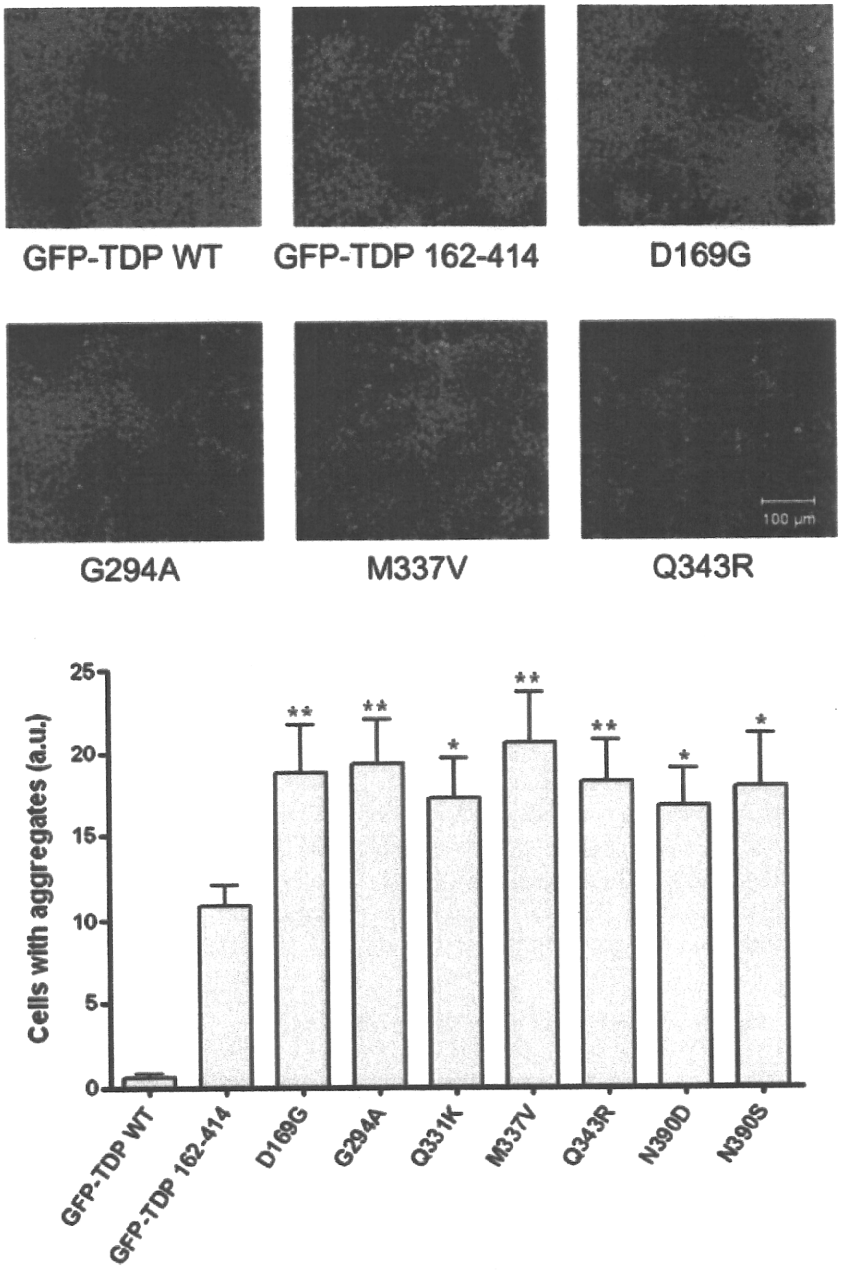

Fig. 6 The effect of transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) mutations on aggregates formation of the C-terminal fragment of TDP-43. All seven mutations significantly facilitated the formation of intracellular aggregates of green fluorescent protein (GFP)-TDP 162–414, as compared with those of wild-type GFP-TDP 162–414.

Using our cellular models of TDP-43 proteinopathy described above, we investigated the effects of MB and dimebon on the formation of TDP-43 aggregates.[77] Following treatment with 0.05 μM MB or 5 μM dimebon, the number of TDP-43 aggregates was reduced by 50% and 45%, respectively (Fig. 7A–C,I). The combined use of MB and dimebon resulted in an 80% reduction in the number of aggregates (Fig. 7D,I), and in the significant reduction of phosphorylated TDP-43 in insoluble fraction of the cell lysate (Fig. 7E–H). These results suggest that MB and dimebon may be useful for the treatment of ALS, FTLD-TDP and other TDP-43 proteinopathies.

CONCLUSION

Intracellular aggregation of TDP-43 takes place in brains of patients with ALS, FTLD and a variety of other neurodegenerative diseases, suggesting the possibility that TDP-43 has wide influence on neuronal dysfunction and neurodegeneration. Phosphorylated and truncated forms of TDP-43 are major species accumulated in diseased brains, and the proteolytic cleavage of TDP-43 may play an important role for the pathological process of TDP-43 proteinopathy. In cultured cells, expression of the TDP-43 C-terminal fragments results in accelerated aggregate formation and in

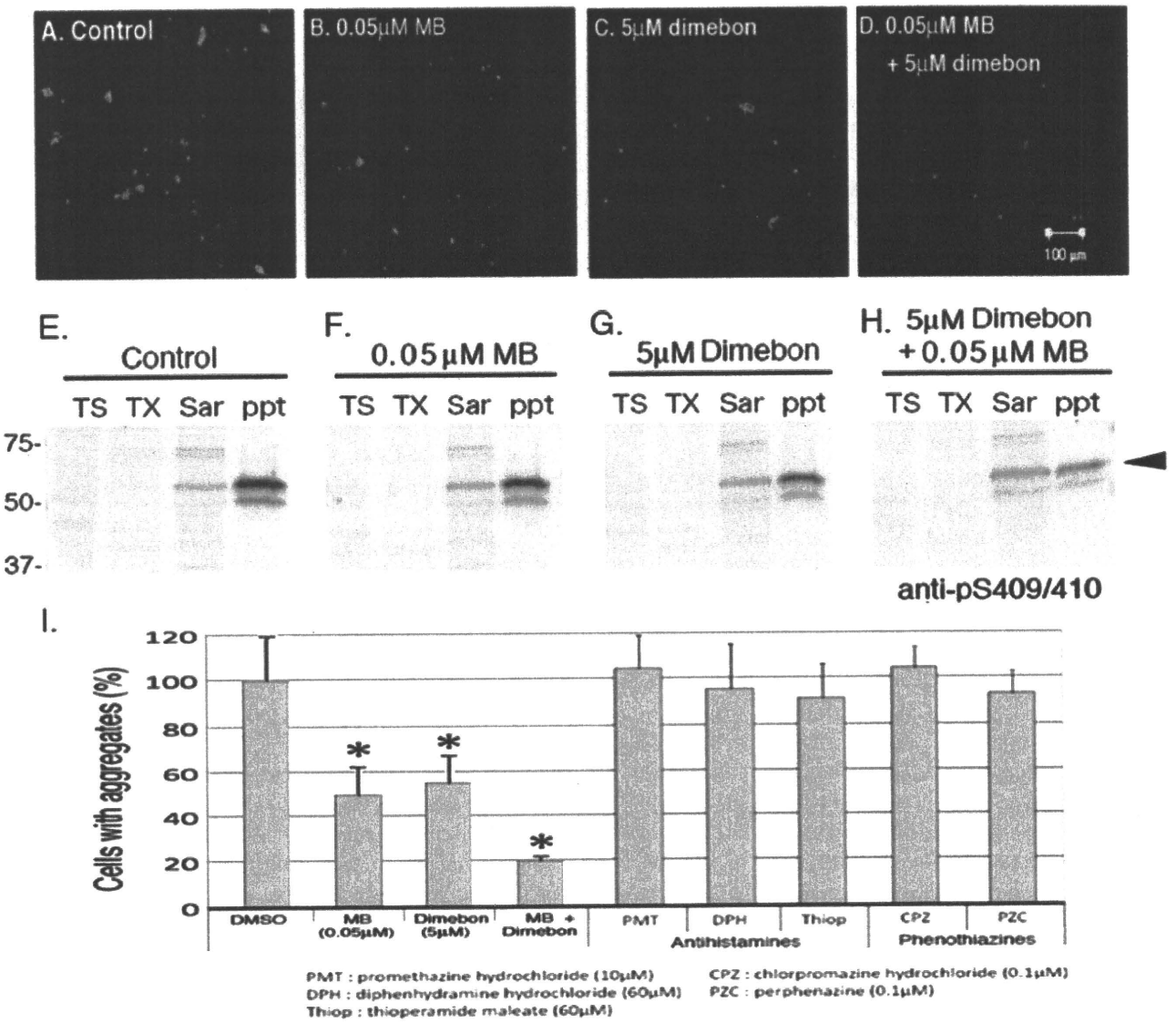

Fig. 7 Inhibition of aggregates formation of transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) in cellular models by methylene blue (MB) and dimebon. (A–D) Immunohistochemical analysis of the effects of MB and dimebon on the aggregation of TDP-43 in SH-SY5Y cells expressing TDP-43 (ΔNLS and 187–192). TDP-43 inclusions were stained with anti-pS409/410 antibody and detected with Alexa Fluor 488-labeled secondary antibody. Representative confocal images from cells treated with control (dimethyl sulfoxide + distilled water) (A), 0.05 μM MB (B), 5 μM dimebon (C) and 0.05 μM MB + 5 μM dimebon (D) are shown. (E–H): Immunoblot analysis of the effects of MB and dimebon on the aggregation of TDP-43 in SH-SY5Y cells expressing green fluorescent protein (GFP)-tagged TDP-43 C-terminal fragment (162–414). Tris saline (TS)-soluble material, Triton X-100 (TX)-soluble material, Sarkosyl (Sar)-soluble material and the remaining pellet (ppt) were prepared from control cells (E) and from cells treated with 0.05 μM MB (F), 5 μM dimebon (G), and 0.05 μM MB + 5 μM dimebon (H), run on SDS-PAGE and immunoblotted with anti-pS409/410 antibody. (I) Quantitation of cells with TDP-43 aggregates. The number of cells with intracellular TDP-43 aggregates was counted and expressed as the percentage of cells with aggregates in the absence of compound (taken as 100%). Data are means ± SEM $^*P < 0.01$ by Student's *t*-test.

failure of nuclear localization of endogenous TDP-43. At present, it is unknown whether loss of function, toxic gain of function, or a combination of both mechanisms contributes to neurodegeneration. Cultured cells or animal models expressing those abnormal TDP-43 species are expected to be useful tools to investigate the pathogenesis of TDP-43 proteinopathy and to develop effective diagnostics and therapeutics.

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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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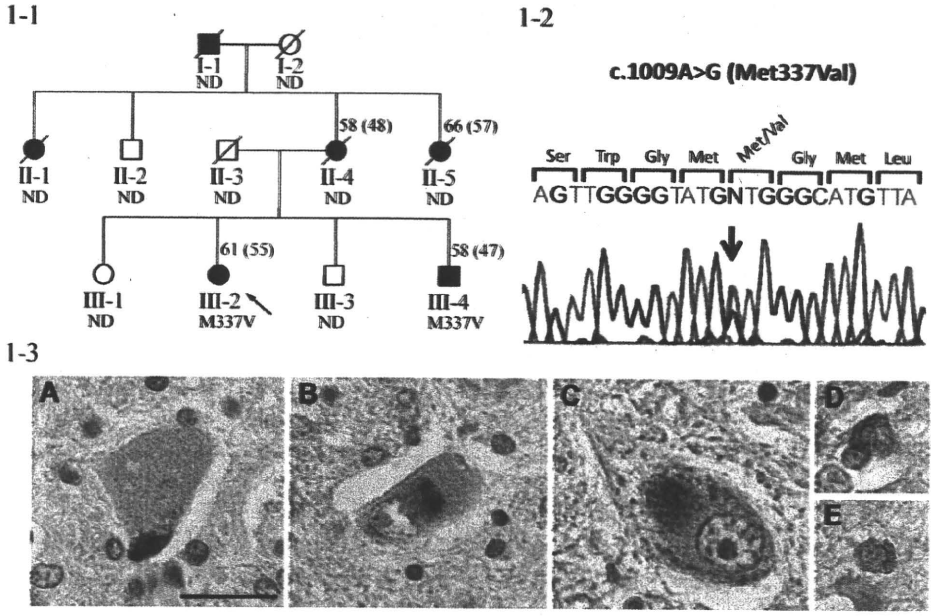

Figure 1. 1-1. Pedigree of the present family. Circles represent women and squares represent men. The slashed symbols indicate deceased subjects. Known affected persons are shown as filled symbols. The arrow represents the proband. Age at death or current age and age at disease onset in parenthesis are 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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Characterization of Inhibitor-Bound α-Synuclein Dimer: Role of α-Synuclein N-Terminal Region in Dimerization and Inhibitor Binding

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α-Synuclein is a major component of filamentous inclusions that are histological hallmarks of Parkinson's disease and other α-synucleinopathies. Previous analyses have revealed that several polyphenols inhibit α-synuclein assembly with low micromolar IC_{50} values, and that SDS-stable, noncytotoxic soluble α-synuclein oligomers are formed in their presence. Structural elucidation of inhibitor-bound α-synuclein oligomers is obviously required for the better understanding of the inhibitory mechanism. In order to characterize inhibitor-bound α-synucleins in detail, we have prepared α-synuclein dimers in the presence of polyphenol inhibitors, exifone, gossypetin, and dopamine, and purified the products. Peptide mapping and mass spectrometric analysis revealed that exifone-treated α-synuclein monomer and dimer were oxidized at all four methionine residues of α-synuclein. Immunoblot analysis and redox-cycling staining of endoproteinase Asp-N-digested products showed that the N-terminal region (1–60) is involved in the dimerization and exifone binding of α-synuclein. Ultra-high-field NMR analysis of inhibitor-bound α-synuclein dimers showed that the signals derived from the N-terminal region of α-synuclein exhibited line broadening, confirming that the N-terminal region is involved in inhibitor-induced dimerization. The C-terminal portion still predominantly exhibited the random-coil character observed in monomeric α-synuclein. We propose that the N-terminal region of α-synuclein plays a key role in the formation of α-synuclein assemblies.

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Abbreviations used: PD, Parkinson's disease; Exi-monomer, exifone-bound monomer; Exi-dimer, exifone-bound dimer; HSQC, heteronuclear single quantum coherence; NBT, nitroblue tetrazolium; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry.

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Introduction

Parkinson's disease (PD) and other α-synucleinopathies are progressive neurodegenerative diseases characterized by the selective loss of dopaminergic neurons and deposition of filamentous Lewy bodies, of which α-synuclein is the major component. Formation of amyloid fibrils and/or intermediate oligomers of α-synuclein is a complex process, and small-molecular inhibitors have been used to investigate the pathways involved. Conway *et al.* reported that catechol-containing compounds, including dopamine, inhibited the formation of α-synuclein fibrils, causing the accumulation of α-synuclein protofibrils.[1] It was also reported that α-synuclein fibrillization was inhibited by dopamine analogues, and α-synuclein oligomers were stabilized by these compounds.[2] We have previously reported that several polyphenols inhibited α-synuclein assembly with IC_{50} values in the low micromolar range, and that noncytotoxic, SDS-stable α-synuclein oligomers were formed in the presence of inhibitory compounds.[3]

Analyses of the interactions between small-molecular inhibitory compounds and α-synuclein have been reported by many groups, but the mechanisms involved remain controversial. It was proposed that amyloid fibril formation is inhibited by polyphenol compounds *via* noncovalent aromatic interactions with the amyloidogenic core.[4] A recent report showed that chemical aggregates inhibited amyloid formation of the yeast and mouse prion proteins in a manner characteristic of colloidal inhibition, suggesting a nonspecific mechanism.[5] Mutagenesis and competition studies with specific synthetic peptides suggested α-synuclein residues 125–129 (YEMPS) as an important region for dopamine-induced inhibition of α-synuclein fibrillization, and the inhibition was proposed to be due to conformational alterations of α-synuclein induced by noncovalent interaction with oxidized dopamine.[6] Molecular dynamics simulations suggest that dopamine binds to the YEMPS region, and the bound dopamine is further stabilized by long-range electrostatic interactions with E83 in the NAC region.[7] A recent NMR analysis indicated that a polyphenol compound, epigallocatechin gallate, noncovalently binds to the C-terminal region of α-synuclein (D119, S129, E130, and D135).[8] NMR analysis showed that A53T mutant α-synuclein, which is linked to autosomal dominant forms of PD, has a greater propensity to aggregate in the presence of dopamine, compared to wild-type α-synuclein.[9] Meanwhile, NMR characterization of the interaction between α-synuclein and various small molecules indicated that residues 3–18 and 38–51 act as noncovalent binding sites for inhibitory compounds.[10]

Covalent attachment of inhibitors to α-synuclein, on the other hand, has been proposed by several groups. Conway *et al.* suggested that 5–10% of dopamine was covalently incorporated into α-synuclein by radical coupling (dopamine-derived orthoquinone to Tyr) and/or nucleophilic attack (e.g., Lys forming a Schiff base with the orthoquinene).[1] Mass spectrometry (MS) and NMR characterization suggested that the oxidation product (quinones) of a dopamine analogue was covalently linked to the amino groups of the α-synuclein chain, thereby generating α-synuclein–quinone adducts.[2] Thus, the binding mode and binding site(s) of small-molecular inhibitors remain controversial.

The conformation of inhibitor-induced α-synuclein oligomers is also a matter of debate. Norris *et al.* reported that spherical oligomers of dopamine-modified α-synuclein take a predominantly random-coil structure with some β-pleated sheets on the basis of CD and Fourier-transform infrared spectroscopy studies.[6] Another group demonstrated that in the presence of small inhibitory molecules, α-synuclein is still dominated by random-coil character.[10] Ehrnhoefer *et al.* proposed that epigallocatechin prevented the conversion of monomeric α-synuclein into toxic on-pathway aggregation intermediates and resulted in the formation of unstructured, nontoxic α-synuclein oligomers that they considered to be off-pathway.[8] On the other hand, it has recently been reported that a flavonoid, baicalein, stabilized β-sheet-enriched oligomers based on CD and Fourier-transform infrared spectroscopy analysis.[11] The baicalein-stabilized oligomers were characterized as quite compact globular species based on small-angle X-ray scattering data and atomic force microscopy.

Masuda *et al.* isolated α-synuclein dimers formed in the presence of inhibitory compounds,[3] and the isolated soluble dimers were recently characterized using a panel of epitope-specific α-synuclein antibodies.[12] The reactivities of the antibodies indicated that the conformations of polyphenol-bound α-synuclein dimers differ from those of unbound monomers, but resemble those of amyloid fibrils, suggesting that inhibitor-bound molecular species are on-pathway intermediates.

This situation prompted us to carry out a comprehensive analysis of inhibitor-treated α-synucleins by means of NMR spectroscopy in conjunction with other biochemical methods, such as peptide mapping, immunoblotting, and redox-cycling staining. We have previously analyzed the antibody binding and site-specific phosphorylation of α-synuclein using ultra-high-field NMR spectroscopy.[13] For the structural characterization, we prepared and purified a ^{15}N-labeled α-synuclein dimer in the presence of polyphenol inhibitors on a milligram scale and analyzed it by ultra-high-field NMR spectroscopy recorded at a proton observation frequency of 920 MHz.

Results and Discussion

Isolation and characterization of inhibitor-bound α-synuclein dimer and monomer

SDS-stable, noncytotoxic α-synuclein oligomers were detected in the soluble fraction in the presence of inhibitory compounds such as polyphenols.[3] For detailed characterization of inhibitor-induced α-synuclein oligomers, we attempted to prepare exifone-, gossypetin-, and dopamine-induced α-synuclein dimer and monomer (for inhibitor structures, see **Fig. 1**) and to separate them by gel-filtration chromatography as described.[12] **Fig. 2a** shows the HPLC patterns of control and exifone-treated α-synucleins. The HPLC fractions of exifone-treated α-synuclein were analyzed by SDS-PAGE and Western blotting (**Fig. 2b**). The data indicate that the exifone-treated α-synuclein dimer (Exi-dimer) was successfully purified by gel-filtration chromatography. The homogeneity of inhibitor-induced monomer and dimer was also checked by diffusion NMR experiments (data not shown). α-Synuclein monomer and dimer treated with exifone, as well as control monomer (without inhibitor), were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS measurements (**Fig. 2c**). α-Synuclein monomer (control) showed a major signal at 14,460 Da, which matched the predicted mass (14,460 Da). On the other hand, exifone-treated monomer (Exi-monomer) gave a major signal at 14,524 Da, which corresponded to that of α-synuclein plus 64 Da. Exifone-bound α-synuclein (molecular mass of exifone, 278.2 Da) was not detected, presumably because exifone binding was noncovalent. The MS spectrum of Exi-dimer showed a broad peak at around 30 kDa and 15 kDa, and we could not obtain an accurate molecular mass. The peak at 15 kDa might be the doubly charged ion of the Exi-dimer and/or the monomer released from the Exi-dimer in the ionization process. To estimate the ratio of exifone bound to α-synuclein dimer and monomer, absorption of exifone at 385 nm was measured for Exi-dimer and Exi-monomer. The results indicate that Exi-dimer contains around 3

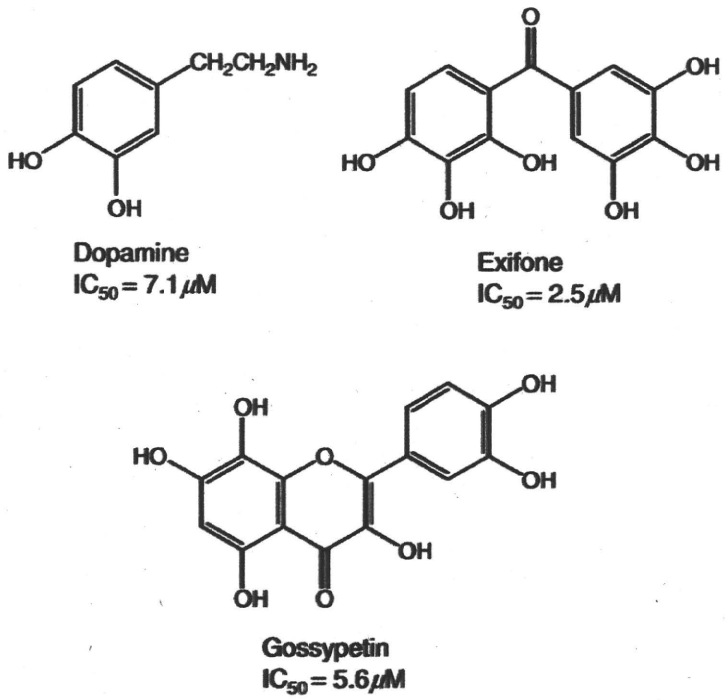

Fig. 1. Chemical structures of dopamine, exifone, and gossypetin with their IC_{50} values for inhibition of α-synuclein filament assembly.[3]