Research article

Spatial patterns of phosphorylation-dependent TDP-43-immunoreactive neuronal cytoplasmic inclusions (NCI) in frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP)

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Abstract

The transactive response (TAR) DNA-binding protein of 43kDa (TDP-43) is an RNA binding protein encoded by the *TARDPB* gene. Abnormal aggregations of TDP-43 in neurons in the form of neuronal cytoplasmic inclusions (NCI) are the pathological hallmark of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). To investigate the role of TDP-43 in FTLD-TDP, the spatial patterns of the NCI were studied in frontal and temporal cortex of FTLD-TDP cases using a phosphorylation dependent anti-TDP-43 antibody (pTDP-43). In many regions, the NCI formed clusters and the clusters were distributed regularly parallel to the tissue boundary. In about 35% of cortical regions, cluster size of the NCI was within the size range of the modular columns of the cortex. The spatial patterns of the pTDP-immunoreactive inclusions were similar to those revealed by a phosphorylation-independent anti-TDP-43 antibody and also similar to inclusions characterized by other molecular pathologies such as tau, a-synuclein and 'fused in sarcoma' (FUS). In conclusion, the data suggest degeneration of cortical and hippocampal anatomical pathways associated with accumulation of cellular pTDP-43 is characteristic of FTLD-TDP. In addition, the data are consistent with the hypothesis of cell to cell transfer of pTDP-43 within the brain.

Introduction

The transactive response (TAR) DNA-binding protein of 43kDa (TDP-43) is an RNA binding protein encoded by the highly conserved *TARDPB* gene. TDP proteins have a glycine-rich domain and are believed to carry out essential cellular functions including the regulation of transcription, alternate splicing, and acting as a framework for nuclear bodies (Wang *et al*. 2004).

Recent studies suggest a significant role for TDP-43 in neurodegenerative disease. Hence, TDP-43 is a major pathological protein in frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) (Cairns *et al*. 2007a), a disorder that accounts for approximately 10% of early-onset dementias. FTLD-TDP is characterized by a widespread atrophy of the brain largely affecting the frontal, temporal, and parietal lobes. In addition, there is the presence of abnormal aggregates of TDP-43 protein within the cytoplasm of neurons (Cairns *et al*. 2007b) termed neuronal cytoplasmic inclusions (NCI).

Various genetic defects have been identified in FTLD-TDP cases, the majority being caused by mutation of the *progranulin* (*GRN*) gene (FTLD-TDP-GRN) (Baker *et al*. 2006, Behrens *et al*. 2007, Cruts *et al*. 2006, Mukerjee *et al*. 2006, Rademakers & Hutton 2007). A less prevalent disorder, FTLD with *valosin-containing protein* (*VCP*) gene mutation (Forman *et al*. 2006) has TDP-43-immunoreactive inclusions, and familial cases have also been shown to be caused by *ubiquitin-associated binding protein 1* (*UBAP1)* (Rollinson *et al.* 2009) and the *chromosome-9 open reading frame 72* (*C90RF72*) gene (Luty *et al*. 2008, Renton *et al*. 2011).

NCI characterized by various molecular pathologies are a common feature of neurodegenerative disease (Goedert *et al*. 2001). Most disorders are associated with abnormal cellular aggregations of tau (tauopathies), a-synuclein (synucleinopathies), TDP-43, or 'fused in sarcoma' (FUS) proteins (Armstrong *et al*. 2011). In the cerebral cortex of many disorders, the various molecular types of NCI have a distinct spatial pattern, i.e., they occur in clusters which exhibit a regular periodicity parallel to the pia mater (Armstrong *et al*. 1997, 1998, 1999, 2004, 2011). This spatial pattern suggests that the NCI develop in relation to clusters of cells associated with the columnar structure of

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the cortex and specifically, the anatomical projections which connect different cortical gyri (cortico-cortical projections) and the cortex with the hippocampus (cortico-hippocampal projections). In previous studies, the spatial patterns of the NCI have been studied using a phosphorylation independent anti-TDP-43 antibody (Armstrong *et al.* 2010). However in FTLD-TDP, TDP-43 is redistributed from the nucleus to the cytoplasm, is ubiquinated, hyperphosphorylated, and then cleaved to generate C-terminal fragments (Neumann *et al*. 2007). Hence, the objective of the present study was to determine in a larger series of FTLD-TDP cases whether the phosphorylated TDP-43-immunoreactive NCI (pTDP-43) exhibit a similar spatial pattern to that revealed by anti-TDP-43.

Materials and Methods

Cases

Thirty-two clinically and neuropathologically verified cases of FTLD-TDP (16 male, 16 female) (see Table 1) were obtained from the Knight Alzheimer's Disease Research Center, Department of Neurology, Washington University School of Medicine, St. Louis, MO, USA. All cases exhibited FTLD with neuronal loss, varying degrees of microvacuolation of the superficial cortical laminae, and a reactive astrocytosis consistent with proposed diagnostic criteria for FTLD-TDP (Cairns *et al.* 2007b). Of the 32 cases, 20 were identified as familial (at least one or more first degree relatives affected) and of these, 10 cases were identified as having *GRN* mutations (Baker *et al.* 2006, Behrens *et al.* 2007, Cruts *et al.* 2006, Muckerjee *et al.* 2006,), one had a *VCP* gene mutation and one case was associated with *ubiquitin-associated binding protein 1* (*UBAP1)* (Rollinson *et al*. 2009). The majority (N = 7) of the *GRN* cases come from a single hereditary dysphasic disinhibition dementia (HDDD) family (HDDD2) (Mukherjee *et al.* 2006) and the remainder (N = 3) from the HDDD1 family (Behrens *et al.* 2007). No genetic defects have been identified to date in the remaining familial cases (N = 8) and none of these had a strong autosomal dominant pattern of inheritance.

Histological methods

After death, the consent of the next-of-kin was obtained for brain removal, following local Ethical Committee procedures and the 1995 Declaration of Helsinki (as modified in Edinburgh, 2000). Tissue blocks were taken from the frontal lobe at

Table 1. Summary of demographic features, gross brain weight, and familial status of the 32 cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Abbreviations: M/F = male/female, Fm/S = Familial/Sporadic, - indicates data not available, *GRN* = cases caused by *progranulin* gene mutations, *VCP* = case caused by *valosin-containing protein, UBAP1* = case caused by *ubiquitin-associated binding protein 1*

Case	M/F	Onset (years)	Duration (years)	Age (years)	BW (gm)	Fm/S
1.	M	57	8	65	960	S
2.	F	72	12	84	900	S
3.	F	61	16	77	950	S
4.	F	68	6	74	975	*GRN*
5.	M	52	13	65	1300	*GRN*
6.	M	66	16	82	-	S
7.	F	69	15	84	970	*GRN*
8.	M	60	6	66	-	*GRN*
9.	F	65	12	77	810	*GRN*
10.	M	52	15	67	960	*GRN*
11.	M	74	6	80	1270	Fm
12.	F	-	-	67	990	S
13.	F	59	9	68	650	Fm
14.	M	74	1	75	1360	S
15.	M	60	11	71	1450	Fm
16.	M	43	7	50	1060	Fm
17.	M	55	11	66	1005	*GRN*
18.	F	63	3	66	950	S
19.	F	58	9	67	880	*GRN*
20.	F	64	19	83	720	Fm
21.	F	69	4	71	1070	S
22.	M	38	9	47	1185	*VCP*
23.	F	-	-	73	720	S
24.	M	50	18	68	1170	S
25.	M	58	8	66	1080	Fm
26.	F	65	13	78	960	Fm
27.	M	57	6	63	1080	*GRN*
28.	M	51	11	62	880	*UBAP1*
29.	F	71	13	84	960	S
30.	F	73	9	82	-	*GRN*
31.	F	58	8	66	-	Fm
32.	M	71	8	79	1150	*GRN*

the level of the genu of the corpus callosum to study the middle frontal gyrus (MFG) (B32) and the temporal lobe at the level of the lateral geniculate nucleus to study the superior temporal gyrus (STG) (B20), parahippocampal gyrus (PHG) (B28), CA1/2 sectors of the hippocampus, and dentate gyrus (DG). Tissue was fixed in 10% phosphate buffered formal-saline and embedded in paraffin wax. Immunohistochemistry (IHC) was performed on 4 to 10μm sections with a mouse monoclonal antibody that specifically recognizes phosphorylated pTDP-43 (dilution 1:40,000; pS409/410-1, Clone 11-9, Cosmo Bio USA, Inc., Carlsbad, CA, USA). Sections were counterstained with haematoxylin.

Morphometric methods

NCI (Figure 1) were counted along strips of tissue (1,600 to 3,200 μm in length) located parallel to the pia mater in 250 x 50 μm sample fields arranged contiguously as reported previously (Armstrong 2003). The sample fields were located both in the upper (laminae II/III) and lower (laminae V/VI) cortex, the short edge of the sample field being orientated parallel with the pia mater and aligned with guidelines marked on the slide. In the hippocampus, the features were counted in the cornu ammonis (CA) sectors CA1 and CA2, the short dimension of the contiguous sample field being aligned with the alveus. In addition, NCI have been observed in the dentate gyrus (DG) in FTLD-TDP (Kovari *et al.* 2004, Mackenzie *et al.* 2006, Woulfe *et al.* 2001) and the sample field was aligned with the upper edge of the granule cell layer. The NCI were identified as round, spicular, or skein-like inclusions (Davidson *et al.* 2007, Yaguchi *et al.* 2004).

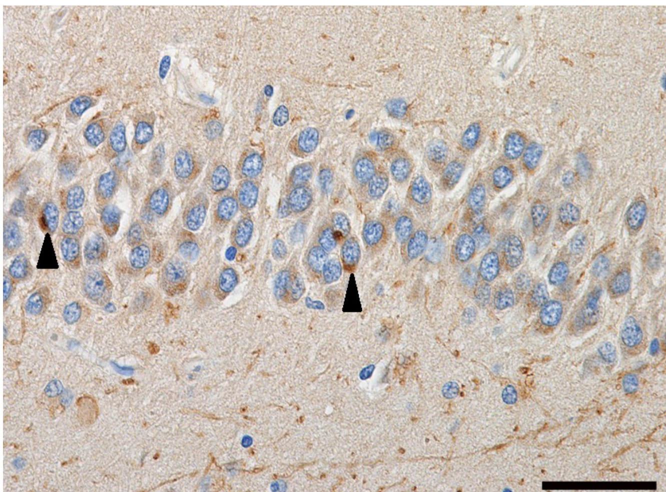

Figure 1. Neuronal cytoplasmic inclusions (NCI) (arrows) comprising aggregations of phosphorylated TDP-43 (pTDP-43) in the dentate gyrus (DG) granule cell layer in frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) (anti-pTDP-43 immunohistochemistry, haematoxylin, bar = 30μm).

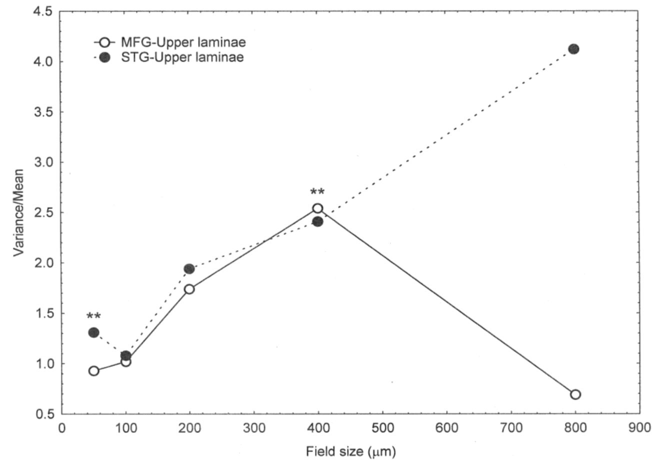

Figure 2. Examples of the spatial patterns exhibited by pTDP-43-immunoreactive neuronal cytoplasmic inclusions (NCI) in frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) (MFG = Middle frontal gyrus, STG = Superior temporal gyrus, ** significant Variance/Mean peaks).

Data analysis

To determine the spatial patterns of the NCI, the data were analysed by spatial pattern analysis described previously (Armstrong 1993a, 1997; 2006). This method uses the variance-mean ratio (V/M) to determine whether the NCI were distributed randomly (V/M = 1), regularly (V/M < 1), or were clustered (V/M > 1) along a strip of tissue. V/M was plotted against various field sizes, e.g., 50 x 250 μm, 100 x 250 μm, 200 x 250 μm etc., to determine whether the clusters of NCI were regularly or randomly distributed and to estimate the mean cluster size. A V/M peak indicates the presence of regularly spaced clusters while an increase in V/M to an asymptotic level suggests the presence of randomly distributed clusters. The statistical significance of a peak was tested using the 't' distribution (Armstrong 1997).

Cluster sizes of the NCI in different brain regions were compared using analysis of variance (ANOVA). First, upper laminae data of the cortical regions (MFG, ITG, PHG) were compared with CA1/2 sectors of the hippocampus and DG using one-way ANOVA. If significant differences were observed between regions then 'Fisher's protected least significant difference' was used as a *post-hoc* test. Second, a similar analysis was carried out but substituting the lower cortical laminae data. Third, the data as a whole were analysed using a two-factor ANOVA to determine whether brain region, cortical lamina, or interaction between the two factors affected cluster size.

Table 2. Summary of spatial patterns exhibited by the pTDP-43-immunoreactive neuronal cytoplasmic inclusions (NCI) in various brain regions lobe (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus, PHG = Parahippocampal gyrus, CA1/2 = Sectors CA/2 of the hippocampus, DG = Dentate gyrus), U = Upper cortex, L = lower cortex) in cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Column 6 indicates the proportion of analyses in which the cluster size of NCI in cortical regions was within the range 400 – 800 mm. (N = number of regions studied, R = Random distribution, Reg = Regular distribution).

Region	N	R	Reg	Regular Clusters	% (400-800μm)	Large cluster (>800 μm)
MFG-U	27	8	3	13	50	3
MFG-L	25	3	7	11	33	4
ITG-U	26	7	1	13	35	5
ITG-L	16	8	1	6	14	1
PHG-U	22	4	5	10	38	3
PHG-L	16	5	0	8	27	3
CA1/2	18	5	3	8	-	2
DG	25	4	5	15	-	1

Chi-square (c^2) contingency table analysis: Between brain regions; c^2 = 25.79 (21DF, P > 0.05)

Results

An example of the typical appearance of pTDP-43-immunoreactive NCI in the granule cell layer of the DG is shown in Figure 1. Statistical analysis is often necessary to reveal the spatial patterns of protein inclusions in the tissue. However, two NCI are present in the centre of the section and a single NCI some distance to its left.

The spatial patterns shown by the pTDP-43 immunoreactive NCI are shown in Figure 2. In the upper laminae of the MFG, there was a V/M peak at a field size of 400 μm, suggesting clusters of NCI of mean dimension 400 μm regularly distributed parallel to the pia mater. Similarly, in the upper laminae of the STG, there was a V/M peak at 50 μm suggesting smaller clusters of regularly distributed NCI.

A summary of the spatial patterns exhibited by the NCI in all cases and regions is shown in Table 2. The commonest spatial pattern exhibited by the NCI was clustering with the clusters regularly distributed parallel to the tissue boundary and evident in 84/175 (48%) of brain regions analysed. In about 35% of cortical regions, cluster size of the NCI was within the range of size of the modular columns which form the cells of origin of the cortico-cortical projections. NCI were also randomly distributed in a total of 44/176 (25%). Clustering on a larger scale (clusters > 800 μm) and a more uniform distribution of NCI relative to the tissue boundary were relatively uncommon. NCI exhibited a similar range of spatial patterns between brain regions and in different cases of FTLD-TDP.

A comparison of cluster sizes of the NCI between brain regions is shown in Figure 3. Mean cluster sizes in all regions were <350 mm, the SE indicating significant variation between cases. A one-way ANOVA of the data comparing upper laminae of the cortical regions studied with sectors CA1/2 and the DG suggested no significant differences in mean cluster sizes of NCI (F = 1.33, P > 0.05). Similarly, when lower laminae data were analysed, there were no significant differences between regions (F = 0.23, P > 0.05). However, the two-factor ANOVA suggested significant differences in cluster size between laminae (F = 6.30, P < 0.05), with larger clusters of NCI in the upper cortical lamiane, a pattern consistent in all cortical areas studied (Lamina x Region interaction, F = 0.27, P > 0.05).

Discussion

The data suggest that in the majority of regions the NCI occurred in clusters that exhibit a regular pattern parallel to the tissue boundary. This spatial pattern is similar to that previously reported using a phosphorylation-independent antibody (Armstrong 2011). The advantage of pTDP-43 antibodies is that they do not label normal physiological TDP-43 (Armstrong *et al*. 2011, Hasegawa *et al*. 2008, Neumann *et al*. 2009, Olive *et al*. 2009, Schwab *et al*. 2009,) thus enabling the TDP-43-immunoreactive inclusions to be more clearly visualized and quantified. In addition, the spatial pattern of the NCI is similar to that reported for different molecular types of cellular inclusion characterized by tau (Armstrong *et al*. 1998, 1999), a-synuclein (Armstrong *et al*. 1997, 2004), and FUS immunoreactivity (Armstrong *et al*. 2011).

A major feature of the anatomical structure of the cerebral cortex is the replicated local neural circuit (represented by ‘columns’ or ‘modules’) (Hiorns *et al*.

1991). The diameter of individual cortical modules varies between 500-1000 µm depending on region and there are specific connections maintained between ordered sets of columns (Hiorns *et al.* 1991). In disorders characterized by tau, a-synuclein, and FUS immunoreactivity, the spatial patterns of the NCI clusters within the cerebral cortex and hippocampus suggested that the inclusions were related to this modular structure, most specifically, the cells of origin of specific cortico-cortical and cortico-hippocampal projections (Delacoste & White 1993, Delatour *et al.* 2004, Hiorns *et al.* 1991). First, the cells of origin of the cortico-cortical projections are themselves clustered and occur in bands that are more or less regularly distributed along the cortical strip. Second, individual bands of cells, approximately 500-800 µm in width traverse the cortical laminae in columns (Hiorns *et al.* 1991). In approximately 44 -66% of cortical regions studied, the clusters of NCI were regularly distributed parallel to the pia mater consistent with an association with these connections. However, in only a proportion of cortical areas (approx. 35%) did the estimated width of the NCI clusters approximate to the dimension of the cells of origin of the cortico-cortical projections. In the majority of cortical areas, the NCI developed in smaller clusters, usually between 50 and 200 µm in diameter, a size similar to the FUS-immunoreactive NCI in neuronal intermediate filament inclusion disease (NIFID) (Armstrong *et al.* 2011). Hence, NCI may affect only a subset of cells within a cortical column and therefore are unlikely to completely explain neurodegeneration in FTLD-TDP. In some regions, however, clusters of NCI larger than 800 µm in diameter were present suggesting that smaller clusters of inclusions could evolve into larger aggregations (Armstrong 1993b). Hence, there may be an increasing burden of pTDP-43 pathology affecting the cortical columns as the disease progresses.

The data suggest degeneration of specific cortico-cortical and cortico-hippocampal anatomical pathways associated with accumulation of cellular pTDP-43 is characteristic of familial and sporadic FTLD-TDP. Several different frame-shift and premature termination mutations have been identified in FTLD-TDP with *GRN* mutation (Beck *et al.* 2008). Abnormal protein products may accumulate within the endoplasmic reticulum of the cell due to inefficient secretion or mutant RNA may have a lower expression within the cell at least in some mutants (Mukherjee *et al.* 2006). TDP-43 is a nuclear protein but in FTLD-TDP, TDP-43 is redistributed from the nucleus to the cytoplasm, is ubiquinated, hyperphosphorylated, and then cleaved to generate C-terminal fragments (Neumann *et al.* 2007). These fragments may then accumulate to form the NCI

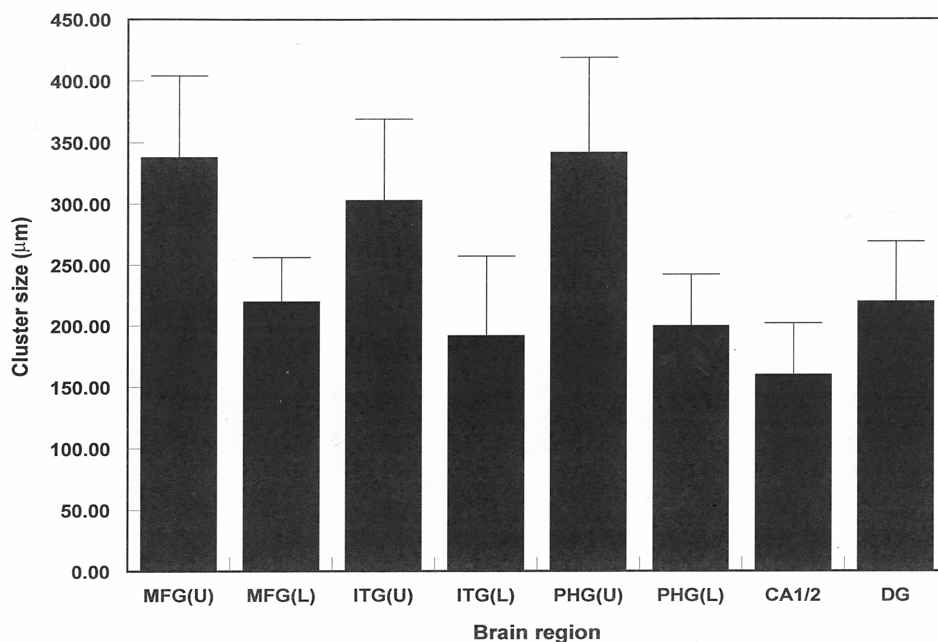

Figure 3. Mean cluster sizes (with SE of the mean) of the neuronal cytoplasmic inclusions (NCI) in frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) in different brain regions lobe (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus, PHG = Parahippocampal gyrus, CA1/2 = Sectors CA/2 of the hippocampus, DG = Dentate gyrus), U = Upper cortex, L = lower cortex). Analysis of variance (ANOVA): One way (upper cortical data, CA1/2, DG) F = 1.33 (P > 0.05), One way (lower cortical data, CA1/2, DG) F = 0.23 (P > 0.05); Two way between regions F = 0.26 (P > 0.05), Between upper and lower cortex F = 6.30 (P< 0.05), Interaction F = 0.27 (P >0.05)

and cause cell death. Similar mechanisms may be involved in FTLD-TDP associated with other types of genetic defect whereas in sporadic cases, the molecular mechanism responsible for TDP-43 accumulation (sFTLD-TDP) remains to be established.

The spatial patterns of the pTDP-43-immunoreactive inclusions in FTLD-TDP could reflect cell to cell transfer of pathogenic proteins as postulated in other neurodegenerative disorders (Steiner *et al.* 2011). Proteins such as tau and a-synuclein can exit host cells, transfer between cells, gain access to new cells, and create pathology within these cells (Steiner *et al.* 2011). For example, a-synuclein taken up from the extracellular space can induce aggregation of other a-synuclein proteins in recipient cells. By analogy with the scrapie form of prion protein (PrP^{sc}), nucleation or seeding activity of a-synuclein may result in a core of an NCI of transferred a-synuclein surrounded by additional layers of cytoplasmic a-synuclein contributed by the host cell. It is possible that pTDP-43 has these properties and therefore, that cell to cell transfer of pTDP-43 may be a mechanism common to all forms of FTLD-TDP. The hypothesis of cell to cell transfer of pTDP-43 makes two predictions regarding the distribution and abundance of the NCI: (1) there should be a distinct spatial pattern of the NCI in brain regions affected by the disease and (2) an increasing burden of the pathology as it spreads from region to region. The observed spatial patterns of the pTDP-43 NCI are consistent with both these predictions.

In conclusion, the spatial pattern of the pTDP-immunoreactive NCI in FTLD-TDP is similar to that reported for cellular inclusions characterized by tau, a-synuclein, and FUS immunoreactivity and also similar to that reported using a phosphorylation independent TDP-43 antibody (Armstrong 2011). In addition, cell to cell transfer of pTDP-43 could be involved in the pathogenesis of FTLD-TDP.

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