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Caspase-Cleaved TAR DNA-Binding Protein-43 in Parkinson’s Disease and Dementia with Lewy Bodies

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Abstract -

Background: TAR DNA-binding protein-43 (TDP-43) proteinopathies are classified based upon the extent of modified TDP-43 and include a growing number of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration with ubiquitin immunoreactive, tau-negative inclusions (FTLD-U) and FTLD with motor neuron disease (FTLD-MND).

Objective: The purpose of the study was to examine whether proteolytic modifications of TDP-43 are a relevant finding in Parkinson’s disease (PD) and dementia with Lewy Bodies (DLB).

Methods: A novel site-directed caspase-cleavage antibody, termed TDP caspase-cleavage product antibody (TDPccp), was utilized based upon a known caspase-3 cleavage consensus site within TDP-43 at position 219.

Results: Application of this antibody to postmortem brain sections from PD and DLB revealed the presence of caspase-cleaved TDP-43 in Lewy bodies and Hirano bodies in all cases examined. Co-localization of TDPccp with an antibody to alpha-synuclein (α-Syn), which served as a general marker for Lewy bodies, was evident within the substantia nigra in both alpha-synucleinopathies. Interestingly, the TDPccp antibody detected a greater number of Lewy bodies in PD and DLB compared to the α-Syn antibody. In addition, a semi-quantitative analysis in both diseases confirmed this finding by indicating that the percent of caspase-cleaved TDP-43 single-labeled Lewy bodies was approximately twice the percent that of α-Syn labeling (in DLB 13.4% vs. 5.5%, while in PD 34.6% vs. 17.6%, respectively).

Conclusion: Collectively, these data have identified caspase-cleaved TDP-43 as a primary component of Lewy and Hirano bodies in PD and DLB, and suggest the TDPccp antibody is an effective marker for the detection of Lewy bodies in these neurodegenerative diseases.
**Introduction**

TAR DNA binding protein-43 (TDP-43) is a highly conserved 414 amino acid protein with an apparent molecular weight of approximately 43 kDa. It is ubiquitously expressed and appears to play a role in regulating RNA transcription and alternative splicing [1]. Findings from a recent study have also linked TDP-43 function to cytoskeletal stability and axonal transport by showing that TDP-43 regulates human neurofilament (hNFL) RNA stability [2].

TDP-43 has been identified as a major component of ubiquitinated tau-negative inclusions in sporadic and familial FTLD-U and ALS [3]. A conspicuous finding in these studies was the presence of 25 and 35 kDa truncated fragments of TDP-43 in brain extracts from affected individuals that were not present in control subjects [3]. For this common pathology, these diseases were grouped together as a new entity of neurodegenerative disorders, classified as TDP-43 proteinopathies [4]. In addition, it has been recently reported that TDP-43 positive inclusions occur in other neurodegenerative disorders including brains of patients with argyrophilic grain disease, Alzheimer’s disease (AD), Lewy body related diseases, Pick’s disease and Huntington’s disease [5-11]. Current understanding suggests modifications to TDP-43 including hyperphosphorylation and proteolytic cleavage by caspases lead to a toxic gain of function. In particular, truncated TDP-43 redistributes from the nucleus to the cytoplasm [12] and this may promote cellular dysfunction by causing altered trafficking of the protein [13]. Therefore, post-translational proteolytic processing of TDP-43 by caspases may be a key step in protein misfolding and aggregation of TDP-43 [13, 14].

In a recent report, Zhang et al. showed that the ectopic expression of a ~25-kDa TDP-43 fragment corresponding to the C-terminal truncation product of caspase-cleaved TDP-43 leads to the formation of toxic, insoluble, and ubiquitin-positive cytoplasmic inclusions within human cell lines. In addition, by generating a conformation-dependent antibody that detects C-terminal fragments, caspase-cleaved TDP-43 was identified in postmortem human brain sections in FTLD-U and ALS [12].

We recently developed a site-directed caspase-cleavage antibody to TDP-43, termed TDPccp, and identified caspase-cleaved TDP-43 in several tauopathies including AD and Pick’s disease [7, 11]. Specifically, caspase-cleaved TDPccp was identified within Hirano bodies in CA1 region of the hippocampus in AD and Pick’s disease suggesting this might be a common feature of tauopathies [7, 11]. These findings support the conclusion that the presence of TDP-43 pathology is not solely restricted to TDP-43 proteinopathies, but may be more widely distributed in a number of neurodegenerative diseases [13]. The purpose of the present study was to determine a possible role for caspase-cleaved TDP-43 in PD and DLB, neurodegenerative disorders classified as alpha-synucleinopathies.

PD and DLB are clinically characterized by progressive dementia and/or motor syndromes and exhibit widespread neuronal cell loss. In PD, patients develop extrapyramidal movement disturbances [15] and the diagnosis is based on presence of two of the three following clinical features: bradykinesia, resting tremor and rigidity [16]. The pathological hallmark of idiopathic PD is loss of dopaminergic neurons from the substantia nigra [15]. In DLB, several groups have recognized distinctive clinical features including impairment of attention, problem solving, and visuospatial skills associated with loss of neurons from the cortex [17, 18]. Microscopically, in PD and DLB cell loss is associated with the presence of Lewy body inclusions that are comprised principally of aggregated alpha-synuclein (α-Syn) [19].

In the present study, application of our site-directed caspase-cleavage antibody to TDP-43 in postmortem brain sections from PD and DLB revealed the presence of caspase-cleaved TDP-43 in Lewy bodies of all cases examined. Co-localization of TDPccp with an antibody to α-Syn was evident within the substantia nigra in both alpha-synucleinopathies. Interestingly, the TDPccp antibody detected on average roughly twice the number of Lewy bodies compared to the α-Syn antibody. Collectively, these data have identified caspase-cleaved TDP-43 as a primary component of Lewy bodies in PD and DLB, and suggest the TDPccp antibody is an effective marker for the detection of Lewy bodies.
Materials and Methods –

Antibodies

The monoclonal mouse α-Syn antibody (clone LB509) was purchased from Zymed (San Francisco, CA). The caspase-cleavage product antibody to TDP-43 (TDPPcp) was an in house antibody that has previously been shown to be a specific marker for caspase-cleaved TDP-43 [7]. This antibody was synthesized based upon a putative caspase cleavage consensus site (DVMD^{219}) within TDP-43.

Human subjects

Autopsy brain tissue from five neuropathologically confirmed Parkinson’s cases and five confirmed dementia with Lewy bodies cases was studied. Human brain tissues used in this study were provided by the Institute for Memory Impairments and Neurological Disorders at the University of California, Irvine.

Immunohistochemistry and immunofluorescence microscopy

For immunohistochemical and immunofluorescence studies, free-floating 40 µm-thick serial sections from PD and DLB subjects were used as previously described [20, 21]. Antibody dilutions were 1:100 for TDPPcp and 1:500 for mAb α-Syn. For single labeling, antigen visualization was accomplished using ABC complex (ABC Elite immunoperoxidase kit, Vector labs), followed by brown/red DAB substrate (Vector Labs). For immunofluorescence co-localization studies, antigen visualization was accomplished using an Alexa fluor 488-labeled tyramide (green, Ex/Em = 495/519) for the first label and streptavidin Alexa Fluor 555 (red, Ex/Em = 555/565) for the second label, both purchased from Invitrogen (Carlsbad, CA).

Western blot analysis

Human brain lysates from DLB and age-matched control subjects were processed for Western blot analysis as previously described [21]. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. To verify equal loading between samples, transferred slabs were stained in coomassie blue. Membranes were incubated in TDPPcp and primary antibody was visualized with a goat anti-rabbit HRP-linked secondary antibody (1:5,000; Jackson’s Laboratory, West Grove, PA), followed by ECL detection. Protein content from all the samples was analyzed using the BCA assay (Pierce) to ensure equal protein loading. In addition, Western blot analysis was performed utilizing a beta-actin antibody (1: 400) as a loading control.

Quantification and statistical analysis

A semi-quantitative analysis was performed by first taking 20X immunofluorescence, overlapping images (labeled with both TDPPcp and α-Syn) from the substantia nigra area in five separate Parkinson’s and five DLB cases. Photographs were then analyzed by counting the number of Lewy bodies and calculating the percent of inclusion bodies labeled for either α-Syn or TDPPcp antibodies in the entire tissue section for each case. Data were than averaged ±S.E.M. Additionally, α-Syn labeling was utilized as a general marker for Lewy bodies and compared with the number of α-Syn-labeled Lewy bodies that co-localized with TDPPcp. Statistical difference between percentage of α-Syn single-labeled, TDPPcp single-labeled and α-Syn-TDPPcp double-labeled Lewy Bodies was analyzed by 1-way ANOVA (GLM procedure with contrast statement) with statistical significance established at a p-value < 0.05. Data were normally distributed with no significant difference in variances across the antibody classes. The calculations were performed using a SAS program version 9.1.3 Service Pack 3 for Windows XP-Pro.

Results –

A critical feature of TDP-43 proteinopathies is the presence of cytoplasmic C-terminal fragments of TDP-43 (~25 and 35 kDa) that have ability to aggregate [22]. One candidate protease that may be involved in the proteolytic processing of TDP-43 is caspase-3. Zhang et al. have shown in vitro that TDP-43 is a substrate for caspase-3.
leading to the generation of 25 and 35 kDa species [23]. The goal of the present study was to examine whether caspase-cleaved TDP-43 is a relevant finding in the PD and DLB brain.

Five confirmed cases of PD and DLB were examined by immunohistochemistry utilizing our site-directed caspase-cleaved antibody to TDP-43, termed TDPccp. As an initial approach, immunoreactivity to TDPccp was assessed in two different brain regions including the substantia nigra and hippocampus. Application of the TDPccp antibody using bright-field microscopy revealed caspase-cleaved TDP-43 labeling in the SN region within Lewy bodies (arrowheads, Fig. 1, A and B). In hippocampal sections, caspase-cleaved TDP-43 was found in Lewy bodies and within Hirano bodies in both diseases (arrows, Fig. 1, C and D). To confirm the immunohistochemical results, Western blot analysis was carried out using the TDPccp antibody. The results confirmed the presence of the predicted caspase-cleaved fragment of TDP-43 (25 kDa) in all four DLB cases examined (Fig. 2). As shown, the antibody also recognized a fainter band in DLB subjects running at 35 kDa. This may represent an additional caspase-cleaved fragment of TDP-43 as a previous study has shown that caspase cleavage of TDP-43 results in the generation of both 35 kDa and 25 kDa fragments [12]. There was no immunoreactivity observed at 43 kDa corresponding to full-length TDP-43 in any of the samples analyzed (control or DLB), suggesting a lack of immunoreactivity of TDPccp to full-length TDP-43.

A common pathological feature of PD and DLB is the presence of α-Syn, and numerous studies employ the use of a full-length antibody to α-Syn as a general marker for Lewy bodies. We examined the extent of co-localization of TDPccp with a well-characterized full-length antibody to α-Syn (LB509, Zymed Labs). Immunofluorescence double-labeling experiments indicated a colocalization of both antibodies within Lewy bodies in PD and DLB (Fig. 3). Of interest was the relative uniform distribution of α-Syn and TDPccp labeling within Lewy bodies in PD, while in DLB both α-Syn and the TDPccp labeling appeared to be more peripherally distributed. These findings confirmed the presence of caspase-cleaved TDP-43 within Lewy bodies in PD and DLB. Although co-localization was evident within the SN of both diseases, single-labeling of Lewy bodies with either antibody was also evident (Fig. 4). Moreover, it appeared more Lewy bodies were singly labeled with TDPccp than with the α-Syn antibody (Fig. 4). Quantification experiments indicated that the majority of Lewy bodies identified within SN brain sections were labeled with both TDPccp and the α-Syn antibody (blue bars, Fig. 5). However, quantification of the number Lewy bodies labeled by α-Syn and TDPccp antibodies in PD and DLB cases revealed the average percent of caspase-cleaved TDP-43 single-labeled Lewy bodies was approximately twice the percent that of α-Syn labeling in both alpha-synucleinopathies (Fig. 5A: 13.4% vs. 5.5% in DLB, Fig. 5B: 34.6% vs. 17.6% in PD). Statistical differences between the percentages of α-Syn single-labeled, TDPccp single-labeled and α-Syn-TDPccp double-labeled Lewy Bodies was analyzed by 1-way ANOVA (GLM procedure with contrast statement) with statistical significance established at a p-value < 0.05. Data were normally distributed with no significant difference in variances across the antibody classes. For DLB, the overall F_{2, 12} was 284.07 with the p-value* < 0.05 and for PD, the overall F_{2, 12} was 26.41 with the p-value* < 0.05.

**Discussion**

TDP-43 has been identified as a major component of ubiquitinated tau-negative inclusions in sporadic FTLD-U and ALS [3]. For this common pathology, FTLD-U and ALS are now grouped together as a new entity of neurodegenerative disorders, classified as TDP-43 proteinopathies [24]. Recent advances have suggested a key event that may promote disease progression in TDP-43 proteinopathies is the redistribution of truncated forms of TDP-43 from the nucleus to the cytoplasm [25]. In support of this hypothesis are data demonstrating the presence of smaller (~25 kDa) phosphorylated fragments of TDP-43 in brain samples from both FTLD-U and ALS [26]. These findings suggest that post-translational modification of TDP-43 may signal a shift to a toxic gain of function. One candidate protease that may be involved in the proteolytic processing of TDP-43 is caspase-3. Zhang et al. have shown that TDP-43 is a substrate for caspase-3 leading to the generation of 25 and 35 kDa species [23]. Our lab has also supported the hypothesis that caspases may play a major role in the processing of TDP-43 in other neurodegenerative disorders: utilizing a site-directed caspase-cleavage antibody to a known consensus cleavage site within TDP-43 (DVMD^{219}), we identified caspase-cleaved TDP-43 in two tauopathies, AD and Pick’s disease [7, 11]. Specifically, caspase-cleaved TDP-43 was identified within Hirano bodies in CA1 region of the hippocampus in AD and Pick’s disease.
These findings support the conclusion that the presence of caspase-cleaved TDP-43 is not solely restricted to TDP-43 proteinopathies, but may be more widely distributed in a number of neurodegenerative diseases. Thus, to confirm and extend these findings, our goal in the present study was to examine whether caspase-cleaved TDP-43 is a relevant finding in PD and DLB, collectively known as α-synucleinopathies. In PD and DLB, neurodegeneration is believed to be associated with the presence of intraneuronal inclusions termed Lewy bodies that are composed of α-synuclein [27]. Numerous studies support the hypothesis that α-synuclein aggregation is the key step driving pathology, cellular damage, and subsequent neuronal dysfunction [28-31]. In the present study, we examined whether an accumulation of caspase-cleaved TDP-43 is a major finding associated with these α-synucleinopathies.

Five confirmed cases of PD and DLB were examined by immunohistochemistry utilizing our site-directed caspase-cleaved antibody to TDP-43, termed TDPccp. As an initial approach, immunoreactivity to TDPccp was assessed in two different brain regions including the substantia nigra and hippocampus. Application of the TDPccp antibody using bright-field microscopy revealed the presence of caspase-cleaved TDP-43 within Lewy bodies in the SN of DLB and PD samples. In hippocampal sections, caspase-cleaved TDP-43 was found in Lewy bodies and within Hirano bodies in both diseases. Hirano bodies are characterized as rod-shaped, paracrystalline structures in the neurons of the central nervous system and are commonly found in numerous neurodegenerative diseases within the CA1 region of the hippocampus [32]. These results parallel our findings of caspase-cleaved TDP-43 within Hirano bodies in AD and Pick’s disease [7, 11] and support a general role for caspase activation and cleavage of TDP-43 within these eosinophilic structures. It is important to note that although structures labeled by our TDPccp antibody morphologically resembled Hirano bodies, no experiments were carried out to confirm this finding using specific markers to Hirano bodies. Therefore, further studies will be necessary to confirm the finding of caspase-cleaved TDP-43 within these structures.

A central feature of TDP-43 proteinopathies is the presence of cytoplasmic fragments of TDP-43 (~25 and 35 kDa) that have ability to aggregate [22]. Current understanding suggests that the 25 kDa TDP-43 fragment corresponding to the truncation product of caspase-cleaved TDP-43 leads to a toxic gain of function. For example, caspase cleavage of TDP-43 is a critical step leading to the aggregation and redistribution of TDP-43 from the nucleus to the cytoplasm [12]. In addition, a recent study by Johnson et al. has demonstrated that only aggregating forms of TDP-43 are toxic [33]. Therefore, post-translational proteolytic processing of TDP-43 by caspases may be a key step in protein misfolding, aggregation and toxicity of TDP-43 in ALS and FTLD-U [13, 14]. Our data showing the presence of caspase-cleaved TDP-43 (~25 kDa fragment) within Lewy bodies extends these findings to α-synucleinopathies.

A common pathological feature of PD and DLB is the presence of α-Syn, and numerous studies employ the use of a full-length antibody to α-Syn as a general marker for Lewy bodies. We examined the extent of co-localization of TDPccp with a well-characterized full-length antibody to α-Syn (LB509, Zymed Labs). Immunofluorescence double-labeling experiments indicated a colocalization of both antibodies within Lewy bodies in PD and DLB, thereby confirming the presence of caspase-cleaved TDP-43 within Lewy bodies in PD and DLB. Quantification experiments indicated that the majority of Lewy bodies identified within SN brain sections were labeled with both TDPccp and the α-Syn antibody. However, a small percentage of Lewy bodies were found that were only labeled with either TDPccp or the α-Syn antibody, respectively. Quantification of the number Lewy bodies single-labeled by TDPccp was approximately twice the percent that of the full-length α-Syn antibody in both α-synucleinopathies. Statistical differences were detected between the percentages of α-Syn single-labeled, TDPccp single-labeled and α-Syn-TDPccp double-labeled Lewy Bodies by 1-way ANOVA (GLM procedure with contrast statement) with statistical significance established at a p-value < 0.05. The identification of Lewy bodies that only labeled with TDPccp and are alpha-synuclein-negative can be interpreted in several ways. One interpretation is the existence of Lewy bodies in the absence of α-Syn pathology. Although possible, and in fact previously reported [34] these structures probably represent a minority of total number of Lewy bodies present following tissue analysis. A more likely interpretation of our data is the presence of Lewy bodies that labeled with TDPccp, but had insufficient levels of α-Syn to be detected by the α-Syn antibody at the titer employed (1:500). In either case, our data clearly show that the majority of Lewy bodies identified contain both α-Syn and caspase-cleaved TDPccp.

In conclusion, results from the present study identify caspase-cleaved TDP-43 as a primary component of Lewy bodies in α-synucleinopathies, and suggest the TDPccp antibody is an effective marker for the detection of Lewy bodies. Future studies are necessary, however, to address whether the presence of caspase-cleaved TDP-43 in α-
synucleinopathies is a cause or effect of the underlying pathological events associated with these neurodegenerative diseases.

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References –


Figure legends -

Figure 1. Caspase-cleaved TDP-43 in PD and DLB. Representative bright-field single labeling utilizing the TDPccp antibody from a Parkinson’s case (A, C) and Dementia with Lewy Bodies case (B, D). Application of the TDPccp antibody revealed labeling in the SN region within Lewy bodies (arrowheads, A and B) and in the CA1 region of the hippocampus within Hirano bodies (arrows, C and D) as well as Lewy bodies (arrowheads, C and D). All scale bars represent 10 µm.

Figure 2. Western blot analysis utilizing the TDPccp antibody in representative DLB or control cases. Western blot analysis using the TDPccp antibody confirmed the presence of the predicted caspase-cleaved fragment TDP-43 (25 kDa) in all four DLB cases. Bottom panel represents the same blot stripped and reprobed with a β-actin antibody, which served as a loading control. Data are representative of three independent experiments.

Figure 3. Caspase-cleaved TDP-43 co-localizes with alpha-synuclein within Lewy bodies in PD and DLB. Full-length α-Syn and caspase-cleaved TDP-43 antibodies co-localized within Lewy bodies in PD (A-C) and DLB (D-F). Representative immunofluorescence double-labeling employing an α-Syn monoclonal antibody (green, A and D) and TDPccp (red, B and E) and the overlap image for both markers (yellow, C and F). Note the relative uniform distribution of α-Syn and TDPccp labeling within the Lewy body in PD case (C), while in DLB case both α-Syn and the TDPccp appear to be more peripherally distributed (F). All scale bars represent 10 µm.

Figure 4. The TDPccp antibody detects a greater number of Lewy bodies in PD and DLB than the standardized marker, alpha-synuclein. A-C: Representative immunofluorescence double-labeling utilizing the α-Syn antibody (green), TDPccp (red) and the overlap image (C). D-F: Identical to Panels A-C showing α-Syn labeling (green), TDPccp (red), and the overlap image for both markers within a Lewy body (arrowhead, F). Note the greater number of caspase-cleaved TDP-43-labeled Lewy bodies compared to full-length α-Syn-labeled Lewy bodies in SN in both alpha-synucleinopathies (arrows, C and F). A-C: Scale bars represent 10 µm; D-F: Scale bars represent 20 µm.

Figure 5. Caspase-cleaved TDP-43 as a primary marker for Lewy bodies in DLB and PD. Quantification of Lewy bodies labeled by α-Syn and TDPccp antibodies. A monoclonal antibody to α-Syn was utilized as a marker for Lewy bodies. Data show the average percent of Lewy bodies labeled with α-Syn, TDPccp, and with both antibodies in SN of DLB cases (A) and PD cases (B) (percent given above bars) ± S.E.M. Note in both alpha-synucleinopathies the percent of caspase-cleaved TDP-43 single-labeled Lewy bodies is approximately twice the percent that of full-length α-Syn labeling. Data represent the average percent of Lewy bodies (±S.E.M.) in whole sections from five representative DLB cases (A, overall $F_{2, 12} = 284.07$, p-value* < 0.05) and five Parkinson’s cases (B, overall $F_{2, 12} = 26.41$, p-value* < 0.05).