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Plasma phosphorylated-TDP-43 protein levels correlate with brain pathology in frontotemporal lobar degeneration

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Abstract

In the present study, we have correlated plasma TDP-43 levels, as measured by ELISA, with the presence of TDP-43 pathological changes in the brains of 28 patients with frontotemporal lobar degeneration (FTLD) (14 with FTLD-TDP and 14 with FTLD-tau) and 24 patients with pathologically confirmed AD (8 with, and 16 without, TDP-43 pathological changes). Western blotting revealed full-length TDP-43, including a phosphorylated form, and a phosphorylated C-terminal fragment, in all samples examined. Both ELISA and immunohistochemistry were performed using phospho-dependent and phospho-independent TDP-43 antibodies for detection of phosphorylated and total TDP-43, respectively. Over all 52 cases, plasma levels of TDP-43, and scores of brain TDP-43 pathology, determined using TDP-43 phospho-dependent antibody correlated with the equivalent measure determined using the TDP phospho-independent antibody. In FTLD, but not AD, TDP-43 plasma levels correlated significantly with the pathology score when using the TDP-43 phospho-dependent antibody, but a similar correlation was not seen in either FTLD or AD using the TDP-43 phospho-independent antibody. With the TDP-43 phospho-independent antibody, there were no significant differences in median plasma TDP-43 levels between FTLD, or AD, patients with or without TDP-43 pathology. Using TDP-43 phospho-dependent antibody, median plasma TDP-43 levels were greater in patients with, than in those without, TDP-43 pathology for FTLD patients, though not significantly so, but not for AD patients. Present assays for TDP-43 do not differentiate between FTLD, or AD, patients with or without TDP-43 pathological changes in their brains. However, the levels of phosphorylated TDP-43 in plasma do correlate with the extent of TDP-43 brain pathology in FTLD, and therefore might be a useful surrogate marker for tracking changes in TDP-43 brain pathology during the course of this disease.

Keywords

Frontotemporal lobar degeneration; Alzheimer's disease; TDP-43; ELISA; Plasma

Introduction

Frontotemporal lobar degeneration (FTLD) describes a heterogeneous group of non-Alzheimer forms of dementia with onset of illness usually before 65 years of age arising from the degeneration of the frontal and temporal lobes. The main clinical syndrome in FTLD is frontotemporal dementia (FTD), though related disorders of semantic dementia (SD) and progressive non-fluent aphasia (PNFA) stem from differing topographical distributions of what is considered to be a similar underlying pathology [23]. When the behavioural and personality changes of FTD are accompanied by clinical motor neurone disease (MND), the syndrome of FTD with motor neurone disease (FTD + MND) emerges [23].

About 45% cases of FTLT display insoluble tau proteins in their brains in the form of intraneuronal neurofibrillary tangles or Pick bodies [27,30] (currently known as FTLT-tau [18]); some of these are associated with mutations in the tau (*MAPT*) gene. However, a tau-negative, ubiquitin positive (UBQ-ir) histology (FTLT-U) is the most common histological change underlying FTLT and accounts for about 55% of cases [14,16,20,27,30]. This histology is exemplified by the presence of neuronal cytoplasmic inclusions (NCI) and/or neuritic changes (DN) in cerebral cortex and the hippocampus; and in some FTLT-U cases, neuronal intranuclear inclusions (NII) of a “cat's eye” or “lentiform” appearance [15,32] have been described, especially in cases with autosomal dominant inheritance associated with mutations in progranulin (*PGRN*) gene [4–6,17,26,28]. The major protein component in most cases of FTLT-U has been identified as the TAR DNA-binding protein, TDP-43 [2,24], and such cases are currently referred to as FTLT-TDP [18]. However, TDP-43 pathological changes are also present in the ubiquitinated NCI in patients with MND alone [2,7,8,24], and in NCI in 20–25% patients with either Alzheimer's disease (AD) [1,12,31] or Lewy body disorders [3,21].

In a previous study [9], we investigated whether we could detect the presence, or increased amounts, of TDP-43 in plasma of patients with FTLT and AD compared to normal control subjects. Using ELISA, we detected elevated levels of TDP-43 protein in plasma of 46% patients with clinical FTD and 22% patients with AD, compared to 8% of control subjects. Such proportions of patients with FTD and AD showing raised plasma TDP-43 levels correspond closely to those proportions known from autopsy studies to contain TDP-43 pathological changes in their brains [1,12,14,16,20,27,30,31]. We considered that raised TDP-43 plasma levels might thereby reflect TDP-43 pathology within the brain and provide a biomarker that may help to distinguish those cases of FTLT-TDP from those with FTLT-tau, and those patients with AD harbouring TDP-43 pathological changes from those without such additional pathology. As a predictive test, plasma TDP-43 level could, therefore, have great practical value in directing therapeutic strategies aimed at preventing or removing tau or TDP-43 pathological changes from the brain in FTLT and AD.

However, a major limitation of the previous study was that all measurements of plasma TDP-43 level were made on living patients, and therefore, it was not possible to determine whether TDP-43 pathological changes were indeed present within the brains of those individuals displaying high plasma readings. In this present study, we have been able to examine plasma TDP-43 levels in a series of patients with autopsy confirmed FTLT and AD and thereby directly compare plasma TDP-43 levels with the presence of TDP-43 brain pathology.

Materials and methods

Patients

Brain tissues (paraffin sections) and matching plasma samples were available with Ethical Approval from 52 patients courtesy of Dr E Bigio at Northwestern CNADC Neuropathology Core, Northwestern University Feinberg School of Medicine, Chicago, USA (Table 1). Twenty-eight patients (#1–28) clinically had FTLT, or related disorder. These were classified according to recent consensus criteria [18]. Of these, 14 patients had FTLT-TDP; patients #1–6 bore progranulin gene (*PGRN*) mutations and patients #9–14 clinically had FTD or FTD + MND). Fourteen patients (#15–28) had FTLT-tau, including 1 patient (#15) with FTLT with Pick bodies, 7 patients (#16–22) with corticobasal degeneration (CBD), 4 patients (#23–26) with progressive supranuclear palsy (PSP) and 2 patients (#27 and 28) with an unclassifiable tauopathy. Twenty-four patients (#29–52) had pathologically confirmed AD (CERAD score C, Braak stage V or VI), with additional Lewy body pathology in 1 of these (#31) (Table 1). Patients with AD and FTLT fulfilled the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's disease and Related Disorders Association (NINCDS–ADRDA) criteria for probable AD [19] and International criteria for FTLT [22], respectively.

Immunohistochemistry

Tissue sections had been cut at a thickness of 6 µm from formalin fixed, paraffin embedded blocks of frontal cortex (BA8) and temporal cortex (BA21) to include the hippocampus. Sections were immunostained for TDP-43 protein using a commercial phosphorylation-independent rabbit polyclonal anti-TDP-43 antibody (Proteintech Group Inc, Chicago, USA), as described previously [7] and for phosphorylated TDP-43 using the phospho-dependent anti-TDP-43 antibody, pS409/410 [11] at dilution of 1:1,000, using the same methodology [7].

The severity of TDP-43 pathological changes, as determined by each anti-TDP-43 antibody, was separately assessed on a 5-point scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe) in layer II of frontal and temporal cortex, in dentate gyrus, areas CA4/5, CA2/3 and CA1/subiculum, entorhinal cortex and fusiform gyrus. A total severity score for each case was determined by summation of individual scores over these 8 regions giving a maximum possible score of 32 per case per antibody.

Preparation of recombinant protein

TDP-43 was expressed in *E. coli* as a His-tagged protein. The cDNA for TDP-43 was PCR amplified from EST IMAGE clone 5498250 with TDP-43-*Bam*HI sense primer 5'-AGAGGATCCATGTCTGAATATATTCGGGTAAC-3', TDP-43-*Hind*III antisense primer 5'-AGAGAAAGCTT CTACATTCGCCAGCCAGAAG-3' and subcloned into pET30a via *Bam*HI and *Hind*III restriction sites in-frame with the vector encoded N-terminal polyhistidine tag. His-tagged TDP-43 was overexpressed in *E. coli* Rosetta-gami 2(DE3)pLysS. Cells were lysed by sonication in 20 mM Tris-HCl pH8.0, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF, 0.02% Triton X-100, 10% glycerol and soluble proteins applied to a HiTrap™ chelating column charged with nickel. Proteins were eluted from the column with a linear 20–500 mM imidazole gradient, TDP-43 containing fractions analysed by SDS-PAGE and immunoblotting, and pooled. Purity of His-TDP-43 in pooled fractions was estimated as >95%.

In order to create a standard curve for the phosphorylated TDP-43 ELISA, His-TDP-43 was coupled to the antigen peptide, CMDSKS(p)S(p)GWGM (as used for immunization to produce pS409/410–2 antibody). The conjugation involved Sulfo-KMUS (Pierce) as a heterobifunctional cross-linker to prepare the protein-peptide conjugate in a two-step reaction, as per the manufacturer's instructions.

ELISA assays

Whole blood samples (5 ml blood with EDTA acting as anti-coagulant) had been taken 1–15 years after onset of illness. From these samples, plasma was separated by routine methods, and stored in deep freeze (–80°C) until assay. Levels of TDP-43 within plasma samples were determined by ELISA, as described [9]. The ELISA plates (96-well PVC assay plates, Iwaki, Japan) were coated by overnight incubation at 4°C with 0.2 µg/ml anti-TDP monoclonal antibody (H00023435-M01, clone 2E2-D3, Abnova Corporation, Taiwan), 100 µl/well, diluted in 200 mM NaHCO₃ buffer, pH 9.6, containing 0.02% (w/v) sodium azide (final concentration 1:1,000). The plates were washed 3 times with PBST (PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) containing 0.05% Tween 20), and incubated with 200 µL/well of blocking buffer (PBS containing 2.5% gelatin and 0.05% Tween 20) for 2 h at 37°C. The plates were again washed 3 times with PBST, and 100 µl of the plasma samples to be tested, diluted 1:5 with PBS, were added to each of three wells. After 2 h incubation at 37°C the plates were washed 3 times with PBST, and 100 µl of the detection antibody, TDP rabbit polyclonal antibody (BC001487, Proteintech Group, Chicago) diluted to 0.2 µg/ml (1:1,000) in blocking buffer, was added per well and the plates were incubated at 37°C for 2 h. After another wash (as before), the plates were incubated with 100 µl/well of goat anti-rabbit secondary antibody coupled to horseradish peroxidase (HRP) (Sigma, UK),

diluted 1:5,000 in blocking buffer, at 37°C for 1 h. The plates were then washed again with PBST, before adding 100 µl/well Sure Blue TMB Microwell Peroxidase Substrate (KPL Inc, Maryland, USA) and leaving the colour to develop for 30 min at room temperature. Finally, 100 µl/well of stop solution (0.3 M H₂SO₄) was added and absorbance values were read at 450 nm in a Victor² multi-function microtitre plate reader. Net absorbance was calculated by deducting the mean value obtained for a triplicate of “blank” wells containing PBS only. The recombinant His-TDP-43 protein was used to create the standard curve (Fig. 1).

This basic antibody-sandwich ELISA [9] was modified to detect only phosphorylated forms of TDP-43 protein by replacing the Proteintech phospho-independent polyclonal TDP-43 antibody as the detection antibody with the phospho-dependent polyclonal TDP-43 antibody, pS409/410-2 (Cosmobio Co Ltd, Tokyo), used at a dilution of 1:5,000: the monoclonal antibody (2E2-D3) was again chosen as the preferred capture antibody (1:1,000). The TDP-43-phosphorylated peptide/His-TDP-43 protein conjugate was used in the standard curve for detection of phosphorylated forms of TDP-43 (Fig. 2).

All data were analysed using SPSS v 14.0. Because OD values from ELISA were not normally distributed (according to Kolmogorov–Smirnov test). Mann–Whitney test was employed to compare plasma TDP-43 levels between FLTD and AD groups of patients. Correlations involving ELISA data, TDP-43 pathological scores, age at onset of disease, and duration of illness when sampled, were made using Spearman rank correlation statistic.

Immunoblotting

Four plasma samples that gave relatively high absorption values (cases #12, 32, 19 and 42 detailed in Table 1) and four samples with low values (cases #5, 29, 21 and 38 detailed in Table 1), were immunodepleted of albumin and IgG (Sigma Product no. PROTIA-1KT) according to manufacturer's instructions. For SDS-PAGE, these samples were run on 12.5% polyacrylamide gels and the separated proteins were electrotransferred onto nitrocellulose membranes (0.45 µm, Invitrogen), at 25 V, 125 mA for 75 min, which were then blocked with 5% powdered, skimmed milk dissolved in PBST for 1 h. Membranes were incubated overnight with (a) phospho-independent, monoclonal anti-TDP-43 antibody (0.2 µg/ml) (H00023435-M01, clone 2E2-D3, Abnova Corporation, Taiwan), or (b) the phospho-dependent polyclonal anti-TDP-43 antibody, pS409/410-2 (Cosmobio Co Ltd, Tokyo), at a dilution of 1:5,000. The membranes were washed three times in PBST, followed by incubation with HRP-conjugated rabbit anti-mouse or goat anti-rabbit (Sigma), as appropriate, at 1:5,000 in PBST, for 1 h. The protein bands were visualised using ECL reagents (Pierce, Rockford, IL, USA) as described by the manufacturer.

Results

Immunohistochemistry

Using the phosphorylation-independent (Proteintech) TDP-43 antibody, 23/52 patients displayed TDP-43 pathological changes (Table 1). In patients #1–8, there were pathological changes in frontal and temporal cortex, and hippocampus, typical of FTLD-TDP type 3 (Fig. 3a), whereas in patients #9–14 FTLD-TDP type 2 changes were seen (Fig. 3b): patient #15 with Pick-type histology showed no TDP-43 immunoreactive changes. Two of the 4 patients with PSP (patients #24 and 25), and 8 of the 24 patients with AD (patients #29–36), showed TDP-43 pathological changes within the entorhinal cortex and fusiform gyrus (Fig. 3c) and dentate gyrus granule cells (Fig. 3d). One patient with PSP (#25) and 6 with AD (#29–31, 33, 34 and 36) showed TDP-43 pathological changes in temporal neocortex, but only AD patients #30 and #36 showed similar changes in frontal cortex. None of the seven patients with CBD

showed TDP-43 pathological changes, neither did the patient with FTLT-tau with Pick bodies nor did the two FTLT-tau patients with an unclassifiable tauopathy.

Similar results were obtained with the polyclonal phospho-dependent TDP-43 antibody, pS409/410-2, with the exception that the normal diffuse pattern of nuclear staining was not present with this antibody (Table 1). All patients and sections displaying TDP-43 pathological changes with Proteintech antibody were also immunoreactive with pS409/410 antibody, with no additional patients/sections displaying TDP-43 immunoreactivity using the latter antibody (Table 1). While in some instances, overall rating scores for TDP-43 pathology were identical using both TDP-43 antibodies, on other occasions these differed though usually only by one or two points either way (Table 1). Consequently, the pathological score using pS409/410 antibody correlated significantly with that using Proteintech antibody ($p < 0.001$).

ELISA analysis

Phospho-independent TDP-43 ELISA—Plasma TDP-43 levels, as detected by this ELISA, did not differ overall between FTLT and AD cases. Because TDP-43 immunohistochemistry demonstrated TDP-43 pathological changes in 2 cases of FTLT-tau (cases #24 and 25), as well as in all 14 cases of FTLT-TDP, it was decided, for ELISA analysis, to group the FTLT cases into those with or without TDP pathological changes, irrespective of the underlying histological type, or what morphological form or topographic distribution the TDP pathological changes undertook. Hence, plasma TDP-43 levels did not differ between those 16 FTLT cases with TDP pathological changes (subsequently known as FTLT TDP+ve cases) from those 12 FTLT cases without TDP-43 pathological changes (subsequently known as FTLT TDP-ve cases), nor did they differ in those 8 AD cases with TDP-43 pathological changes from those 16 AD cases or without TDP-43 pathological changes (Fig. 4; Table 2). There was no correlation between plasma TDP-43 levels and the severity of brain TDP-43 pathology, either over all 52 cases, or in only those 16 FTLT or 8 AD cases displaying TDP-43 pathological changes.

Phosphorylated (pS) TDP-43 ELISA—Plasma pS-TDP-43 levels, as detected by this ELISA, were generally higher in FTLT TDP+ve than in FTLT TDP-ve cases (Fig. 5; Table 2) but this did not reach statistical significance. This trend was not apparent when comparing the AD TDP-43 positive and AD TDP-43 negative cases. There was no significant correlation between pS-TDP-43 levels and the severity of brain pS-TDP-43 pathology over all 52 cases. However, there was a significant correlation between plasma pS-TDP-43 levels and pS-TDP-43 histopathology scores for all 28 FTLT cases ($p = 0.037$) and for the 16 FTLT TDP +ve cases alone ($p = 0.010$) (Fig. 6). There was no such correlation for the 24 combined AD cases, or for those 8 AD cases with TDP-43 pathology.

There was a highly significant ($p < 0.0001$) correlation between plasma TDP-43 levels as determined by both assays. However, as in our previous study [8], there were 3 patients with FTLT (patients #2, 12 and 16) and 2 patients with AD (patients #32 and 42) who showed 'high' plasma TDP-43 levels in each assay (Figs. 4, 5). Two of these three FTLT 'outliers', and both of the AD 'outliers' (patients #2 and 12, and patients #32 and 42, respectively) were 'outliers' as measured by both TDP-43 ELISAs. They did not, however, match up exactly with cases showing TDP-43 pathological changes in the brain. In FTLT, patients #2 and 12 had TDP-43 pathology while patient #16 did not. In AD, patient #32 had TDP-43 pathology while patient #42 did not.

There were no significant correlations between plasma TDP-43 levels and age at onset, or duration of illness when sampled, for all 52 cases, or when these were stratified into FTLT or AD cases, using either the phospho-independent or the phospho-dependent antibody. There were also no significant correlations between TDP pathological scores and age at onset, or total

duration of illness, for either FTLN or AD cases, overall or when considering only those cases that were TDP-43 positive, using either the phospho-independent or the phospho-dependent antibody.

Western blotting

Western blots were carried out in order to confirm the presence of TDP-43 and ps-TDP-43 in selected plasma samples, which were depleted of albumin and IgG (see Fig. 7). The phospho-independent, anti-TDP-43 monoclonal antibody 2E2-D3 raised against the N-terminal end of TDP-43 (aa205–222) [33], which potentially recognises phosphorylated and non-phosphorylated forms of TDP-43, was able to detect a full-length form of TDP-43, migrating at ~45 kDa, in all samples examined (Fig. 7a). In addition, all samples also showed a fainter and more diffuse higher molecular weight band, migrating at ~55 kDa. The phospho-dependent polyclonal TDP-43 antibody, pS 409/410–2, raised against the C-terminal end of TDP-43 phosphorylated at Serine 409/410 [11], detected a full-length phosphorylated form of TDP-43, migrating at ~45 kDa, and a phosphorylated C-terminal fragment (~25 kDa) in all samples (Fig. 7b). There was also a suggestion of a higher molecular weight band (~55 kDa) in some samples (see lanes 6 and 7 in Fig. 7b). In general, the relative band densities of all of these forms of TDP-43 were greater in the plasma samples with high TDP-43 ELISA scores compared to those with low TDP-43 ELISA scores.

Discussion

In this present study, we confirm previous findings [1,12,31] of the presence of TDP-43 pathological changes within the medial temporal lobe of about one-third of patients with AD. However, we also noted a similar (to AD) pattern of TDP pathology in the brains of two patients with PSP (FTLD-tau). To our knowledge, this has not been reported previously. Uryu et al. [31] investigated 77 cases of PSP but did not detect TDP-43 pathology in any case. These same authors analysed 39 cases of CBD and found TDP-43 pathological changes in 6 cases. These resembled the changes of AD in two cases, but in four others, a more widespread pathology was seen which included clusters of threads resembling astrocytic plaques. In the present study, we did not observe TDP-43 pathology in any of the seven CBD cases investigated.

In our previous study [9], we presented data inferring that plasma TDP-43 levels might have utility in differentiating patients with FTLN with a TDP-43-based pathology from those who have tau-based pathology, and patients with AD who were harbouring TDP-43 pathological changes in their brains from those without such changes. However, these findings were based on living patients for whom there had been no pathological confirmation of disease diagnosis or histological characteristics. In the present study, we have investigated plasma TDP-43 levels in patients with autopsy-confirmed FTLN and AD and thereby have been able to relate plasma TDP-43 levels directly to the presence and amount of TDP-43 brain pathology.

Using the same commercial polyclonal phospho-independent TDP-43 antibody as in our previous study [9], we have shown that plasma levels of TDP-43 do not correlate with either the presence or the amount of TDP-43 brain pathology. However, TDP-43 protein can also be detected in cerebrospinal fluid (CSF) using the phospho-independent TDP-43 antibody and, on average, CSF TDP-43 levels are raised in patients with FTLN [29] and MND [13,29] relative to control subjects, although there is wide variation in individual TDP-43 levels, with substantial overlap between diagnostic groups. Since the protein composition of CSF is more likely to reflect changes in brain pathology than that of peripheral blood plasma, further studies of TDP-43 as a CSF biomarker are warranted. It is possible that levels of CSF TDP-43 will correlate better with the formation of TDP-43 pathology in the brain and spinal cord than those in plasma.

TDP-43 proteins accumulate in phosphorylated form (pS-TDP-43) in the diseased brain [2, 24] and so it is possible that the presence or levels of pS-TDP-43 will correlate more accurately with brain pathology than the non-phosphorylated protein. With this in mind, we modified our existing ELISA protocol [9] to apply to pS-TDP-43 by using the phospho-specific antibody, pS409/410-2 [11], as detection antibody, instead of the phospho-independent antibody. In the present series of patients, the ELISA signal obtained for pS-TDP-43 in blood plasma correlated strongly with total TDP-43 levels as determined using the phospho-independent TDP-43 antibody. This suggests that our modified assay does indeed detect pS-TDP-43 and that a broadly consistent proportion of the total TDP-43 present in plasma exists in phosphorylated form. It should be noted that we have also been able to confirm, by western blot, the presence of pS-TDP-43 proteins in human plasma. With the ELISA for pS-TDP-43, we could not, on a group basis, differentiate patients with FTLN (or AD) with TDP-43 brain pathology from those without such pathological changes. Although the median values of pS-TDP-43 were higher in FTLN TDP positive cases than in FTLN TDP-negative cases, this did not reach statistical significance. Therefore, it seems unlikely that this particular methodology can be employed for diagnostic purposes in blood plasma, although the utility of pS-TDP-43 as a diagnostic marker in CSF remains to be determined. However, our data show a positive correlation between plasma levels of pS-TDP-43 and the extent of brain pS-TDP-43 pathology in FTLN.

One potential role for any putative biomarker would be to aid the initial diagnosis of disease, and this can be divided into two separate objectives: (1) the differentiation of individuals who have acquired a particular disease from normal subjects even before any symptoms develop; and (2) the differentiation of a particular disorder from other diseases with similar symptoms after they appear. It may be the case that plasma TDP-43 levels do not fulfil either of these criteria, although our data still do not rule out the first possibility. However, once a clinical diagnosis is firmly established, then the role of the biomarker changes. One further valuable role of any biomarker is in longitudinal studies where it may be used (as a surrogate) to track the course of disease progression. This is potentially valuable in clinical trials, for example, where changes in a particular molecular marker can provide a level of objectivity, which may enable a reduction in the duration of a trial, and/or the number of patients required for significance. There is a great need for this type of biomarker in the neurodegenerative diseases, clinical trials and longitudinal studies of which are overly reliant on clinical rating scales (which often fluctuate and are at best only semi-quantitative) as a measure of disease progression. For many of these diseases, the development of biomarkers that can detect pathological progression independently of clinical symptoms would be a major advantage. Our data showing a positive correlation in FTLN overall, and in FTLN cases with TDP pathology, between plasma levels of pS-TDP-43 and the extent of pS-TDP-43 brain pathology, as detected by immunohistopathology, suggest that this protein could serve as marker of disease progression in already diagnosed patients. Given the advent of putative neuroprotective agents, there will be an increasing need for this type of biomarker in future clinical trials. This will be especially relevant if drugs are developed to inhibit the accumulation of TDP-43 in the brain.

We have shown previously [13] that, in MND, CSF TDP-43 levels are only raised in the initial stages of the disease (i.e. within 10 months of onset), falling back to control levels thereafter. In the present study, we found no correlation between plasma TDP-43 levels (either phosphorylated or non-phosphorylated) and duration after disease onset, but the minimum time from initial diagnosis to take a blood sample was 12 months and so it remains possible that testing of CSF or plasma from patients with FTLN or AD earlier than this would have been more informative.

As in our previous study [9], we observed, using both types of TDP-43 capture antibodies, a number of patients with FTLN or AD who had very high plasma TDP-43 levels. In other studies, we have detected polymorphic variations within regulatory regions of the *TARDBP*

gene that influence the rate of transcription of TDP-43 mRNA. However, analysis of the patients in the present study with high plasma TDP-43 levels indicates that they were not those with a 'high expressing' TDP-43 haplotype (Pickering-Brown, personal communication). Why some patients show such high plasma TDP-43 levels remains unclear.

The data from the western blots supports the ELISA results, and reveals, for the first time, the presence of phosphorylated full-length (~45 kDa) and truncated forms of TDP-43 (~25 kDa C-terminal fragment) in plasma samples, as demonstrated with the phospho-dependent antibody. The phospho-independent antibody also detected a band at ~45 kDa, which appeared to co-migrate with the full-length form of the protein detected by the phospho-dependent antibody. This could likewise represent the phosphorylated form of the protein. The identity of the higher molecular weight band (at ~55 kDa) is unclear, but could be due to additional post-translational modification, possibly glycosylation. The development of these phospho-dependent and phospho-independent antibodies has provided powerful tools for the specific detection and discrimination of disease-associated abnormal TDP-43 species within tissues and body fluids in both immunohistochemical and biochemical studies, clarifying the histological and biochemical features of TDP pathology within the full spectrum of FTL and MND [25], and characterising the TDP proteinopathy in other disorders such as AD, DLB and Argrophilic grain disease [1,3,10,12,31]. They will prove to be extremely useful for the routine neuropathological diagnosis of TDP-43 proteinopathies, and for the investigation of emerging cellular and animal models of such. Further studies employing antibodies of these kinds will determine if these truncated forms are more useful as a disease marker than full-length forms of either TDP-43 or pS-TDP-43.

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Fig. 1.
Standard curve for the recombinant His-TDP-43 ELISA (data shown $\pm$ SD)

Fig. 2.
Standard curve for the TDP-43, phosphorylated peptide conjugate ELISA (data shown mean $\pm$ SD)

Fig. 3.

Using the phosphorylation-independent (Proteintech) TDP-43 antibody, 23/52 patients displayed TDP-43 pathological changes. In FTLD-TDP patients #1–8 there were TDP-43 pathological changes in temporal cortex typical of FTLD-TDP type 3 (**a**), whereas in FTLD-MND patients #9–14 FTLD-TDP type 2 changes were seen (**b**). Two patients with PSP (patients #24 and 25) and 8 patients with AD (patients #30–37) showed TDP-43 pathological changes within the entorhinal cortex and fusiform gyrus (**c**) and dentate gyrus granule cells (**d**).

Fig. 4.

Box-whisker plots for the plasma phospho-independent (Proteintech) TDP-43 ELISA results (absorbance values). The length of each *box* represents the interquartile range (75–25%) of the sample, the *solid line* drawn across the box the median, the *dashed line* the mean, and outliers are denoted by *dots*

Fig. 5.

Box-whisker plots for the plasma phosphodependent (pS409/410-2) TDP-43 ELISA results (absorbance values). The length of each *box* represents the interquartile range (75–25%) of the sample, the *solid line* drawn across the *box* the median, the *dashed line* the mean, and outliers are denoted by *dots*

Fig. 6.
Correlation ($p < 0.001$) between pS-TDP-43 ELISA and pS-TDP-43 pathological score for the 16 FTLD patients with TDP pathology

Fig. 7.

Western blot analysis of plasma samples. **a** Analysis with the phospho-independent monoclonal antibody 2E2-D3 (Abnova), raised against the N-terminal end of TDP-43 (aa205–222), which recognises phosphorylated and non-phosphorylated forms of TDP-43. **b** Analysis with a polyclonal antibody pS409/410-2 (Cosmo Bio Co Ltd), raised against the C-terminal end of TDP-43 phosphorylated at Serine 409/410, which recognises full length and C-terminally truncated, phosphorylated forms of TDP-43. *Lanes 1, 3, 5 and 7* are samples from cases #12, 32, 19 and 42, respectively (detailed in Table 1), all with relatively high TDP-43 ELISA absorbance values. *Lanes 2, 4, 6 and 8* are from cases #5, 29, 21 and 38, respectively (Table 1), all with low TDP-43 ELISA values

Table 1

Clinical and pathological details of cases

Case	Sex	Onset age (years)	Age at death (years)	Duration when sampled (years)	Total duration of disease (years)	Clinical diagnosis	Pathological diagnosis	TDP pathology score (Ptech)	TDP pathology score (pS409/410)	Ptech ELISA A450 nm	pS 409/410 ELISA A450 nm
1	F	63	68	1.3	5	CBD/PPA	FTLD-TDP (type 3)	10	12	0.25	1.842
2	M	58	65	4	7	FTD	FTLD-TDP (type 3)	11	10	1.062	2.370
3	M	60	64	3	4	FTD	FTLD-TDP (type 3)	5	5	0.498	0.840
4	M	49	53	1.5	4	FTD	FTLD-TDP (type 3)	5	6	0.248	0.952
5	M	57	61	1.3	4	PPA	FTLD-TDP (type 3)	8	10	0.252	0.478
6	F	56	61	1.7	5	PPA	FTLD-TDP (type 3)	12	11	0.252	0.691
7	M	53	61	4	8	PPA	FTLD-TDP (type 3)	10	11	0.581	1.890
8	M	55	62	3	7	PPA	FTLD-TDP (type 3)	6	9	0.323	1.200
9	M	54	60	4	6	ALS + FTD	FTLD-TDP (type 2)	8	7	0.339	0.660
10	M	58	61	1.5	3	ALS + FTD	FTLD-TDP (type 2)	12	16	0.301	1.462
11	M	54	58	3.5	4	FALS + dementia	FTLD-TDP (type 2)	8	12	0.629	1.695
12	F	65	68	2.1	3	FALS + PPA	FTLD-TDP (type 2)	14	19	1.434	2.406
13	M	64	66	1	2	FTD	FTLD-TDP (type 2)	12	18	0.561	1.446
14	M	58	61	4	3	PPA + ALS	FTLD-TDP (type 2)	6	10	0.553	1.402
15	M	50	55	5	5	FTD	FTLD-tau (Picks)	0	0	0.174	0.462
16	M	67	77	4	10	CBD	FTLD-tau (CBD)	0	0	0.741	1.357
17	F	63	74	10	11	CBD	FTLD-tau (CBD)	0	0	0.195	0.509
18	M	36	44	2	8	FTD	FTLD-tau (CBD)	0	0	0.394	1.327
19	M	50	54	2.3	4	FTD	FTLD-tau (CBD)	0	0	0.505	1.660
20	M	67	78	7	11	PPA	FTLD-tau (CBD)	0	0	0.29	0.898
21	F	59	65	4	6	PPA	FTLD-tau (CBD)	0	0	0.15	0.345
22	F	58	67	2	9	PPA	FTLD-tau (CBD)	0	0	0.513	1.671
23	F	70	81	6	11	FTD	FTLD-tau (PSP)	0	0	0.665	1.823
24	M	80	87	4.6	7	MIXED	FTLD-tau (PSP)	3	6	0.271	1.228
25	F	70	84	3	14	PRAD	FTLD-tau (PSP)	9	9	0.188	0.384
26	M	81	87	5	6	PSP	FTLD-tau (PSP)	0	0	0.21	1.089
27	M	62	73	8	11	FTD	FTLD-tau (unclassifiable)	0	0	0.239	0.942

Case	Sex	Onset age (years)	Age at death (years)	Duration when sampled (years)	Total duration of disease (years)	Clinical diagnosis	Pathological diagnosis	TDP pathology score (Ptech)	TDP pathology score (pS409/410)	Ptech ELISA A450 nm	pS 409/410 ELISA A450 nm
28	F	70	74	2.9	4	FTD	FTLD-tau (unclassifiable)	0	0	0.29	0.761
29	M	66	79	8	13	FTD	Mixed VaD + AD with NII	11	12	0.239	0.441
30	F	65	72	4	7	FTD	AD with NII and HS	10	9	0.171	0.318
31	F	75	84	4	9	PRAD	AD with NII + DLB	13	14	0.26	0.983
32	F	76	89	7	13	PRAD	AD with HS	3	3	1.166	2.564
33	F	74	85	5	11	PRAD	AD	9	6	0.559	1.325
34	F	59	77	7	18	PRAD	AD with HS	12	11	0.234	0.505
35	M	70	80	6	10	PRAD	AD	1	2	0.589	1.579
36	F	71	85	5	14	PRAD	AD	14	13	0.213	0.696
37	F	70	79	4	9	PRAD	AD	0	0	0.124	0.274
38	M	54	71	10	17	PRAD	AD	0	0	0.225	0.444
39	F	73	84	1.3	11	PRAD	AD	0	0	0.187	0.450
40	F	73	81	4	8	PRAD	AD	0	0	0.225	0.833
41	M	71	77	4	6	PRAD	AD	0	0	0.398	1.244
42	M	62	69	1.3	7	PRAD	AD	0	0	1.463	2.451
43	F	76	82	3	6	PRAD	AD	0	0	0.217	0.490
44	M	64	73	5	9	PRAD	AD	0	0	0.7	1.984
45	M	59	68	5.9	9	PRAD	AD	0	0	0.335	1.103
46	M	45	50	3	5	FTD	AD	0	0	0.364	1.346
47	M	59	76	9	17	FTD	AD	0	0	0.115	0.285
48	M	53	60	1	7	FTD	AD	0	0	0.284	0.729
49	M	53	60	2	7	FTD	AD	0	0	0.18	0.758
50	M	51	72	15	21	PPA	AD	0	0	0.488	1.746
51	F	71	77	1.4	6	PPA	AD	0	0	0.225	0.841
52	F	57	64	3	7	PPA	AD	0	0	0.875	1.479

AD Alzheimer's disease, ALS amyotrophic lateral sclerosis, CBD corticobasal degeneration, DLB dementia with Lewy bodies, FALS familial motor neurone disease, FTD frontotemporal dementia, FTLD frontotemporal lobar degeneration, HS hippocampal sclerosis, NII neuronal intranuclear inclusions, PPA primary progressive aphasia, PRAD probable Alzheimer's disease, PSP progressive supranuclear palsy, VaD vascular dementia

Table 2

Details of groups of patients with FTLD-TDP and FTLD-tau, and AD patients with or without TDP-43 pathological changes, at the time when blood samples were taken

	FTLD TDP+ve	FTLD TDP-ve	AD TDP+ve	AD TDP-ve
Patients (<i>n</i>)	16	12	8	16
Age at onset range (years)	49–80	36–81	59–76	45–73
Mean age at onset (years $\pm$ SD)	59.6 $\pm$ 7.5	61.1 $\pm$ 11.8	69.5 $\pm$ 5.8	61.9 $\pm$ 9.5
Age at death range (years)	53–68	44–87	72–89	50–84
Mean age at death (years $\pm$ SD)	65.0 $\pm$ 8.8	69.1 $\pm$ 12.6	65.9 $\pm$ 9.2	71.4 $\pm$ 9.3
Mean disease duration when sampled (years $\pm$ SD)	2.7 $\pm$ 1.2	4.8 $\pm$ 2.5	5.8 $\pm$ 1.5	4.6 $\pm$ 3.8
Total disease duration range (years)	2–14	4–11	7–18	5–21
Mean total disease duration (years $\pm$ SD)	5.4 $\pm$ 2.9	8.0 $\pm$ 2.9	11.9 $\pm$ 3.4	9.5 $\pm$ 4.7
Gender; Male:Female % (<i>n</i>)	79:11 (11:3)	57:43 (8:6)	25:75 (2:6)	63:37 (10:6)
Mean absorbance value (pS-independent) ^a	0.52 $\pm$ 0.35	0.34 $\pm$.18	0.43 $\pm$ 0.34	0.40 $\pm$ 0.35
Median absorbance value (pS-independent) ^a	0.42	0.29	0.25	0.25
Mean absorbance value (pS409/410-2) ^a	1.31 $\pm$ 0.63	1.07 $\pm$ 0.50	1.03 $\pm$ 0.64	1.05 $\pm$ 0.75
Median absorbance value (pS409/410-2) ^a	1.32	1.02	0.84	0.84

Mean and median plasma TDP-43 levels as determined by phospho-independent (Proteintech) and phospho-dependent (pS409/410-2) TDP-43 antibodies are shown

AD Alzheimer's disease, *FTLD* frontotemporal Lobar Degeneration

^aIncludes 2 FTLD-tau cases positive for TDP-43 pathological changes