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CASE REPORT

Mixed tau, TDP-43 and p62 pathology in FTLD associated with a *C9ORF72* repeat expansion and p.Ala239Thr *MAPT* (tau) variant

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Abstract A massive intronic GGGGCC hexanucleotide repeat expansion in *C9ORF72* has recently been identified as the most common cause of familial and sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). We have previously demonstrated that *C9ORF72* mutant cases have a specific pathological profile with abundant p62-positive, TDP-43-negative cytoplasmic and intranuclear inclusions within cerebellar granular cells of the cerebellum and pyramidal cells of the hippocampus in addition to classical TDP-43 pathology. Here, we report mixed tau and TDP-43 pathology in a woman with behavioural variant FTLD who had the *C9ORF72* mutation, and the p.Ala239Thr variant in *MAPT* (microtubule associated protein tau) gene not previously associated with tau pathology. Two of her brothers, who carried the *C9ORF72* mutation, but not the *MAPT* variant, developed classical ALS without symptomatic cognitive changes. The dominant neuropathology in this woman with FTLD was a tauopathy with Pick's disease-like features. TDP-43 labelling was mainly confined to Pick bodies, but p62-positive, TDP-43-negative inclusions, characteristic of *C9ORF72* mutations, were present in the cerebellum and hippocampus. Mixed pathology to this degree is unusual. One might speculate that the presence of the *C9ORF72* mutation might influence tau deposition in what was previously thought to be a "benign" variant in *MAPT* in addition to the aggregation of TDP-43 and other as yet unidentified proteins decorated with ubiquitin and p62.

Keywords ALS · FTLD · *C9ORF72* · p62 · TDP43 · Tau

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Introduction

A hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (*C9ORF72*) has been associated with both familial and sporadic amyotrophic lateral sclerosis (ALS), and frontotemporal lobar degeneration (FTLD) [3, 13, 16, 22]. We, and others, have demonstrated distinctive pathology in *C9ORF72* mutant ALS and FTLD cases with p62 positive, TDP-43 negative neuronal cytoplasmic and intranuclear inclusions in cerebellum and hippocampus in addition to TDP-43 positive neuronal cytoplasmic inclusions (NCIs) in the frontal and temporal lobes that typify the TDP-43 proteinopathies [1, 7, 8, 10, 15, 17, 21]. Mutations in the *MAPT* (microtubule associated protein tau) lead to tau deposition and manifest as tauopathies, with varying clinical and pathological profiles but often presenting with frontotemporal dementia [4, 6, 19]. Here,

we describe a patient with a frontotemporal dementia but with mixed p62, TDP-43 and tau pathology probably due to the concurrence of a *C9ORF72* mutation and a rare *MAPT* sequence variation.

Case report

The index case was a 57-year-old female nurse who presented with progressive obsessional and ritualistic behaviour involving repetitive hand cleaning and inappropriate patient care. As her disease progressed she developed marked personality change, with disinhibition, perseveration, anti-social behaviour, memory loss and language deficits resulting in profound global dementia and mutism. Examination elicited no evidence of extrapyramidal or frontal release signs such as pout or grasp reflex. CT scan revealed asymmetrical frontal and temporal lobe atrophy with preferential involvement of the left side and a clinical diagnosis of frontotemporal dementia was made. She continued to deteriorate and she died 12 years after symptom onset and underwent a consented autopsy, which was limited to removal and examination of the brain and spinal cord.

Two of the patient's brothers developed rapidly progressive lower limb weakness and were diagnosed with ALS. Their age at symptom onset was 51 and 60 years, respectively, and both died within 2 years from respiratory failure without symptoms of cognitive dysfunction. Their mother developed symptoms of anxiety, depression and dementia diagnosed as Alzheimer's disease at the age of 72 and died 3 years later. One maternal aunt was reported to have developed dementia in her 80s. The father of the index case was reported to have had Parkinson's disease and died at the age of 84. He had four brothers who had a normal life span and were not thought to have had any neurological problems. Apart from the index case none of the other family members had had post mortem examinations.

A pedigree summarising the affected family members is shown in Fig. 1.

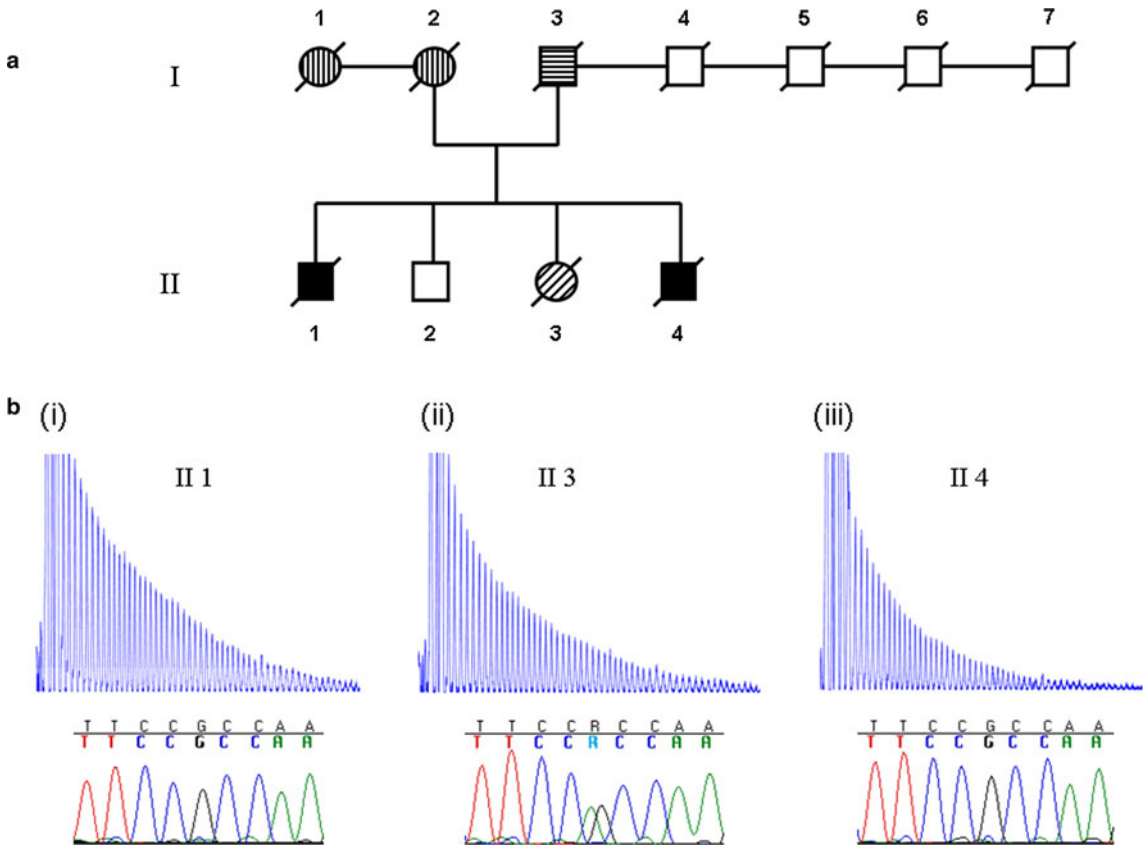


Fig. 1 Genetic analyses. **a** Illustrates the family pedigree with the index case II-3, in addition to the two brothers who had ALS-II-1 and II-4. The father with Parkinsonism is depicted as I-3 and the mother and aunt with dementia I-2 and I-1, respectively. **b** The fragment analysis traces taken from Genemapper clearly show the presence of the expanded GGGGCC repeat (in chromosome 9) from the repeat primed PCR reaction in all three cases. The *vertical axis* represents fluorescent intensity with the *horizontal axis* displaying product size. An expansion positive case manifests as a saw-tooth pattern of individual peaks. The *lower diagram* illustrates the corresponding G>A variation in position 715 in the tau gene (chromosome 17) of the index case, but not present in the two brothers

Genetic analysis

Blood had been taken from the index case and the two brothers and DNA extracted after fully informed consent in a study approved by our Research Ethics Committee. Samples were screened for the GGGGCC Hexanucleotide repeat expansion mutation (HREM), using repeat primer PCR [13]. Fragment analysis was conducted on an ABI 3130 DNA Analyser and peaks visualized using Genemapper 4.0 revealing expanded alleles in all three siblings as shown in Fig. 1. Direct sequencing of the exons and exon/intron boundaries of the MAPT gene (Refseq Transcript NM_001203252.1) was performed using Big Dye V1.1 chemistry and an ABI 3130 genetic analyser. Only the index case was shown to have a G>A variant at nucleotide position 715 that codes for an alanine to threonine amino acid substitution (Ala239Thr) (Fig. 1). This variant was absent in the two brothers with ALS. The variant is present in dbSNP135 (rs137861668) at a frequency of 0.002 in 4,288 control chromosomes (approximately 1/500 individuals).

Biochemistry

Sarkosyl-insoluble tau extraction

For insoluble tau extraction human brain was homogenised in A68 buffer (10 mM Tris–HCl pH7.4, 0.8 M NaCl, 1 mM EGTA and 10 % sucrose) as previously indicated [18]. The homogenate was centrifuged at 14,000 rpm for 20 min at 4 °C and the supernatant collected into a new tube while the pellet was re-suspended in 5 volumes of A68 buffer. The new suspension was centrifuged at 14,000 rpm for 20 min at 4 °C and the two supernatants pooled. The supernatant mixture was incubated with 1 % *N*-Lauryl-Sarkosyl and complete EDTA-free protease inhibitor cocktail for 1 h at RT. The solution was then ultracentrifuged at 100,000 g for 1 h at 4 °C (Optima™ MAX Ultracentrifuge, Beckman Coulter, High Wycombe, UK). The pellet was reconstituted in 50 mM Tris–HCl (0.1 ml/g) and an aliquot used for western blotting. The blot was qualitative in nature and performed to detect which of the groups of tauopathy this case belonged to.

Soluble tau extraction and dephosphorylation

Human brain was homogenised in 25 % percloric acid, the homogenate was kept on ice for 20 min and then centrifuged at 15,000*g* for 20 min at 4 °C. The supernatant was collected and dialysed over night at 4 °C against 50 mM Tris–HCl pH 7.4, 0.1 mM EDTA and 1 mM PMSF. Soluble tau extracts were dephosphorylated with 18 U/ml Alkaline Phosphatase (AP) from *Escherichia coli* (*E. coli*) (Sigma Aldrich) in 50 mM Tris–HCl pH 7.4 5 mM $MgCl_2$ (Promega) for 4 h at 65 °C.

Western blotting

Aliquots of phosphorylated and de-phosphorylated soluble tau extracts and sarkosyl-insoluble tau were run on a 10 % PAGE and transferred electrophoretically onto a PVDF membrane. Total tau antibody (Dako) and phosphorylation-dependent anti-tau antibody AT8 were used (both diluted 1:1,000) to detect soluble and insoluble tau protein.

Biochemical analyses of cerebral cortex extracts revealed no significant differences between soluble tau in control and patient brain (Fig. 2a), and an insoluble Sarkosyl tau preparation from the patient demonstrated a typical Pick's disease pattern with the lower 60 and 64 kDa bands (Fig. 2b).

Neuropathology

The fixed left half brain weighed 333 g, and there was marked atrophy of the left cerebral hemisphere especially the frontal lobe which revealed a "knife edge"-like gyral

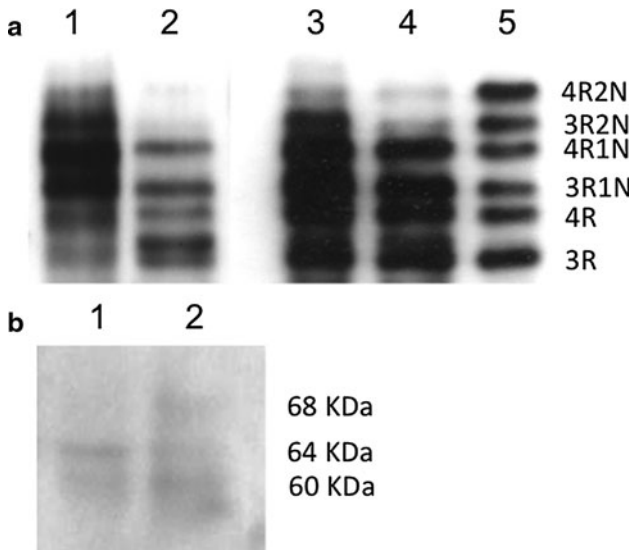


Fig. 2 Western blot analysis of soluble and insoluble tau protein. **a** Soluble tau extracts from the patient (*lanes 1*,*2*) and a control brain (*lanes 3*, *4*) before (*lanes 1*, *3*) and after alkaline phosphatase (AP) (*lanes 2*, *4*) treatment were immunoblotted and stained with the anti-tau antibody (DAKO). Following AP treatment both samples resolved in six tau isoforms that aligned with recombinant human tau proteins (*lane 5*). No significant difference was observed in the expression of tau isoforms between patient and control brains. **b** Sarkosyl-insoluble tau extract from a patient with Alzheimer-like tau pathology used as positive control and showing that the 60, 64 and 68 kDa tau bands (*lane 2*) were compared with sarkosyl-insoluble tau extract from the FTD patient (lane 1), who showed the Pick's disease characteristic 60 and 64 kDa tau bands when stained with the phosphorylation-dependent anti-tau antibody AT8

Table 1 The antibody pretreatments, dilutions and methods for immunohistochemistry in the brain and cord

Antibody	Pretreatment	Dilution	Source
p62	Citrate mw	1:200	BD Biosciences, Belgium
p-TDP-43 (Ser409/410-2)	Citrate mw	1:4,500	CosmoBio, Japan
Tau (AT-8)	Citrate mw	1:1,000	Autogen Biosciences, UK
3-R Tau (8E6/C11)	80 % formic acid and TBS mw	1:1,500	Millipore, UK
4-R Tau (1E1/A6)	80 % formic acid and TBS mw	1:100	Millipore, UK
Fluorescence			
Tau (AT-8)	Citrate mw	1:1,000	Autogen Biosciences, UK
p62 (polyclonal)	Citrate mw	1:500	Cell Signalling, USA

All sections 7 μm thickness. All blocked in normal serum before antibody application. Antibodies applied for 1 h at room temperature except fluorescent antibodies applied at 37 °C for 1 h. For non-fluorescence, sections washed then biotinylated secondary antibody (DAKO, UK) was applied, followed by avidin:biotinylated enzyme complex (ABC kit, Vector, UK). Finally sections were incubated with 3,3'-diaminobenzidine chromagen (Sigma, UK). For double immunofluorescence sections, washed and secondary Alexa Fluor antibody (Invitrogen, UK) was applied in the dark. Autofluorescence was quenched by incubating the sections in Sudan Black for 10 min, followed by washing

TBS tris-buffered saline, *mw* microwave pretreatment

pattern and the temporal lobe. Coronal slices confirmed severe atrophy of the frontal and temporal cortex with loss of white matter. The hippocampus was small. There was also moderate atrophy of the cingulate gyrus, parietal lobe, insular cortex and mild atrophy of the occipital lobe. The caudate appeared flattened, partially due to atrophy and partially secondary to the marked dilatation of the lateral ventricle. The putamen and globus pallidus showed mild atrophy. The substantia nigra and locus coeruleus were pale, and the cerebellum revealed no atrophy. The immunohistochemistry protocols are outlined in Table 1, and histology findings summarised in Tables 2 and 3.

The hippocampus revealed some neuronal loss in the dentate gyrus. Immunohistochemistry for hyperphosphorylated (HP-) tau (with the phosphorylation dependent anti-tau antibody AT8) showed a number of Pick bodies in this layer (Fig. 3a) and these were also positive for 3 repeat tau (3-R tau) (Fig. 3b) and p62. The phosphorylated TDP-43 (p-TDP-43) also labelled many of these inclusions (Fig. 3c). The 4 repeat tau (4-R tau) labelled only occasional pretangle-like structures. The pyramidal cell layer revealed neuronal loss and again Pick bodies with immunopositivity for HP-tau, 3-R tau and p62 (Fig. 3d). The p62, however, gave additional NCIs in the pyramidal cell layer, especially in the CA4 sector. These inclusions were mainly in separate neurons from the Pick bodies and were often globular or star-shaped (Fig. 3d), and were negative for HP-tau and p-TDP-43. This pattern was confirmed using double immunofluorescence (Fig. 3e). One neuron indeed showed both a Pick body and separate star-shaped NCI (Fig. 3f). Occasional p62 positive, HP-tau and p-TDP-43 negative neuronal intranuclear inclusions (NIIs) were seen (Fig. 3g).

The frontal and temporal neocortex showed severe atrophy with superficial spongiform change and marked neuronal loss amounting in some areas to a status spongiosis pattern. There were a number of Pick bodies demonstrated on HP-tau, 3-R tau and p62 and to a lesser degree with p-TDP-43 but not 4-R tau. The Pick bodies were seen throughout the cortex, but appeared particularly associated with layers II and VI in the temporal cortex. The 4-R tau did reveal some NCIs, which were either thorn-like or granular in nature, these also appeared to be detected with p62. Because the TDP-43 pathology was mainly in the form of NCIs with few neurites and no NIIs, then one could argue that it most closely fitted the so-called type B pattern of the "harmonization criteria" for FTLD-TDP; however, since the neuronal TDP-43 pathology was also actually almost completely associated with Pick bodies it would not appear entirely appropriate to subtype it [9]. The white matter showed that both 3-R tau and 4-R tau (Fig. 3b inset) labelled glial cytoplasmic inclusions (GCIs) and neurites. Other areas of the cerebral cortex showed neuronal loss, varying from moderate in the parietal lobe to relatively mild in the occipital lobe. Of particular note was the anterior cingulate cortex, which exhibited marked neuronal loss including depletion of Von Economo neurons. The cerebellum showed no obvious neuronal loss, and had numerous p62 positive, p-TDP-43 negative, HP-tau negative NCIs (Fig. 3h) and neurites and occasional NIIs in the granular cell layer. Occasional NCIs with the same immunohistochemical profile were seen in Purkinje cells and moderate numbers seen in the molecular layer. The dentate nucleus revealed NCIs positive for p62, HP-tau and 3-R tau but not 4-R tau or p-TDP-43. No obvious neuronal loss was seen in the anterior horns of the cord and no skeins or neuronal p-TDP-43 positive NCIs. The HP-tau and 3-R tau, did show occasional NCIs in the posterior horn only. One p-TDP-43 positive neurite was seen. There was also preservation of the corticospinal tracts. The medulla

Table 2 Distribution of p62 and p-TDP-43 immunopositivity in the brain and cord

	p62			pTDP-43		
	Neuronal	Glial	Neurites	Neuronal	Glial	Neurites
Hippo (CA4)	+++ (gran, Pi, NII)	+	+++	+ (Pi)	−	+
Hippo (Dentate)	+++ (Pi)	−	+	+++ (Pi)	−	+
Temporal (Cx)	+++ (Pi)	+++	+++	+++ (Pi)	−	+
Temporal (Wm)	−	+++	+++	−	−	−
Frontal (Cx)	+++(Pi, th)	+++	+++	+++/++ (Pi)	+	+
Frontal (Wm)	−	++	+	−	+	+
BG (C)	+++ (Pi)	++	+	+++ (gran)	++	++
BG (P)	++ (th, Pi)	+	++	+ (gran)	−	++
BG (GP)	+++ (th, Pi)	++	+++	+ (gran)	+	++
Int capsule	−	++	++	−	−	+
Amygdala	+++ (Pi)	+++	+++	+++ (ro, gran)	−	+
Pons	+++ (Pi, gran)	+	+++	−	± (1)	−
Medulla	++ (glob)	+	+	−	++	+ (gran)
Cerebellum (G-L)	+++ (+NII)	+	++	−	−	−
Cerebellum (Mol)	++	+	−	−	−	−
Cerebellum (Purk)	+	−	−	−	−	−
Cerebellum (Wm)	−	++	+	−	−	−
Cerebellum (Dentate)	+ gran	+	−	−	−	−
Spinal cord	++ (glob) interneurones	−	+	−	−	± (1)

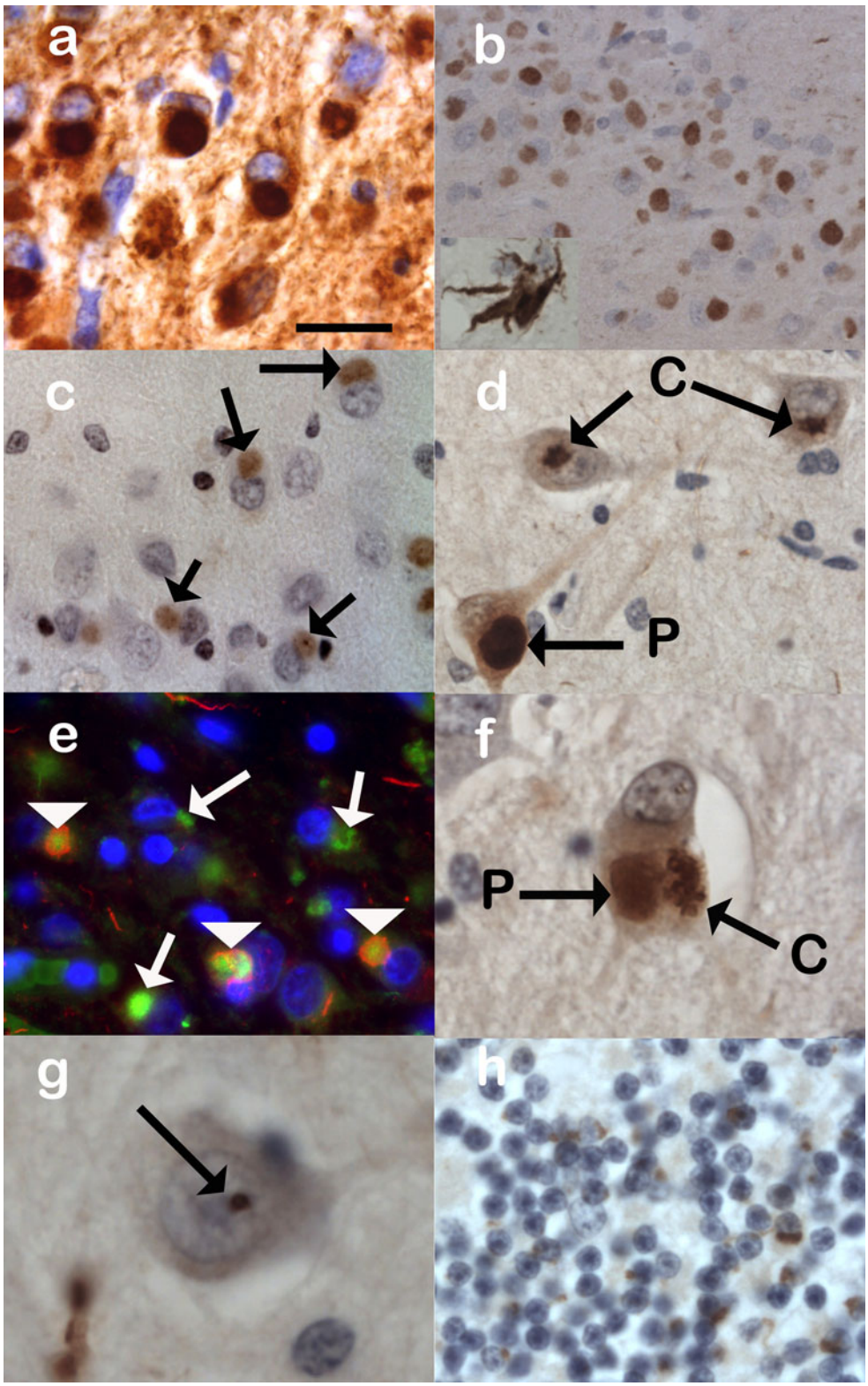
−, Negative; ±, 1–2 structures only; +, occasional; ++, moderate numbers; +++, frequent numbers of inclusions/neurites

BG basal ganglia, *C* caudate, *Cx* cortex, *G-L* granular layer, *GP* globus pallidus, *glob* globular inclusions, *gran* granular inclusions, *Hippo* hippocampus, *Int* internal, *mol* molecular layer, *NII* neuronal intranuclear inclusions, *P* putamen, *Pi* Pick bodies, *p-TDP-43* phosphorylated TDP-43, *Purk* Purkinje, *ro* rounded inclusions, *th* thorn like inclusions, *Wm* white matter

revealed 3-R tau positive NCIs, globular p62-positive NCIs in the XIIth nerve nucleus (but no skeins), and some p-TDP-43 positive GCIs but no p-TDP-43 positive NCIs. The substantia nigra revealed mild neuronal loss with scattered 3-R tau and 4-R tau positive neurites and glial cytoplasmic inclusions. The locus coereleus exhibited severe neuronal loss with occasional globular NCIs positive for 3-R tau (but not 4-R tau).

Discussion

The *C9ORF72* hexanucleotide repeat expansion has been linked to a high proportion of familial cases of ALS and FTLD-TDP as well as a proportion of sporadic cases [3, 13]. We and others have described a characteristic pathological profile including numerous p62 positive, TDP-43 negative NCIs and NIIs (neuronal intranuclear inclusions) in the granular layer of the cerebellum and in the hippocampus [1, 7, 8, 10, 15, 17, 21]. These features were present in our index case, which is consistent with the *C9ORF72* mutation but the pathology in her frontal and temporal lobes was predominantly that of 3-R tau deposition appearing as numerous Pick bodies in the hippocampus and cerebral cortex. Whilst tau positive glial inclusions are certainly seen in sporadic Pick's disease, the abundance and nature of them here would be atypical for the condition [20]. Previous mutations in the *MAPT* gene have been associated with a Pick's disease-type pattern with atypical glial inclusions [11, 18]. It is therefore certainly possible that a sequence variant Ala239Thr may present similarly. However, the same sequence variant has been reported previously in a familial FTLD case associated with a progranulin mutation and ubiquitin pathology but without tau deposition [12]. The TDP-43 pathology that was present in our case appeared to be predominantly associated with tau positive Pick bodies. This phenomenon has previously been described in Pick's disease before, but is certainly atypical for FTLD-TDP [2, 5]. No p-TDP-43 positive skeins were detected in the spinal cord and there was no evidence of spinal motor neuron loss and this would be consistent with the clinical diagnosis of frontotemporal dementia (FTD). We previously included a brief description of this case in a series of cases of FTLD-TDP and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◀ **Fig. 3** Immunohistochemical features of the patient's brain. Immunohistochemistry from the brain revealing **a** large numbers of tau positive, Pick bodies in the dentate gyrus (anti-tau). **b** These are strongly positive for 3-R tau (anti 3-R tau). *Inset* focal 4-R tau glial positivity is seen, here in the temporal lobe (anti 4-R tau). **c** Phosphorylation-dependent TDP-43 staining in the dentate gyrus showing neuronal cytoplasmic inclusions in the form of Pick-like bodies (*arrows*) (anti-p-TDP-43). **d** p62 staining in the CA4 region of the hippocampus reveals both Pick bodies (P) and star-like neuronal cytoplasmic inclusions (C) (anti-p62). **e** Double immunofluorescence in the hippocampus with tau (*red*) and p62 (*green*) showing separate p62-only neuronal cytoplasmic inclusions (*arrows*) and some combined *red* and *green* inclusions (*arrowheads*) (anti-tau and p62). **f** p62 immunohistochemistry reveals one neuron in the CA4 region which shows both a Pick body (P) and separate granular/star-like inclusion (C) (anti-p62). **g** p62 positive dot-like neuronal intranuclear inclusion in the CA4 region (anti-p62). **h** Numerous p62 positive (TDP-43 and tau negative) neuronal cytoplasmic inclusions identified in the granular layer of the cerebellum (anti-p62). *Scale bar* **a**, **c** 20 μm; **b**, **d** 30 μm; **e** 25 μm; **f** 20 μm; **g** 15 μm; **h** (**b** *inset*) 40 μm

ALS, which had unusual p62-positive cerebellar inclusions prior to the identification of *C9ORF72* and *MAPT* variation [8]. Interestingly, one patient in a recently published pathological series on *C9ORF72* cases had corticobasal degeneration-like pathology, although it is not clear whether there were cerebellar granular p62-positive NCIs present [17]. Aside from this finding our case would be the only other reported case to our knowledge, which expressed predominant tau pathology in the background of the *C9ORF72* repeat expansion.

The family data suggest that an obligate carrier of the *C9ORF72* mutation is associated with late onset of the disease (either the mother with features of Alzheimer's disease or the father with Parkinsonian symptoms). Whilst according to one recent study this finding appears to be unusual in that there was a suggestion of earlier age of onset in carriers [14], we have conducted a extensive characterisation of British ALS ± FTD cases due to the repeat expansion and did not find an earlier age of onset [16].

Our patient presented with symptomatic and radiological features typical of FTLD, which on genetic and pathological grounds appear likely to be caused by a concurrent *C9ORF72* mutation and *MAPT* sequence variation. It is intriguing that the dominant pathology is that of a tauopathy, which overshadows the TDP-43 and p62 pathology linked to the *C9ORF72* mutation. It is of course

Table 3 Distribution of 3-R and 4-R Tau immunopositivity in the brain and cord

	3-R Tau			4-R Tau			
	Neuronal	Glial	Neurites	Neuronal	Glial	Neurites	Other
Hippo (CA)	+++ (CA1-3) Pi; CA4; (Pi, tang)	+	+	+ (pret)	±	+++ (CA4)	1 astro plaque
Hippo (Dentate)	+++ (Pi); ± (tang)	+	++	± (pret)	+	++	
Temporal (Cx)	+++ (Pi); + (pret)	++	+	++ (thorn/gran)	++ (coil)	++	No astro plaques
Temporal (Wm)	−	+++	+++	−	+	+	
Frontal (Cx)	+++ (Pi); +(pret)	+	++	++ (thorn, pret)	++ (coil)	++	No astro plaques
Frontal (Wm)	−	+++	+++	−	+	+	
BG (C)	+++ (Pi)	+	++	+ (gran, coil)	+	+	
BG (P)	++ (Pi)	+	±	+ (coil)	++	+	
BG (GP)	+++ (Pi)	+++	++	+ (coil)	++	++	
Int capsule	−	+	+	−	+ (gran)	+	
Amygdala	+++ (Pi); ++(pret)	+++	+++	+++ (glob, tang, pret)	++	+++	Astro plaques
Midbrain (SN)	−	+	+++	± (mild gran, glob)	++	+++	
Pons	+++ (glob, gran)	+	++	−	± (1)	± (1)	
Medulla	+ (glob)	−	−	± (1 tang)	−	+	
Cerebellum (Cx)	−	−	−	−	−	−	
Cerebellum (Wm)	−	−	−	−	−	−	
Cerebellum (Dentate)	+++ (Pi,tang)	++	+++	−	−	−	
Spinal cord	+ (gran)	−	+	−	−	−	

−, Negative; ±, 1–2 structures only; +, occasional; ++, moderate numbers; +++, frequent numbers of immunopositive structures

astro astrocytic, *BG* basal ganglia, *C* caudate, *coil* coiled inclusions, *Cx* cortex, *glob* globular, *GP* globus pallidus, *gran* granular, *Hippo* hippocampus, *Int* internal, *P* putamen, *Pi* Pick bodies, *pret* pretangle, *SN* substantia nigra, *tang* tangle, *thorn* thorn-like inclusion, *Wm* white matter

possible that the *MAPT* variation is not pathogenic and that this case represents the chance concurrence of the *C9ORF72* mutation and a sporadic tauopathy. The previous case report of FTLD associated with Ala239Thr tau variant and a progranulin mutation had only ubiquitin (and therefore probable TDP-43) pathology [12]. It is interesting to note, however, that the two brothers who presented with limb-onset ALS without cognitive problems did not have the *MAPT* variant.

One could speculate that the presence of the mutant 3-R tau promoted dysfunction of cortical neurons due to the *C9ORF72* mutation rather than spinal motor neurons. Conversely aberrant RNA processing due to TDP-43 deposition linked to the *C9ORF72* mutation could have promoted tau deposition. It is also of interest that having dual pathology did not lead to an earlier symptomatic presentation or a more aggressive disease course than her brothers. The mechanism by which *C9ORF72* leads to TDP-43 mislocalisation and aggregation is unknown and its influence on tau deposition requires further investigation.

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Conflict of interest The authors declare they have no conflict of interest.

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