Familial tauopathy with P364S MAPT mutation: clinical course, neuropathology and ultrastructure of neuronal tau inclusions

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Aims: This report presents the clinical course, neuropathology and ultrastructure of neuronal tau inclusions of four Slovene relatives with P364S MAPT mutation.

Methods: The clinical history of three out of four P364S MAPT mutation carriers was taken. After formalin fixation, thorough sampling of the central nervous system was followed by paraffin embedding, H&E, Gallyas, Bielschowsky and immunostaining with AT8, anti-3R, anti-4R tau, anti-amyloid-β, anti-TDP43 and anti-alpha-synuclein antibodies. The distribution and density of different types of neuronal tau inclusions were semiquantitatively assessed. In addition, the ultrastructure of neuronal tau inclusions was analysed.

Results: Macroscopic examination of the brains was unremarkable. Microscopically, neuronal tau inclusions of almost all known types were widespread and distributed fairly uniformly in all cases. Pick bodies and swollen neurones were found in only one family member. Mutant tau was composed of 3R and 4R isoforms, with a slight predominance of 3R tau. Composite neuronal tau inclusion (CNTI), found in all four relatives, was a hallmark of the P364S MAPT mutation. CNTI showed compartmental differences in H&E and Gallyas staining, tau isoforms immunolabelling and ultrastructure, displaying fuzzy fibrils in the core and paired twisted tubules at the periphery.

Conclusions: P364S MAPT mutation is characterized clinically by a variable combination of frontotemporal dementia, parkinsonism and motor neurone disease of short duration, and neuropathologically by a widespread uniform distribution of all known neuronal tau inclusions in one family member. Two-compartment CNTI is a unique characteristic of the P364S MAPT mutation.

Keywords: composite neuronal tau inclusion, fibrillary tau inclusions, FTDP-17, motor neurone disease, parkinsonism, ultrastructure

Introduction

Tauopathies are a group of neurodegenerative disorders characterized by microtubules associated tau protein
(MAPT) hyperphosphorylation and misfolding, resulting in various neuronal and/or glial cytoplasmic inclusions [1, 2]. In addition to sporadic tauopathies, familiar tauopathies due to mutation in the MAPT gene at chromosome 17, with autosomal dominant inheritance, are well established [3, 4].

Over 50 pathological mutations of the MAPT gene have been described [5]. Recently, a P364S mutation in exon 12 was reported in a living male patient with frontotemporal dementia (FTD), without family history [6]. An in vitro experiment using recombinant P364S mutant tau revealed its increased aggregation rate and fibril formation relative to both wild-type tau and representative MAPT P301L mutant tau [6]. A peculiar composite neuronal tau inclusion (CNTI) was subsequently described in two female siblings of a Slovene family [7]. We describe here the clinical presentation and neuropathological findings in four carriers of the P364S MAPT mutation of a family (Figure 1). Additionally, the ultrastructure of the neuronal tau inclusions is provided. Analysis of all siblings’ brains revealed CNTI as a neuropathological hallmark of the P364S mutation.

**Materials and methods**

**Neuropathology**

After autopsy, brains and spinal cords from three relatives and only the brain from the fourth family member (III.1) were fixed in 10% buffered formalin for 2 weeks. After macroscopic examination, extensive sampling was performed and tissue samples were paraffin embedded. Five-μm-thick sections were cut from all tissue blocks and stained with haematoxylin and eosin (H&E) and the Gallyas silver method. Selected tissue blocks were stained with Klüver-Barrera and Bielschowsky.

**Figure 1.** Five generations family tree with missense P364S MAPT mutation first identified in index female patient IV.2. The same mutation was confirmed in another three deceased family members with similar neuropathology (IV.3, IV.1 and III.1 in blue). Based on some clinical data and the relatively young age of death, the predicted carries of P364S MAPT mutation are marked in orange. Six additional family members were also tested, but were negative for P364S MAPT mutation (marked T).
Additional immunostaining was also performed on selected sections, with mouse monoclonal antibodies against hyperphosphorylated tau (AT8; Pierce Biotechnology, Rockford, IL, USA; 1:20, no pretreatment), 3R and 4R tau (RD3; 8E6/C11; RD4; 1E1/A6; Merck-Millipore, Darmstadt, Germany; 1:100 and 1:80, respectively; autoclaved in distilled water for 15 min and 96% formic acid for 10 min), antibodies against phosphorylated TDP-43 (pS409; Cosmo Bio, Tokyo, Japan; 1:100; microwaved for 10 min in citrate buffer), α-synuclein (LB509; Zymed Laboratories, South San Francisco, CA, USA; 1:80; microwaved in citrate buffer for 10 min followed by 96% formic acid for 2 min), β-amyloid (6F/3D; DakoCytomation, Glostrup, Denmark; 1:10; microwaved in EDTA for 25 min followed by 96% formic acid for 5 min), neurofilament protein (2F11; DakoCytomation, Glostrup, Denmark: 1:100; standard CC1) and alpha B crystallin (polyclonal antibody; Novocastra, New Castle, UK; 1:200; AR for 20 min in citrate buffer). Antigene/antibody reactions were visualized with the automatic stainer Ventana Benchmark XL from Roche, by the peroxidase-polymer method, using a Ventana ultraview universal DAB detection kit. In the case of double RD3/RD4 immunostaining, an additional Ventana ultraview universal AP red detection kit was used for visualization of anti-RD3 antibodies.

The regional distribution of tau neuronal inclusions was observed and the highest density per mm² of each type of inclusion was noted on HE- and Gallyas-stained sections. Semiquantitative scores ranging from 0 (no inclusions), + (1–3 inclusions/mm²), ++ (4–9 inclusions/mm²) to +++ (10 or more inclusions/mm²) were given (Table 2).

Electron microscopy

Samples for electron microscopy were taken from the subiculum, dentate gyrus and anterior horn of the spinal cord of case IV.2 (Figure 1). Specimens were postfixed in osmium tetroxide, Epon embedded, cut into semithin slices and stained with Azur II. After selection of representative structures, ultrathin sections were cut, contrasted with lead citrate and uranyl acetate and studied on a transmission electron microscope Jeol 1200EX. Ultrastructural analysis included 5 CNTI, 5 globose neurofibrillary tangles (gNFT), 2 Pick bodies (PiB) and 1 swollen neurone (SN). Flame-shaped neurofibrillary tangles (NFT) were not found in the semithin sections. Instead, analysis of 5 NFT from an unrelated case of Alzheimer’s disease (AD) was performed for comparison with gNFT.

Molecular analysis

Genomic DNA was extracted from whole blood (IV.1) using a Qiagen DNA Maxi Kit (Qiagen, Hilden, Germany) or from formalin-fixed and paraffin-embedded tissue (FFPE) (III.1, IV.2, IV.3) using a Qiagen QiaAmp FFPE DNA Kit (Qiagen, Hilden, Germany). Exons 1, 9, 10, 11, 12 and 13 of the MAPT gene were amplified by PCR from genomic DNA and sequenced using BigDye 1.1 chemistry (Applied Biosystems, Foster City, CA). The results were analysed using SeqScanner software (Applied Biosystems, Foster City, CA) and compared to reference sequence NM_005910.5.

Additionally, a specific two-step protocol for the detection of a hexanucleotide repeat expansion mutation (HREM) in the C9ORF72 gene was performed in four family members, as previously described [8–10]. Fragment length analysis was performed on an ABI310 Genetic Analyzer (Applied Biosystems), and results were analysed by Gene Scan 3.7 software.

Results

Clinical presentation

The clinical features of the four family members, for which autopsy, neuropathological examination and genetic analysis were performed, are summarized in Table 1.

Relative III.1 never visited a neurologist. His widow, a registered nurse, noted that he had become forgetful, had developed a hand tremor and a forward bent posture. She said he had sleep apnoea, was constipated and lost weight in the months before he died of acute myocardial infarction at the age of 57.

The other male (IV.1) had been treated for depression. A year and a half before death, progressive dyspnoea was noted, he also had walking difficulties and had begun to experience memory problems. One month before dying of respiratory failure at the age of 66 years, he was admitted to hospital due to hypercapnic respiratory insufficiency. Severe cognitive decline with aphasia anomia was observed, and he started to
experience psychotic episodes. Hand muscles were atrophic. There were fasciculations in the interosseous muscles. Muscle tone was spastic. Myotatic reflexes were very brisk and the Babinski sign was present bilaterally. No extrapyramidal signs were present. Ultrasound revealed bilateral diaphragm paresis. Electromyography revealed denervation of the upper and lower extremities. A CT scan of the head was unremarkable.

Affected sisters (IV.2 and IV.3) have been already described [7]. Their mother (III.3 in Figure 1) died at the age of 48 years, allegedly with dementia and displaying forward-bent gait.

Neuropathology

On macroscopic examination, no obvious lobar atrophy was present in any of the brains. Coronal sections revealed a small pseudocyst of the left subpallidal region in relative IV.2 with dilated temporal horn of the left lateral ventricle. In relative IV.1, both hippocampi were small and both lateral ventricles were moderately widened. All four members of the family had pale substantia nigra and locus coeruleus bilaterally. Only case IV.3 had obvious atrophy of the anterior spinal roots.

Table 1. Clinical presentation of four affected family members

| Clinic/case | IV.2 | IV.3 | III.1 | IV.1 |
|-------------|------|------|-------|------|
| Gender      | F    | F    | M     | M    |
| Age at death| 60   | 55   | 57 (COD: AMI) | 66 |
| Duration of illness (months) | 18 | approx. 20 | No neurological evaluation | 18 |
| Cognitive |
| MMSE        | 26/30 | ND   | /     | ND   |
| Depression  | +     | +    | /     | +    |
| Dementia    | Mild  | +    | /     | +    |
| Parkinsonism|
| Rigidity    | +     | +    | /     | -    |
| Bradykinesia| +     | +    | /     | -    |
| Tremor      | +     | +    | /     | -    |
| Forward bent| +     | +    | /     | -    |
| MND |
| Muscle atrophy | -   | +    | /     | +    |
| Fasciculation| -    | +    | /     | +    |
| Respiratory  | +     | +    | /     | +    |
| insufficiency|       |       |       |       |
| EMG         | -     | +    | /     | +    |
| Other       | Upward gaze impairment, uncontrolled crying | ND | / | UMN signs (hyperreflexia, spasticity, pos. Babinski sign), psychotic episodes |
| MRI         | Moderate diffuse brain atrophy | Nonspecific | / | ND |
| CSF         | Decreased amyloid beta, normal tau | ND | / | ND |

AMI, acute myocardial infarction; COD, cause of death; CSF, cerebrospinal fluid; EMG, electromyography; MMSE, mini mental state examination; MND, motor neurone disease; MRI, magnetic resonance imaging; ND, not done; UMN, upper motor neuron; /, not observed.

Light microscopic findings

Similar tau inclusions and glial deposits were found in all brains and spinal cords. The distribution and density of the various tau inclusions are presented in Table 2. In Figure 2, a morphologic catalogue of all neuronal inclusions visualized with different staining methods is shown.

gNFTs of up to 50 μm were the most frequent and dispersed neuronal tau inclusions in cortical and subcortical regions, especially in the subiculum, transentorhinal cortex, dentate nucleus, amygdala, Meynert nucleus, thalamus, subthalamic nucleus, nuclei of the cranial nerves, substantia nigra, locus coeruleus, anterior horn of the spinal cord and motor cortex of all
Table 2. Neuronal tau inclusion distribution throughout CNS in all four relatives

| Region/nucleus       | gNFT     | fNFT     | GT       | CNTI     | PiB | SN |
|----------------------|----------|----------|----------|----------|-----|----|
|                      | IV.2     | IV.3     | III.1    | IV.1     | IV.2 | IV.3 | III.1 | IV.1 | IV.2 | IV.3 | III.1 | IV.1 | IV.2 | IV.3 | III.1 | IV.1 |
| CAI-CAIV             | +++      | ++       | ++       | +++      | +   | -   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Subiculum            | ++       | ++       | ++       | +        | +   | +   | +     | +     | +    | +    | ++    | ++   | ++   | ++   | ++   | ++   |
| Dentate gyrus        | -        | -        | -        | -        | -   | -   | -     | -     | -    | -    | -     | -    | -    | -    | -    | -    |
| Transentorhinal cortex| +++      | ++       | ++       | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Dentate nucleus      | +++      | +++      | +++      | +++      | +++  | +++  | +++   | +++   | +++  | +++  | +++   | +++  | +++  | +++  | +++  | +++  |
| Amygdala             | +++      | +++      | ++       | ++       | +   | +   | +     | +     | +    | +    | ++    | ++   | ++   | ++   | ++   | ++   |
| Temporal cortex      | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | ++    | ++   | ++   | ++   | ++   | ++   |
| Visual cortex        | +        | +        | -        | -        | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Cingulate cortex     | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Insula               | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | ++    | ++   | ++   | ++   | ++   | ++   |
| Frontobasal cortex   | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | ++    | ++   | ++   | ++   | ++   | ++   |
| Motor cortex         | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | ++    | ++   | ++   | ++   | ++   | ++   |
| Sensory cortex       | +        | +        | +        | +        | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Striatum             | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Globus pallidus      | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Claustrum            | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Meynert's nucleus    | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Subthalamic nucleus  | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Thalamus             | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Oculomotor nucleus   | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Substantia nigra     | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Locus coeruleus      | +++      | +++      | +++      | +++      | +   | +   | +     | +     | +    | +    | +++   | +++  | +++  | +++  | +++  | +++  |
| Braingstem tegmentum | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Pontine nuclei       | ++       | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Spinal cord, AH      | n.a.     | ++       | ++       | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |
| Spinal ganglion      | +        | +        | n.a.     | ++       | +   | +   | +     | +     | +    | +    | +     | +    | +    | +    | +    | +    |

AH, anterior horn; CA, cornu amonis; CNTI, composite neuronal tau inclusions; fNFT, flame-shaped neurofibrillary tangles; gNFT, globose neurofibrillary tangles; GT, ghost tangles; PiB, Pick bodies; SN, swollen neurones; not assessed (n.a., grey brackets); no inclusions (−, white brackets), low (+, yellow brackets), moderate (+++, orange brackets), high density (+++, red brackets).
family members. All of them had rare gNFT in visual cortex, except the case IV.1, who did not have any. gNFT were Gallyas, AT8, 3R and 4R tau positive. fNFT were also found in high density in the hippocampus and transentorhinal cortex of all relatives, but in moderate density in frontobasal, cingulate cortex and insula, and also in lesser density in the visual and primary sensory cortex. In relative IV.3, fNFT were also present in tegmental cranial nerve nuclei. fNFT showed the same staining characteristics as

|   | HE | Gallyas | AT8 | RD4 | RD3 | RD3/RD4 |
|---|----|---------|-----|-----|-----|---------|
| CNTI | ![Image](CNTI.png) | ![Image](Gallyas.png) | ![Image](AT8.png) | ![Image](RD4.png) | ![Image](RD3.png) | ![Image](RD3/RD4.png) |
| fNFT | ![Image](fNFT.png) | | | | | |
| gNFT | ![Image](gNFT.png) | | | | | |
| GT | ![Image](GT.png) | | | | | |
| PiB | ![Image](PiB.png) | | | | | |
| SN | ![Image](SN.png) | | | | | |

**Figure 2.** Catalogue of neuronal tau inclusions. Inclusions are organized by type in rows and by staining methods in columns (except the bottom right plate, for which alpha B crystallin was used). The basophilic periphery of double composite neuronal tau inclusion (CNTI) is Gallyas, RD3 and RD4 positive, the pale eosinophilic core is RD3 positive only. AT8 labels both the core and periphery of CNTI. Flame neurofibrillary tangles (fNFT) and globose NFT (gNFT) are labelled with all methods used, as are ghost tangles (GT). Pick bodies (PiB), best shown by AT8 and RD3 immunolabelling, are occasionally RD4 slightly positive and are always Gallyas negative. Swollen neurones (SN) are AT8, RD3 and alpha B crystallin positive. Note that neural threads are more numerous in RD3 than RD4 immunolabelling (scale bar = 30 μm).
gNFT. Ghost tangles (GT) were extensively present, especially in the Meynert nucleus, cornu amonis (CA), the brainstem nuclei of the cranial nerves, the substantia nigra, the locus coeruleus and the anterior horn of the spinal cord, expressing both 3R and 4R tau, 3R diffusely and 4R locally. PiB and SN were found only in relative IV.2. PiB were most frequent in the hippocampal dentate gyrus and subiculum, while SN were present in the amygdala and insula, and very rarely in the subiculum. PiB and SN were preferentially 3R tau positive and very few PiB were faintly 4R tau positive. These two structures were always Gallyas negative, but Bielschowsky positive (figure not shown). SN were also alpha B crystallin positive, but never displayed 4R tau immunolabelling. The most fascinating neuronal tau inclusion type, CNTI, present in all relatives’ brains was found mostly in the subiculum, transentorinal cortex, subthalamic nucleus, thalamus, motor and cingulate cortex and very rarely in the substantia nigra, locus coeruleus or other grey matter. In H&E, CNTI displayed a pale eosinophilic globoid core and a narrow basophilic ring or crescent-shaped periphery. Very rarely, double CNTI were found in the same neurone (Figure 2). The entire CNTI was labelled by AT8 and anti-3R tau, while anti-4R tau antibodies and Gallyas silver impregnation labelled the basophilic periphery only.

In addition to neuronal inclusions, numerous neuropil threads, pretangles and a very few glial inclusions of coiled body type were present in all four brains and were highlighted with AT8, 3R and 4R immunostaining, 3R being more intensive than 4R. There was no other glial tau pathology, such as tufted or thorn astrocytes or astrocytic plaques.

Spinal cord examination showed no obvious corticospinal tracts affection in Klüver-Barrera stain. There were, however, numerous neuronal inclusions, mostly of a gNFT type, as well as GT, especially in the anterior but also in the posterior horns. AT8 immunostaining also revealed numerous neuropil threads, some dystrophic neurites and pretangles (Figure 3).

Spinal ganglia, visual and sensory cortex and inferior olivary nuclei were the least affected regions in all four cases with regard to neuronal tau pathology using AT8 immunostaining.
Amyloid-β, alpha-synuclein and TDP-43 immunohistochemistry performed on the hippocampi of all cases did not show pathology. Alpha B crystallin labelled SNs in relative IV.2.

Neuronal loss and reactive gliosis was prominent in the nucleus basalis of Meynert, substantia nigra, locus coeruleus, transentorhinal and motor cortex, and to a lesser extent in the subthalamic nucleus, leaving behind GT with diameters of up to 50 μm. Additional findings were numerous Hirano bodies and granulovacuolar degeneration in all hippocampi. Mild spongiform degeneration was observed focally in the upper cortical layers.

**Molecular analysis**

Sanger sequence analysis of the MAPT gene revealed a mutation in exon 12 (c.1090C>T; cDNA numbering starting at the A of the ATG translation initiation codon of the National Center for Biotechnology Reference Sequence NM_005910.5) at codon 364 (National Center for Biotechnology Reference Sequence NP_005901.2) resulting in proline to serine change (P364S) in four family members, marked in blue, while the sequences from other tested family members were normal (Figure 1).

HREM analysis of the C9ORF72 gene did not reveal pathogenic expansion of the repeats in any of the analysed family members.

**Ultrastructural features**

Electron microscopic analysis, performed on ultrathin brain sections from relative IV.2, enabled us to additionally characterise neuronal inclusions (Figure 4). In the specimens from the subiculum, five CNTI and one SN were observed. The CNTI were composed of an electron lucent core and electron-dense periphery (Figure 4A and B). In the core, there were randomly oriented straight but fuzzy filaments, 7- to 11-nm thick (Figure 4C), similar to ones we found in the SN from the same region (figure not shown). Between them there were rare paired helical tubules (PHT) with a maximum diameter of 16–23 nm and minimum diameter of 7–13 nm at constrictions with approximately 70 nm intervals, identical to those found in the peripheral part of the CNTI, where numerous PHT were tightly packed and in some places cross-transected, showing a hollowed, tubular appearance (Figure 4D, inset). PHT of the same ultrastructure were present in the gNFT of the neurones in the anterior horn of the spinal cord (Figure 4F) and in the fNFT of the control case of AD (Figure 5A and B). In the CNTI core, there were also very rare straight filaments (SF) of 8–13 nm diameter, similar to the majority of the filaments in the PiB of the dentate gyrus neurones (Figure 4E). PiB also contained some thicker 13–20 nm SF.

**Discussion**

Clinical manifestations and neuropathology with the ultrastructure of different neuronal tau inclusions are described in four members of a Slovene family bearing the P364S MAPT mutation. Although various mutations in MAPT are associated with clinical features of FTDP, there are distinctive clinical and pathologic presentations, which seem to be associated with particular mutations even though the phenotype of FTDP-17 MAPT varies not only between families carrying different mutations, but also between and within families carrying the same mutation [1, 11]. Diverse and variable clinical features of FTD with parkinsonism and/or motor neurone disease in the presented relatives are in agreement with these findings. Motor neurone disease, described only in a minority of MAPT mutations [12–14], was obvious in relatives IV.2 and IV.4. Extrapyramidal symptoms are rarely reported in patients with MND, presumably due to overshadowing of extrapyramidal signs by the pyramidal disorder that limits abnormal movement [15] as may have been the case in relative IV.1, who had severely affected substantia nigra but no clinically obvious extrapyramidal signs. Of note, some clinical features (e.g. forward bent posture or camptocormia) can be caused by both MND and extrapyramidal disorder [16], which may be the case in relative IV.2 with the described extrapyramidal signs but without clinically recognized motor neurone signs, except for the respiratory failure resulting in death. Notwithstanding the obvious lack of motor neurone disease, this patient’s spinal cord anterior horns were severely and very similarly affected to those of other presented relatives with MND.

The duration of illness in the four family members was uniformly short, close to just 2 years. In comparison, the average duration of illness in FTDP-17 MAPT is 9 years [17, 18].
Respiratory insufficiency due to severely affected lower motor neurones in P364S MAPT mutation is probably responsible for the relatively short duration of illness. It is plausible that there was not enough time for frontotemporal brain atrophy to develop.

In all of the described cases, fibrillary tau inclusions had a similar distribution throughout the brain and spinal cord. The most frequent type of neuronal tau inclusion in all brains is large gNFT, which is otherwise a hallmark of progressive supranuclear palsy (PSP) [1]. An important difference is that gNFT in PSP express preferentially 4R tau [19], while in the P364S MAPT mutation, gNFT expressed 3R and 4R tau with a recognizable prevalence of 3R tau in GT. fNFT showed the same immunoreactivity on 3R and 4R tau as gNFT. Following neuronal death, the incompletely degraded extracellular remnants of NFT, the GT, are expected to be found in greatest abundance in sites in which the earliest tau deposits were formed [20, 21]. In the Slovene P364S MAPT mutation relatives, GT were especially abundant in the Meynert nucleus, substantia nigra, hippocampus, neocortex, midbrain, and inferior olivary nuclei.

**Figure 4.** Ultrastructure of various neuronal inclusions in the brain of relative IV.2. (A) Neurone with composite neuronal tau inclusion (CNTI). (B) Electron lucent CNTI core (on the right) and electron-dense periphery (on the left) at higher magnification. (C) Straight 7- to 11-nm-wide filaments in the CNTI core show somewhat fuzzy appearance. Inset: Cross-sectional view of filaments. (D) Tightly packed and mostly parallel paired helical tubules (PHT), 16- to 23-nm wide, with 7–13 nm constrictions at approximately 70 nm interval in the periphery of CNTI. Inset: Cross-sectional view showing paired tubules. (E) Pick body is composed of straight 8- to 13-nm-wide filaments intermingled with amorphous material. (F) Globular neurofibrillary tangle is composed of twisted 16- to 23-nm-wide tubules with 7–13 nm constrictions at approximately 70 nm intervals, the same ultrastructure as the peripheral part of CNTI. Inset: Cross-sectional view of PHT. Magnifications are 4000x (A), 25 000x (B), 200 000x (C–F) and 300 000x (insets).
locus coeruleus, brainstem nuclei and the anterior horns of the spinal cords, the regions in which the disease probably started.

The mutation-specific CNTI, which bears both isoforms of tau protein, partly separated into core and periphery, was similarly distributed in all relatives, being most frequent in the subiculum, thalamus, subthalamic nucleus and motor cortex [7]. In a single relative (IV.2), PiB and SN were found in regions typically affected in Pick’s disease (PiD) and some FTDP-17 with PiB, and had the same staining characteristics as these structures in PiD [11, 22].

Considering the cell-type localization of the tau inclusions, the P364S MAPT mutation can be classified as a neuronal predominant form [23]. MAPT mutations in exons 9, 11, 12 and 13 lead to a predominant neuronal tau pathology, whereas MAPT mutations in exons 1 and 10 and introns following exons 9 and 10 produce neuronal and glial deposits [11].

In addition to the spatial distribution of the tau deposits, biochemical classification based on tau isoforms expression is another way of defining tauopathies. The ratio between 3R and 4R tau isoforms in an adult unaffected brain is considered to be approximately 1 [24]. Sporadic and familial tauopathies can thus be classified as tauopathies having equimolar 3R and 4R tau isoforms (AD, primary age-related tauopathy), those with predominant 3R tau (PiD) and those with predominant 4R tau isoform expression and deposition (PSP, corticobasal degeneration, argyrophilic grain disease and globular glial tauopathy) [25, 26]. Based on immunohistochemical reactivity to RD3 and RD4 antibodies in NFT, the P364S MAPT mutation could tentatively be grouped into tauopathies with expression of both R3 and R4 tau isoforms. Although tau isoforms composition is determined by immunoblot, protein sequence and mass spectrometric analysis of sarkosyl-insoluble and trypsin-resistant tau, tau isoform-specific antibodies used in immunohistochemistry, such as anti-RD3 and anti-RD4 antibodies, are proving to be more precise in determining the exact location of neuronal and glial deposits of specific tau isoforms [27, 28]. Contingent on that, an interesting feature of all brains in our study is the low immunohistochemical co-localization of both isoforms within CNTI, PiB and SN: 3R tau labelling was found in the core of CNTI in all brains in which 4R tau was practically undetectable. In addition, PiB and SN in the brain of relative IV.2 were almost exclusively 3R tau immunoreactive.

Ultrastructural analysis of neuronal and glial tau deposits in various forms of sporadic and familial tauopathies revealed that hyperphosphorylated and misfolded tau proteins formed paired helical filaments (PHF), sometimes named PHT or twisted ribbons, and SF or tubules [29–32]. The majority of tau filaments in AD are described as PHF that measure 22–24 nm at the maximum and 11–12 nm at the minimum diameter, with a distance of 85–96 nm between constrictions [33, 34]. In the analysed inclusions of our P364S MAPT mutation, gNFT and the periphery of CNTI had a similar kind of PHT as fNFT in AD we used as control, but with somewhat different dimensions and ultrastructure: the maximum diameter was 16–23 nm and the minimum diameter was 7–13 nm at approximately 70 nm intervals. When transversely cut, these structures revealed electron lucent cores of both twisted filaments, suggesting that PHT may be a better descriptive term. The same fuzzy SF of 7–11 nm in diameter

Figure 5. Ultrastructure of paired twisted tubules (PHT) in the flame-shaped neurofibrillary tangles (NFT) of the patient with Alzheimer’s disease, unrelated to the presented P364S MAPT mutation cases. (A) NFT is composed of PHT 16- to 23-nm wide with 7–13 nm constrictions at approximately 70 nm intervals. (B) Cross-sectional view of PHT. Arrows mark electron lucent paired cores. Magnifications are 100 000× (A) and 300 000× (B).
present in the core of CNTI and in the SN have been observed in the SN of some other familiar tauopathies [35]. The ultrastructure of PiB, the hallmark of sporadic PiD, is composed mostly of SF with diameter of 10–20 nm [36, 37]. In the PiB of the dentate gyrus neurones, observed only in relative IV.2 of the presented family, there were SF of 8–13 nm in diameter. Differences in dimensions between the PHT and SF described in the literature and our own are presumably due to different tissue processing.

In conclusion, the present report on four family members with P364S MAPT mutation shows preseniile tauopathy with a short duration of illness, variable clinical presentation of parkinsonism, behavioural changes, cognitive decline and motor neurone disease with eventual respiratory insufficiency, and with abundant and widespread, morphologically diverse neuronal tau inclusions of all types described so far. A novel neuronal inclusion, CNTI, the hallmark of P364S MAPT mutation neuropathology, has two distinct compartments in terms of both staining characteristics and ultrastructure. gNFT, the peripheral part of CNTI, and the fNFT of control AD have the ultrastructure of PHT instead of PHF.

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Authors contribution

Peter Strafela carried out microscopical analysis of the neuronal tau inclusions and wrote the first version of the manuscript; Jerica Pleško analysed the ultrastructure of neuronal tau inclusions; Jožef Magdič and Blaž Koritnik provided the clinical data of presenting cases; Andrej Zupan and Damjan Glavač carried out the molecular analysis; Mara Bresjanac co-wrote the manuscript; Mara Popović designed the study, supervised the results and co-wrote the manuscript. All of the authors contributed to the final version of the manuscript.

Conflict of Interest

The authors report no conflict of interest.

Ethical Approval

The research was given ethical approval.

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