Mutant Valosin-Containing Protein Causes a Novel Type of Frontotemporal Dementia

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Mutations in the *valosin-containing protein* (VCP) gene on chromosome 9p13-p12 recently have been shown to cause autosomal dominant inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia. Here, we report the central nervous system autopsy findings in a 55-year-old German patient with inclusion body myopathy and frontotemporal dementia who harbors a heterozygous R155C missense mutation residing in the N-terminal CDC48 domain of VCP, which is involved in ubiquitin binding. We demonstrate that mutant VCP causes a novel type of frontotemporal dementia characterized by neuronal nuclear inclusions containing ubiquitin and VCP.

Ann Neurol 2005;57:457–461

Autosomal dominant inclusion body myopathy (IBM) associated with Paget's disease of the bone and frontotemporal dementia (FTD) is a rare multisystem disorder caused by mutations in the *valosin-containing protein (VCP)* gene on chromosome 9p13-p12 (OMIM 605382).[1]

VCP, a member of the AAA-ATPase superfamily, has been associated with a wide variety of essential cellular protein pathways comprising nuclear envelope reconstruction, cell cycle, postmitotic Golgi reassembly, suppression of apoptosis, and DNA damage response.[2–6] In addition to binding to expanded polyglutamine protein aggregates,[7] VCP has been postulated to play a pivotal role in ubiquitin-dependent protein degradation.[8]

Analysis of skeletal muscle tissue from patients with heterozygous *VCP* mutations indicated multiple fibers containing cytoplasmic VCP-positive protein aggregates.[1] However, a detailed neuropathological evaluation of the brain pathology in FTD patients who are carriers of the *VCP* mutation has not been described so far.

In this study, we report on the central nervous system autopsy findings in a 55-year-old female German IBM-FTD patient harboring a heterozygous R155C missense mutation in exon 5 of the *VCP* gene.

Subjects and Methods

Valosin-Containing Protein Mutation Analysis

Isolation of DNA and VCP mutation analysis was performed as described previously.[1]

Neuropathology

Blocks from formalin-fixed brain tissue were embedded in paraffin. Sections (5μm in thickness) were stained with hematoxylin and eosin, Luxol fast blue, the Gallyas silver technique for neurofibrillary changes,[9] and the Campbell–Switzer silver technique for amyloid material.[9]

Immunohistochemistry was performed using antibodies directed against glial fibrillary acidic protein (polyclonal rabbit, 1/1,000; DAKO, Carpinteria, CA), abnormal τ-protein (AT-8: directed against phosphorylated serine 202 and threonine 205 of τ-protein, 1/1,000; Pierce-Endogene, Boston, MA; AT-270: directed against phosphoepitope threonine 181 of τ-protein, 1/200; Innogenetics, Gent, Belgium; PHF-1: directed against phosphoepitopes serine 396/404 of τ-protein, 1/200; gift of Dr P. Davies[10]; and TG-3: directed against an abnormal configuration epitope of τ, 1/20; gift of Dr P. Davies[11]), amyloid β-protein (4G8, 1/5,000; Signet, Dedham, MA), α-synuclein (polyclonal goat, 1/20; Alexis Biochemicals, San Diego, CA), VCP (rabbit polyclonal, 1/250; gift of Dr C.-C. Li[8]), and ubiquitin (polyclonal rabbit, 1/100; FPM1, 1/40; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). The primary antibodies were detected using biotinylated secondary antibodies and the avidin–biotin complex. Immunostaining was visualized by 3,3-diaminobenzidine-HCl.

For double immunostaining studies, polyclonal VCP antiserum (1/250) was used in conjunction with antibodies directed against ubiquitin (FPM1, 1/40; Novocastra), amyloid precursor protein (22C11, 1/75; Chemicon International, Temecula, CA), and glial fibrillary acidic protein (G-A-5, 1/25; Boehringer-Mannheim Biochemical, Indianapolis, IN). As secondary antibodies, CY3 fluorescence-labeled anti–mouse antibodies (1/50; Dianova, Hamburg, Germany) and CY2 fluorescence-labeled anti–rabbit antibodies (1/100; Dianova) were used.

Valosin-Containing Protein Immunoblotting

Total protein extraction of brain tissue, 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously.[12] A polyclonal

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Received Sep 17, 2004, and in revised form Dec 28. Accepted for publication Dec 28, 2004.

Published online Feb 24, 2005, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.20407

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antibody was used (rabbit polyclonal, 1/500; gift of Dr C.-C. Li[8]) for the detection of VCP.

Results

Case Report

A 46-year-old woman experienced development of progressive proximal muscle weakness. A muscle biopsy taken from her left biceps brachii muscle showed the classical picture of IBM. At 47 years of age, word finding difficulties and comprehension deficits were noticed. A formal neuropsychological examination showed severe deficits in language comprehension and object naming, as well as a marked psychomotor slowing. Brain magnetic resonance imaging indicated marked frontal and temporal brain atrophy. Her last neurological examination at 48 years of age showed a severely demented patient with a flaccid, predominantly proximal tetraparesis. She finally died of respiratory and cardiac failure at 49 years of age. The patient's mother, her mother's brother, and one of his children suffered from a similar medical condition. Autopsy performed approximately 12 hours after death showed generalized wasting of her skeletal muscles caused by IBM, as well as a dilatative cardiomyopathy. Immunohistological examination of her skeletal and cardiac muscle tissue showed multiple muscle fibers containing VCP- and ubiquitin-positive nuclear and cytoplasmic inclusions. Electron microscopy identified tubulofilamentous nuclear inclusions and areas with filamentous cytoplasmic material. However, there was no evidence of Paget's disease of the bone.

Valosin-Containing Protein Mutation Analysis

VCP mutation analysis of a DNA sample prepared from the muscle biopsy showed a heterozygous 463 C→T nucleotide substitution residing in the N-terminal part of the gene (Fig 1A, B). This missense mutation in exon 5, which leads to an amino acid sub-

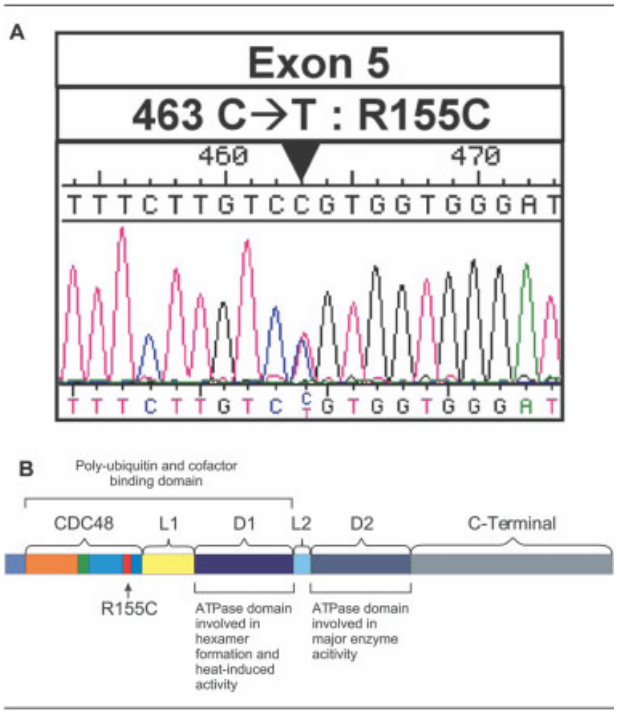
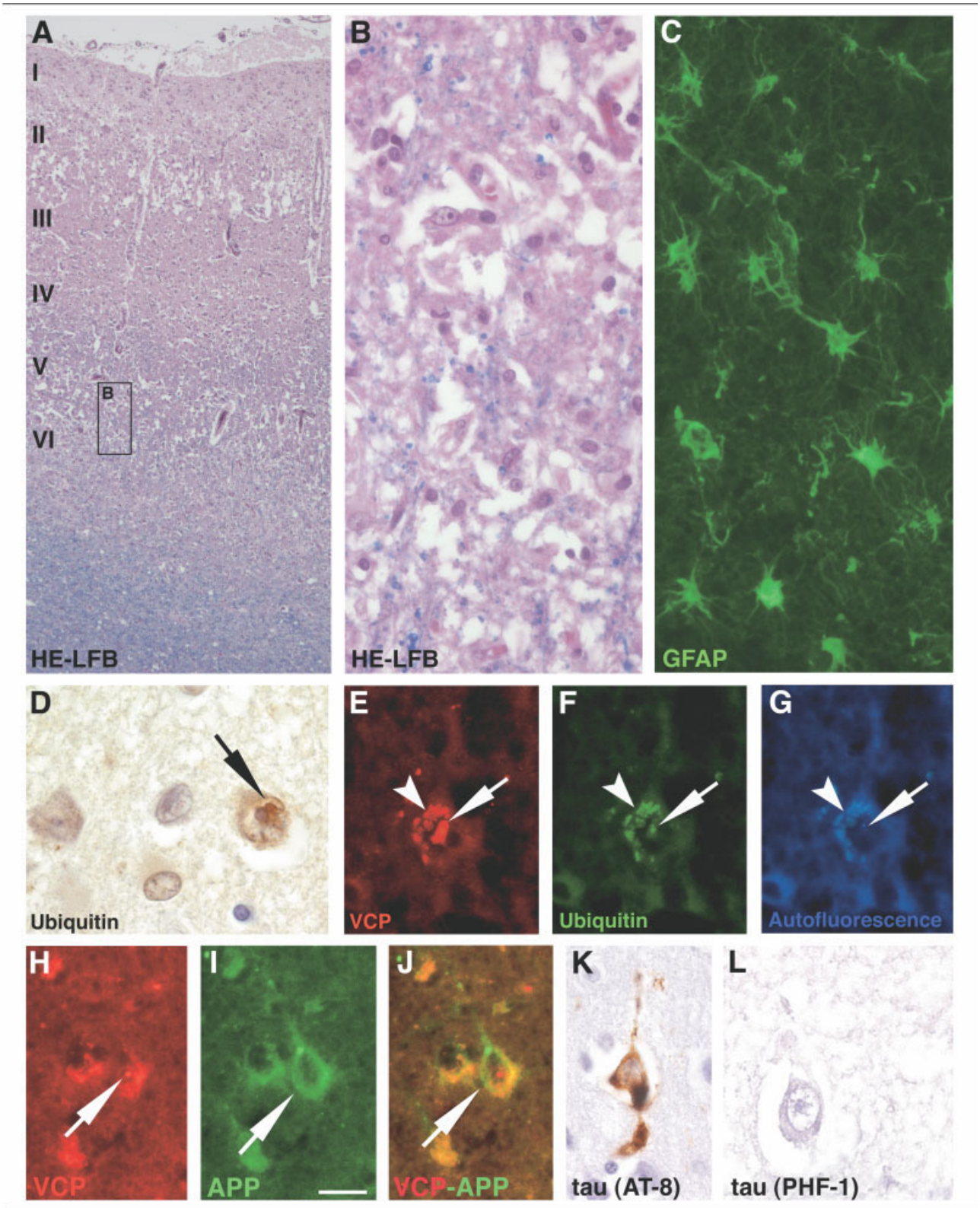


Fig 1. (A) DNA sequencing of DNA extracted from skeletal muscle tissue of the reported patient indicated a 463 C→T nucleotide substitution in codon 155 (exon 5) of the valosin-containing protein (VCP) gene. Because this missense mutation is heterozygous, the DNA sequence analysis shows two overlapping peaks at the same locus (arrowhead). *(B) Schematic representation of the domain structure in VCP: CDC48 domain composed of the double ψ barrel (amino acids 25–106, orange) and the four-stranded β barrel (amino acids 112–186, cyan), connected by a short linker region (amino acids 107–111, green). The CDC48 domain connects the D1 AAA-ATPase domain (amino acids 208–459, dark blue) by a linker region (amino acids 187–208, yellow). The second linker region (L2, blue), second AAA ATPase domain (amino acids 481–761, D2, dark grey), and C-domain (amino acids 762–806, grey) are indicated. The R155 residue, mutated in IBM-FTD, is in red* (arrow).

Fig 2. (A) Hematoxylin and eosin and Luxol fast blue (HE-LFB) staining of cerebral cortex of the occipital lobe displaying spongiosis of layers II, III, and V. (B) High-power view of layer V shows a nearly complete loss of neurons and severe astrogliosis (HE-LFB). (C) Glial fibrillary acidic protein immunofluorescence analysis demonstrates the severe astrogliosis in the temporal neocortex. (D) A surviving layer III neuron of the temporal neocortex contains an ubiquitin-positive nuclear inclusion body (arrow). *(E, F) Valosin-containing protein (VCP) and ubiquitin double-immunofluorescence staining shows cytoplasmic* (arrowhead) *and nuclear* (arrow) *fluorescence. (G) In contrast, analysis of ultraviolet light–induced autofluorescence only shows the cytoplasmic fluorescence pattern* (arrowhead). *This clearly indicates that cytoplasmic fluorescence, but not the nuclear signal seen in E and F (not visible in G as indicated by the* arrow*), represents the characteristic lipofuscin-related autofluorescence.[20] The typical neuronal pattern of the lipofuscin granules clearly identifies this cell as a neuron, most likely a pyramidal cell[20]* (arrowhead). *The nuclear inclusions contain VCP and ubiquitin (E, F,* arrows*). (H–J) The neuronal nature of cells with nuclear inclusion bodies is further confirmed by double labeling with antibodies directed against amyloid precursor protein (APP) and VCP. Note that VCP is present in the nuclear inclusion (H, J,* arrow*), whereas APP shows the typical cytoplasmic staining pattern of neuronal cells (I, J,* arrow*). The apical dendrite of the neuron is clearly stained with anti-APP, identifying the neurons as a pyramidal neuron with its characteristic apical dendrite (I). (K) A neuron within the temporal neocortex exhibits abnormal τ-protein detectable with an antibody directed against the phosphoepitopes serine 202 and threonine 205 (AT-8). (L) Other antibodies directed against conformational τ epitopes and other phosphoepitopes do not stain these AT-8–positive neurons, as demonstrated by the negative staining with the PHF-1 anti-τ antibody. Calibration bars = 100μm (A); 13μm (B, D–G, L); 22μm (C, H–K).*


A
I
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GFAP
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Ubiquitin
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F
Ubiquitin
G
Autofluorescence
H
VCP
I
APP
J
VCP-APP
K
tau (AT-8)
L
tau (PHF-1)

Figure 2

stitution from arginine to cystidine (R155C), was not detected in 200 normal control chromosomes.

Brain Pathology

Autopsy showed a highly atrophic brain (840gm) with accentuated atrophy of the frontal and temporal lobes and the striatum. The diencephalic nuclei, midbrain, pons, medulla oblongata, cerebellum, and spinal cord exhibited normal macroscopic appearance. Histopathological examination indicated widespread loss of cortical neurons associated with astrogliosis in the frontal, temporal, parietal, and occipital regions. Neuronal cell loss was most prominent in cortical layers III and V (Fig 2A, B) and in the striatum. Although to a lesser degree, a reduction in the number of neurons was also documented in the hypothalamus, thalamus, substantia nigra, central grey, colliculi inferiores, and cerebellum. Severe astrogliosis was found in the cerebral and cerebellar white matter, which was further confirmed by glial fibrillary acidic protein staining (see Fig 2C).

A few surviving neurons, predominantly pyramidal neurons located in layers III and V of the cerebral cortex, exhibited VCP- and ubiquitin-positive intranuclear inclusions (Fig 2D–F). Double immunolabeling of VCP and ubiquitin demonstrated the colocalization of both proteins within the nuclear inclusions (see Fig 2E, F). Fluorescent material in the cytoplasm of anti-VCP– and anti-ubiquitin–stained sections was also detectable as autofluorescent material under ultraviolet-light excitation, indicating that this material represents normal lipofuscin. The typical neuronal pattern of these autofluorescent lipofuscin particles indicated that the cells with nuclear VCP- and ubiquitin-containing inclusions were pyramidal neurons (see Fig 2G). The staining pattern of amyloid precursor protein in cells with VCP-positive nuclear inclusions confirmed the neuronal nature of these cells (see Fig 2H–J). Nuclear inclusions were also seen in single neurons of the subiculum, hypothalamus, and substantia nigra. Other neurons, especially those of the fascia dentata, did not exhibit nuclear inclusions. Likewise, we did not observe nuclear inclusions within astrocytes, oligodendrocytes, and microglial cells. Cytoplasmic inclusion bodies were not found in the brain, in neurons, or in glial cells. In sections from control brain, no nuclear or cytoplasmic VCP-positive inclusions were detected.

Using the Gallyas and Campbell–Switzer silver methods, we detected no fibrillary material in neurons or glial cells. Extracellular amyloid deposits and anti–amyloid β-protein–immunopositive plaques were absent. Only single cortical and hypothalamic neurons exhibited AT-8–positive τ-protein (see Fig 2K). However, these neurons did not stain with other antibodies directed against pathological forms of the τ-protein (AT-270, PHF-1, TG-3; see Fig 2L). No pathological α-synuclein aggregates were found.

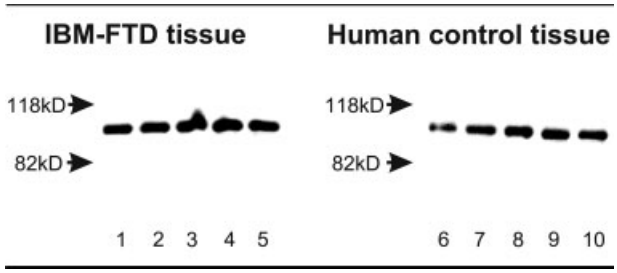

Fig 3. Valosin-containing protein (VCP) Western blot analysis of total protein extracts showed a single band corresponding in size to 97kDa in all samples analyzed with no apparent differences in the signal intensities between diseased (lanes 1–5*) and healthy (lanes* 6–10*) human brain tissue in the hippocampus (lanes* 1, 6*), frontal white matter (lanes* 2, 7*), temporal white matter (lanes* 3, 8*), frontal cortex (lanes* 4, 9*), and temporal cortex (lanes* 5, 10*). Regions containing neurons with nuclear inclusion bodies such as the frontal and temporal cortex (lanes* 4, 5, 9, 10*) showed similar signal intensities for VCP compared with regions without cells exhibiting nuclear inclusion bodies such as the frontal and temporal white matter (lanes* 2, 3, 7, 8*) in diseased (lanes* 2–5*) and control (lanes* 6–10*) human brain.*

Valosin-Containing Protein Expression

Western blot analysis of total protein extracts detected a single 97kDa band in all samples analyzed, thus confirming the specificity of the VCP antibody (Fig 3). There were no apparent differences between healthy and diseased brain tissue. Moreover, our analysis indicated equal amounts of VCP in different brain regions in the patient, as well as in the control brain (see Fig 3).

Discussion

Mutation analysis in the reported patient showed a heterozygous R155C mutation in exon 5 of the *VCP* gene. In agreement with previous data showing that 10 of 13 affected families carry an amino acid change at codon 155 (R155H, n = 7; R155P, n = 1; R155C, n = 2),[1] the identification of the R155C mutation in our IBM-FTD patient further supports the notion that codon 155 is a mutation hot spot of the *VCP* gene.

Neuropathological analysis of the central nervous system indicates that the R155C *VCP* mutation leads to a novel form of FTD characterized by VCP- and ubiquitin-positive neuronal nuclear inclusions. Immunostaining using the AT-8 antibody directed against abnormal phosphorylated τ-protein showed a small number of immunoreactive cortical neurons. These neurons showed no immunoreactivity with various other anti-τ antibodies and no argyrophilic material. Because the AT-8 anti-τ antibody is also capable of staining reactive hyperphosphorylated τ-protein after excitotoxic events,[13] our findings argue against significant τ pathology in VCP-related FTD. The pattern of exclusively intranuclear neuronal VCP and ubiquitin-

positive inclusions and the sparing of the dentate gyrus in nuclear inclusion body pathology indicate that the presented case is distinct from other forms with ubiquitin-positive τ-negative frontotemporal lobar degeneration. These cases show ubiquitin-positive inclusions in the fascia dentata and often also show cytoplasmic inclusions.[14–16] In so doing, this case is different from other forms of FTD in not being associated with IBM.[17,18]

To address the issue whether the VCP immunostaining is paralleled by an altered VCP expression, we performed Western blot analysis of total protein extracts from the VCP-mutant patient and healthy control brain tissue. Immunoblotting showed a single band corresponding in size to 97kDa in all samples analyzed with no apparent differences in the signal intensities between healthy and diseased brain. These findings indicate that the R155C mutation does not lead to significant changes of VCP levels in brain tissue.

Missense mutations in codon 155 lead to an amino acid substitution in the N-terminal CDC48 domain of VCP protein, which is involved in ubiquitin binding.[19] Because VCP has been shown to play a role in the endoplasmic reticulum–associated ubiquitin-dependent proteasome degradation,[6,19] as well as in ubiquitin-dependent nuclear transport processes,[5] the observed inclusion body pathology in brain and striated muscle may indicate that the expression of mutant VCP protein interferes with both ubiquitin-dependent pathways, thereby leading to abnormal protein aggregations in the nucleus and cytoplasm of postmitotic cells.

This study was supported by the Deutsche Forschungsgemeinschaft (TH 624/4-1, D.R.T.) and BONFOR (grant O-154.0041, D.R.T.).

We acknowledge the technical help of B. Söndgen, U. Klatt, K. Kappes-Horn, and M. Stepien-Mering. We also thank Dr P. Davies for his gift of PHF-1 and TG-3 antibodies and Dr C.-C. Li for her gift of the polyclonal VCP antiserum.

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