Title

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Journal

Journal of Neuropathology & Experimental Neurology, 65(6)

ISSN

0022-3069

Authors

Forman, Mark S
Mackenzie, Ian R
Cairns, Nigel J
et al.

Publication Date

2006-06-01

DOI

10.1097/00005072-200606000-00005

J Neuropathol Exp Neurol
Vol. 65, No. 6
June 2006
pp. 571–581

ORIGINAL ARTICLE

Novel Ubiquitin Neuropathology in Frontotemporal Dementia With *Valosin-Containing Protein* Gene Mutations

Mark S. Forman, MD, PhD, Ian R. Mackenzie, MD, Nigel J. Cairns, PhD, Eric Swanson, BS, Philip J. Boyer, MD, PhD, David A. Drachman, MD, Bharati S. Jhaveri, MD, Jason H. Karlawish, MD, Alan Pestronk, MD, Thomas W. Smith, MD, Pang-Hsien Tu, MD, PhD, Giles D. J. Watts, PhD, William R. Markesbery, MD, Charles D. Smith, MD, and Virginia E. Kimonis, MD

Abstract

Frontotemporal dementia (FTD) with inclusion body myopathy and Paget disease of bone (IBMPFD) is a rare, autosomal-dominant disorder caused by mutations in the *valosin-containing protein* (*VCP*) gene, a member of the AAA-ATPase gene superfamily. The neuropathology associated with sporadic FTD is heterogeneous and includes tauopathies and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U). However, there is limited information on the neuropathology in IBMPFD. We performed a detailed, systematic analysis of the neuropathologic changes in 8 patients with *VCP* mutations. A novel pattern of ubiquitin pathology was identified in IBMPFD that was distinct from sporadic and familial FTLD-U without *VCP* gene mutations. This was characterized by ubiquitin-positive neuronal intranuclear inclusions and dystrophic neurites. In contrast to FTLD-U, only rare intracytoplasmic inclusions were identified. The ubiquitin pathology was abundant in the neocortex, less robust in limbic and subcortical nuclei, and absent in the dentate gyrus. Only rare inclusions were detected with antibodies to *VCP* and there was no biochemical alteration in the *VCP* protein. *VCP* is associated with a variety of cellular activities, including regulation of the ubiquitin–proteasome system. Our findings are consistent with the hypothesis that the pathology associated with *VCP* gene mutations is the result of impairment of ubiquitin-based degradation pathways.

Key Words: Frontotemporal dementia, Neurodegenerative disease, Ubiquitin, *Valosin-containing protein*, Inclusion body myopathy, Paget disease of bone.

INTRODUCTION

Inclusion body myopathy (IBM) associated with Paget disease of bone (PDB) and frontotemporal dementia (FTD) or IBMPFD, is a rare autosomal-dominant disorder characterized by variable penetrance of this unusual triad of clinical features (1). Recently, mutations were identified in affected individuals in the *valosin-containing protein* (*VCP*) gene (2). When present, IBM is characterized by adult-onset proximal and distal muscle weakness clinically resembling limb girdle muscular dystrophy. The symptoms of PDB have an early onset with a typical distribution in the spine, pelvis, and skull. In contrast, the dementia associated with IBMPFD presents later than both the IBM and PDB with a mean age at onset of 54 and is clinically typical of FTD characterized by language dysfunction and/or early changes in behavior with relative preservation of memory (1). To date, there is only limited information on the pathology associated with each of the clinical features of this disorder.

VCP (also known as p97, and its homologs TER94 [Drosophila], Cdc48p [yeast], and *VCP*-like ATPase [bacteria]) is a member of the AAA-ATPase gene superfamily (ATPase associated with diverse cellular activities) (3, 4). The expressed protein functions as a molecular chaperone in a plethora of distinct cellular activities, including ubiquitin-dependent protein degradation, stress responses, programmed cell death, membrane fusion, nuclear envelope reconstruction, and postmitotic Golgi reassembly. Notably, almost all of these activities are directly or indirectly regulated by the ubiquitin proteasome system (UPS).

Sporadic cases of the clinical syndrome of FTD are associated with multiple neuropathologic disorders, including tauopathies (i.e. Pick disease, corticobasal degeneration,

From the Departments of Pathology and Laboratory Medicine (MSF, ES) and Medicine (JHK) and Institute on Aging, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; Department of Pathology (IRM), University of British Columbia, Vancouver, British Columbia, Canada; Departments of Neurology (NJC, AP) and Pathology and Immunology (PHT), Washington University School of Medicine, St. Louis, Missouri; Department of Pathology (PJB), University of Texas Southwestern Medical School, Dallas, Texas; Departments of Pathology (TWS) and Neurology (DAD), University of Massachusetts Medical School, Worcester, Massachusetts; Department of Laboratory Medicine (BSJ), St. John's Hospital, Springfield, Illinois; Department of Genetics (GDJW, VEK), Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts; and Departments of Neurology (CDS and WRM) and Pathology and Laboratory Medicine (WRM), University of Kentucky, Lexington, Kentucky.

Send correspondence and reprints requests to: Mark S. Forman, MD, PhD, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 422 Curie Blvd., 605B Stellar-Chance Building, Philadelphia, PA 19104–6140; E-mail: formanm@mail.med.upenn.edu.

This work was supported by grants from the National Institutes of Health (P30 AG10124, P30 AG21300, P50 AG05144, P50 AG05681, R01 AR050236, and R03 AR46869), the Muscular Dystrophy Association, the Paget's Foundation, and the Winspear Family Special Center for Research on the Neuropathology of AD. MSF is supported by a Mentored Clinical Scientist Development Award from the National Institute of Health, K08 AG20073.

progressive supranuclear palsy, and argyrophilic grain disease), dementia lacking distinctive histopathology, neuronal intermediate filament inclusion dementia (NIFID), and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) (5–7). Although much attention has focused on those conditions characterized by an abnormal accumulation of tau protein in the brain (i.e. tauopathies), several recent studies have shown that FTLD-U is one of the most common pathologies associated with clinical FTD (8, 9). This unique pattern of ubiquitin-immunoreactive inclusions was first recognized as the underlying pathology in patients with motor neuron disease (MND) and dementia (10, 11), but was subsequently found in a subset of patients with FTD lacking motor symptoms (i.e., FTLD-U; also designated FTD-MND type or MND inclusion dementia) (12). The pathology in FTLD-U and MND-dementia is characterized by ubiquitin-positive neuronal cytoplasmic inclusions and dystrophic neurites that are not detected with antibodies recognizing other cellular proteins, including tau, α-synuclein, β-amyloid, neuronal intermediate filaments, and expanded polyglutamines (10–13). A genetic etiology is strongly implicated in FTLD-U pathogenesis with a positive family history of a similar neurodegenerative disease reported in up to 40% of patients (14). Recently, the additional pathologic feature of ubiquitin-positive neuronal intranuclear inclusions (NII) was described in a subset of patients with familial FTLD-U (15, 16), and it has been suggested that the presence of frequent NII may distinguish a subset of familial FTLD-U from sporadic cases (17, 18).

There is limited information on the brain pathology associated with IBMPFD. The initial report described only nonspecific neurodegenerative changes (1). More specific pathologic features were described in a recent case report of a 55-year-old German patient with IBM and FTD and a heterozygous $^{R}155^{C}$ missense mutation in the *VCP* gene (19). Postmortem examination of this patient revealed prominent frontotemporal and striatal atrophy and NII that were positive for both ubiquitin and *VCP*. Biochemically, no alteration in the expression of *VCP* was identified. However, both of these studies are incomplete with only limited information on the morphology, regional density, and anatomic distribution of the central nervous system pathology. Moreover, it is unknown whether the findings reported in this single recent case report are a consistent finding in all cases of IBMPFD, regardless of the duration of neurologic deficits and the specific *VCP* gene mutation. In this study, we provide the first detailed neuropathologic description of a large series of patients with this new pathologic entity. We have evaluated the morphology, neuroanatomic distribution, and density of the ubiquitin pathology from several different IBMPFD families with 3 different *VCP* gene mutations and demonstrate unique features of IBMPFD compared with other subtypes of sporadic or familial FTLD-U.

MATERIALS AND METHODS

Patients

We studied postmortem material from 8 affected members from 5 different families with IBMPFD, 6 of whom had a clinical diagnosis of FTD (Table 1). The clinical evaluation and *VCP* gene analysis of several of these families has previously been described in detail (1, 2). Consent for autopsy was obtained from the legal next of kin from all subjects in accordance with state law as well as local Institutional Review Boards. The neuropathology of these cases was compared with that of patients with sporadic and familial FTLD-U from the University of British Columbia which have been previously reported (18). Frozen brain tissue from control and FTLD-U subjects was obtained from the brain bank at the Center for Neurodegenerative Disease Research at the University of Pennsylvania.

Neuropathology

Tissue obtained at the time of autopsy was fixed in neutral buffered formalin, embedded in paraffin, and cut into 6- to 10-μm-thick sections. Sections were stained with hematoxylin and eosin, thioflavin S, and Gallyas silver methods. Immunohistochemistry was performed on sections using a panel of antibodies specific for phosphorylated tau (PHF1 [20]), provided by Dr. P. Davies, Albert Einstein School of Medicine, New York, NY), α-synuclein (Syn303 [21]), β-amyloid (4G8; Senetek, Maryland Heights, MO), phosphorylated neurofilament subunits (RM024 [22]), α-internexin (Zymed Laboratories, San Francisco, CA), ubiquitin (Chemicon

TABLE 1. Clinical Features of Patients With Inclusion Body Myopathy and Paget Disease of Bone

Patient	Sex	Age at Death (years)	Clinical			Mutation	Family*
			IBM	Paget Disease	FTD		
1	M	64	20 years	23 years	No	$^{R}155^{H}$	1
2	F	70	12 years	No	8 years	$^{R}155^{C}$	4
3	M	52	11 years	No	No	$^{R}155^{H}$	16
4	F	60	6 years	6 years	6 years	$^{R}155^{H}$	16
5	F	56	5 years	5 years	5 years	$^{R}155^{H}$	16
6	M	47	6 years	No	9 years	$^{R}155^{H}$	1
7	F	60	No†	25 years	8 years	$^{R}155^{H}$	3
8	M	52	15 years	3 years	5 years	$^{N}387^{H}$	NA

*, Numbering of families as defined by Watts et al (2).
†, Features of IBM identified in muscle at autopsy.
IBM, inclusion body myopathy; FTD, frontotemporal dementia; NA, not applicable.

International, Temecula, CA; Dako Cytomation, Glostrup, Denmark), and expanded polyglutamine repeats (1C2; Chemicon International). In addition, a panel of antibodies specific for *VCP* were used, including 1) monoclonal antibody from BD Pharmingen, San Diego, CA; 2) monoclonal antibody from Affinity BioReagents, Golden, CO; and 3) rabbit polyclonal generously provided by Dr. C.-C. Li, National Cancer Institute, Frederick, MD (23). For *VCP* immunohistochemistry, a variety of antigen retrieval methods were used, including boiling, microwaving, protease digestion, and formic acid pretreatment. The avidin–biotin–peroxidase method with either 3,3-diaminobenzidine or aminoethylcarbizole for color development was used for all immunostaining as described previously (17, 24). The different types of ubiquitin pathology were assessed using a semiquantitative method as previously described (18). Briefly, ubiquitin-immunoreactive pathologic changes were graded as follows: –, none; +, rare (pathologic lesions were only found in a small proportion of 20× microscopic fields examined); ++, mild (a small number of pathologic structures were present in most 20× fields); +++, moderate (moderate numbers of pathologic structures were present in virtually every 20× field examined); and ++++, severe (large numbers of pathologic structures were present in virtually every 20× field examined). The reliability and reproducibility of this grading system has been demonstrated previously (25). The final score was an estimated average for the entire anatomic region being assessed. Alzheimer disease pathology was assessed according to established criteria (26).

Biochemistry

Fresh, frozen brain tissue from neocortex was extracted and analyzed as previously described (27). Briefly, gray matter was separated from white matter and sequentially extracted with buffers of increasing strength at 2 mL/g initial wet tissue weight as follows: 1) high-salt Tris-buffered saline (HS-TBS; 50 mM Tris, pH 7.6, 750 mM NaCl, 1 mM EGTA, 0.5 mM MgS04, 20 mM NaF, 100 μM EDTA); 2) 1% Triton X-100 in HS-TBS; 3) RIPA buffer (0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl, 50 mM Tris Base, pH 8.0); 4) 2% SDS in 50 mM Tris, pH 7.6; and 5) 70% formic acid; each buffer supplemented with a cocktail of protease inhibitors (1 mM PMSF and 100 μg/mL each of pepstatin A, leupeptin, soybean trypsin inhibitor, N-tosyl-L-phenylalanyl chloromethyl ketone, and N-tosyl-lysine chloromethyl ketone). Tissue was homogenized in buffer and centrifuged at 100,000 × g for 30 minutes at 4°C. Each extraction was repeated twice to reduce the risk of carryover of soluble protein. For Western blot analysis, nitrocellulose replicas were prepared from 7.5% SDS-polyacrylamide slab gels and probed with antibodies to VCP and ubiquitin. Primary antibodies were detected with horseradish peroxidase-conjugated antimouse IgG (Jackson Immunoresearch, West Grove, PA) and immunoreactive proteins were revealed using ECL chemiluminescence (NEN Life Science, Boston, MA).

RESULTS

Clinical Features

To characterize the central nervous system pathology of IBMPFD, we analyzed brains from 8 individuals from 5 different families with mutations in the *VCP* gene (Table 1). Four of the 5 kindreds have been previously reported; 3 families (6 individuals) have the $^{R}155^{H}$ mutation, whereas one kindred (patient 2) has the $^{R}155^{C}$ mutation (1, 2). Patient 8 has the novel mutation $^{N}387^{H}$ (V. Kimonis, unpublished data). Of these 8 patients, 6 had clinical FTD (patients 2, 4, 5, and 6) or an uncharacterized dementia (patients 7 and 8). The FTD was characterized by early changes in behavior and personality (patients 2 and 4) or language dysfunction (patients 5 and 6). In all 4 patients with FTD, memory was preserved until late in the course of disease. In addition, 7 of the 8 patients analyzed had a myopathy, whereas 5 had PDB. Three of the patients with dementia also manifested clinical evidence of both muscle and bone disease, whereas 2 had FTD and IBM (patients 2 and 6) and one had both dementia and PDB (patient 7). In this latter patient (patient 7), although no clinical symptoms of myopathy were noted during life,

TABLE 2. Central Nervous System Pathology of Patients With Inclusion Body Myopathy and Paget Disease of Bone

Patient	Brain Weight (g)	Atrophy	CERAD*	Braak†	Tau Pathology	α-Syn Pathology	Other Pathology
1	1,300	None	NA‡	NA	0	0	None
2	1,022	Frontotemporal, moderate	0	0	Rare, focal	0	None
3	1,395	None	0	0	Mild to moderate, focal	Mild to marked	None
4	920	Frontal, moderate	B	II	Mild to marked	0	Microscopic infarct, subiculum
5	940	Frontal mild	A	0	Mild, focal	0	None
6	1,185	Diffuse, mild	A	0	0	0	None
7	890	Diffuse, marked	A	0	Mild, focal	0	None
8	1,190	Medial temporal lobe, mild	0	0	Mild, focal	0	None

*, CERAD, Consortium to Establish a registry for Alzheimer disease and represents a clinicopathologic assessment of senile plaque pathology (59).
†, Braak staging represents a pathologic scoring of neurofibrillary tangle pathology (60).
‡, NA, not applicable; only one section of neocortex was available for examination.

histopathologic evidence of IBM was identified at autopsy. Four of the patients with FTD were female and 2 were male; mean age at death was 57.5 years (range, 47–70 years) with a mean duration of disease of 6.8 years (range, 5–9 years).

Neuropathology of Inclusion Body Myopathy and Paget Disease of Bone

The average brain weight of the 6 FTD subjects was 1,025 g (range, 890–1,160 g) (Table 2). Cerebral atrophy was variable, ranging from mild, focal atrophy involving the frontal or medial temporal lobes to severe, diffuse atrophy (Table 2, Fig. 1). Ventricular enlargement correlated with the degree of cerebral atrophy. In one patient (no. 7), there was striking atrophy of the hippocampus and amygdala. In the more severely affected cases, the cortical ribbon was noticeably thinned. Subcortical nuclei, cerebellum, and brainstem were macroscopically unremarkable and the substantia nigra showed normal pigmentation in all cases. In contrast, the brains of the 2 patients without dementia were grossly unremarkable weighing 1,300 g (patient 1) and 1,395 g (patient 3).

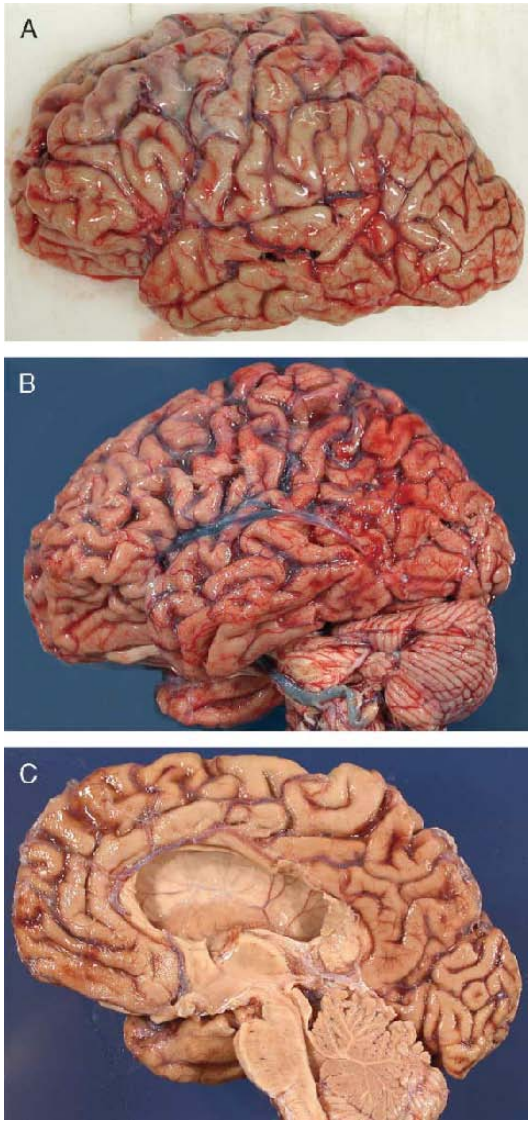

FIGURE 1. Cerebral atrophy in inclusion body myopathy and Paget disease of bone. **(A)** Lateral view of left cerebral hemisphere of patient 2. There was moderate atrophy of frontal lobes with relative sparing of parietal and occipital lobes. Lateral **(B)** and medial **(C)** aspects of the left cerebral hemisphere of patient 7. There was diffuse cerebral atrophy with enlargement of the lateral ventricles. The occipital lobe was relatively well preserved.

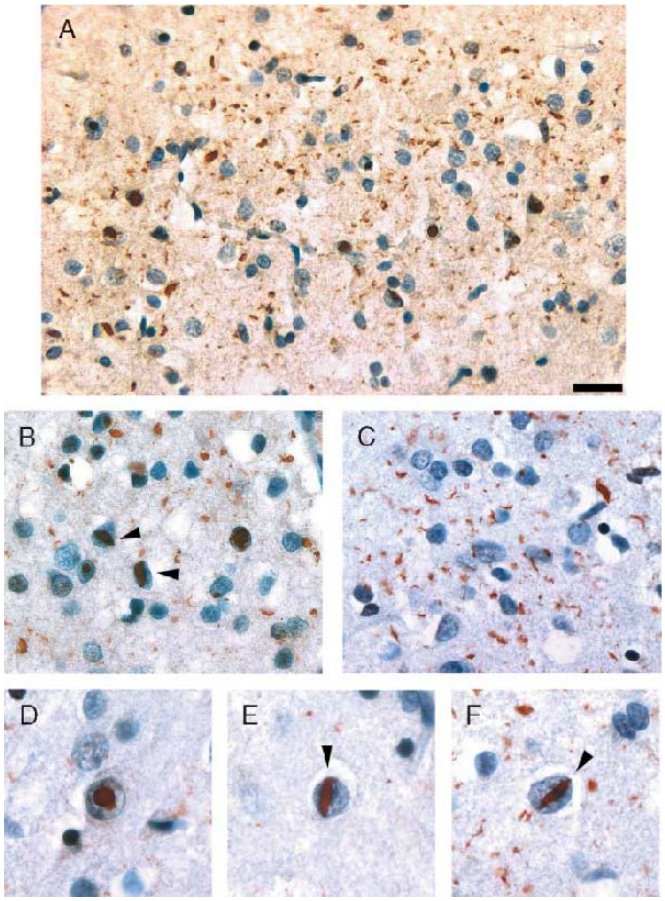

FIGURE 2. Ubiquitin-positive NII and dystrophic neurites in inclusion body myopathy and Paget disease of bone. Neocortex from patient 4 was immunostained with antibodies to ubiquitin. **(A–C)** There is extensive ubiquitin pathology **(A)** consisting of numerous intranuclear inclusions **(B)** and dystrophic neurites **(C)**. **(D–F)** High-power magnification of intranuclear inclusions with round **(D)** and lentiform morphology **(E, F)**. The intranuclear localization of the inclusions was supported by the distension of the nuclear membrane (arrowheads in **[B, E, F]**). Scale bar = **(A)** 40 μm; **(B, C)** 20 μm; **(D–F)** 10 μm.

In the patients with clinical symptoms of dementia, microscopy revealed variable superficial spongiosis, neuron loss, and gliosis in the neocortex and limbic structures with severity corresponding to the pattern of cerebral atrophy seen macroscopically. Hippocampal sclerosis was not identified in any of the cases. There was relative sparing of subcortical nuclei, brainstem, and cerebellum. Immunohistochemical

TABLE 3. Distribution of Ubiquitin Pathology in Inclusion Body Myopathy and Paget Disease of Bone

	Patient 2			Patient 3			Patient 4			Patient 5			Patient 6			Patient 7			Patient 8		
Region	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**
Superior/middle frontal gyrus	+	++	++	0	0	0	++	++++	++++	++	+++	++++	+	+	++	+	++++	+++	0	++	++
Superior/middle temporal gyrus	++	+++	++++	+	+++	++++	+	++++	++++	++	++++	++++	+	+++	++++	+	+++	+++	+	++++	++++
Inferior parietal lobule	0	++	+++	0	0	0	+	++++	++++	+	+++	++++	ND	ND	ND	+	++++	++++	0	+	0
Occipital lobe, calcarine cortex	0	0	0	0	+	0	++	++++	++++	+	++	++++	0	+	++	+	++++	+++	+	+++	+++
Cingulate gyrus, anterior	+	+	++	0	0	0	+	++	+++	0	+	++	ND	ND	ND	+	++	++	0	+	+
Hippocampus, CA1–4	0	0	0	0	0	0	0	0	+	0	0	++	0	0	0	0	0	0	0	0	0
Dentate fascia	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0	0	0	0	0	0	0
Entorhinal cortex	0	+	++	0	0	0	0	+	++	0	0	++	0	0	0	0	+	+	0	++	++
Amygdala	++	++	+++	+	++	+	++	++	+++	+	++	+++	ND	ND	ND	+	++	+++	0	+	+
Caudate*	0	+	+	0	+	+	0	++	+++	0	+	++	ND	ND	ND	++	++	++	ND	ND	ND
Putamen*	+	++	++	0	+	+	0	+	+	0	+	++	ND	ND	ND	0	+	+	0	+	0
Globus pallidus	0	++	+	0	+	0	0	++	++	0	+	+	ND	ND	ND	+	+	+	0	+	0
Nucleus basalis	0	+	++	0	+++	0	0	+	++	0	+	+	ND	ND	ND	ND	ND	ND	0	0	0
Thalamus	+	+	++	0	+	0	0	+	+	0	0	+	ND	ND	ND	0	++	++	0	+	+
Substantia nigra	+	++	+	0	+++	++	0	++	+	0	++	0	ND	ND	ND	0	+	0	0	+	0
Red nucleus	0	++	+	0	+	0	0	+	+	0	0	0	ND	ND	ND	0	0	0	ND	ND	ND
Oculomotor nucleus	0	++	0	0	++	0	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
Locus ceruleus	0	0	0	0	0	0	0	0	0	0	0	0	ND	ND	ND	0	0	0	0	0	0
Basis pontis	0	+	0	0	+	0	0	0	0	0	0	0	ND	ND	ND	0	0	0	0	0	0
Inferior olives	0	+	0	0	++	0	0	0	0	0	0	0	ND	ND	ND	0	0	0	0	0	0
Hypoglossal nucleus	0	+	+	0	0	0	0	0	0	0	0	0	ND	ND	ND	0	0	0	0	0	0
Cerebellum, cortex	0	+	0	0	0	0	0	0	0	0	0	0	ND	ND	ND	0	0	0	0	0	0
Dentate nucleus	0	0	0	0	0	+	0	0	0	0	0	0	ND	ND	ND	0	0	0	0	0	0

No ubiquitin pathology was identified in the single tissue section of unspecified neocortex that was available from patient 1.
*, Occasional nuclear inclusions were detected in cells morphologically consistent with glia in white matter bundles of the basal ganglia.
NCI, neuronal cytoplasmic inclusions; DN, dystrophic neurites; NII, neuronal intranuclear inclusions; ND, not done.
0, None; +, rare; ++, low density; +++, moderate density; ++++, high density.

analysis for β-amyloid and tau demonstrated a low density of senile plaques in 3 patients with FTD as well as a low density of tau-positive cytoplasmic inclusions in 4 patients with FTD consisting predominantly of pretangles in neurons that were not detected with thioflavin S stains. In these 4 patients, rare pretangles were detected with Gallyas silver stains. More extensive tau pathology was found in one FTD subject (patient 4, Table 2). In this individual, there was a moderate density of neocortical neuritic senile plaques and neurofibrillary tangle pathology restricted to the mesial temporal lobe (Braak stage II). In addition, patient 4 showed accumulation of tau-positive inclusions in both neurons (pretangles) and glia in a number of different brain regions (severe in amygdala, moderate in pontine tegmentum, and mild in midbrain and frontal cortex and white matter). Only a subset of the tau-positive lesions was identified with Gallyas silver stains and the lesions were not detected using thioflavin S or Bielschowsky silver stains. The tau pathology did not meet neuropathologic criteria for any of the defined tauopathies (6).

No pathologic inclusions were identified with immunohistochemistry for neurofilament or α-internexin. α-synuclein immunohistochemistry was negative in the patients with dementia but did demonstrate pathology in one individual with IBM who did not clinically manifest either dementia or parkinsonism (patient 3). In this case, Lewy bodies and Lewy neurites were present in the nucleus basalis (high density), limbic structures, including cingulate gyrus and amygdala (moderate density), and brainstem (moderate density in substantia nigra, pontine tegmentum/locus ceruleus, and medulla). Only rare Lewy body pathology was identified in the neocortex. There was no appreciable neuron loss in the regions affected by Lewy body pathology. Thus, in the absence of both dementia and extrapyramidal symptoms, patient 3 most likely represents preclinical limbic Lewy body disease (28).

Ubiquitin Pathology in Inclusion Body Myopathy and Paget Disease of Bone

The most striking feature in the brains of all of the FTD subjects with 3 different *VCP* gene mutations (Table 1) was the presence of abundant ubiquitin-positive NII and dystrophic neurites in a variety of brain regions (Fig. 2; Table 3). These NII and dystrophic neurites were not labeled with antibodies to tau, α-synuclein, β-amyloid, neurofilament, α-internexin, or polyglutamine expansions, and they were not argyrophilic (data not shown). The NII had a characteristic lentiform or rod shape and were either straight or slightly curved (Fig. 2). NII with a round shape may represent lentiform inclusions viewed in cross-section. Alternatively, the round inclusions may represent a second, less abundant type of NII. The intranuclear localization of these inclusions was supported by distension of the nuclear membrane (Fig. 2, arrowheads). Although the majority of the intranuclear inclusions were observed in small neurons, rare lentiform intranuclear inclusions were identified in small nuclei within white matter. Although these inclusions could be in ectopic small neurons, the nuclear morphology was most consistent with astrocytes (Fig. 3). Unfortunately, this association was too infrequent to allow more definitive characterization. The ubiquitin-positive neuritic pathology was more robust than that detected in some cases of normal aging (29) and had a characteristic laminar cortical distribution, similar to that observed in cases of sporadic FTLD-U. Dense oval ubiquitin-immunoreactive neuronal cytoplasmic inclusions were only infrequently identified and were not present in the dentate gyrus of the hippocampus (Fig. 3; Table 3). This pattern of ubiquitin pathology was present in all 6 patients with IBMPFD with dementia, as well one subject (patient 3) who was cognitively intact at the time of his last clinical examination (Table 3). In this nondemented individual, the ubiquitin pathology had the same morphology and distribution as that seen in patients with dementia, but was less robust, suggesting a preclinical stage of disease. There was no ubiquitin immunoreactivity seen in the brain of the other nondemented individual evaluated (patient 1); however, only a single section of neocortex was available for examination from this individual.

The severity of the ubiquitin pathology was assessed using a semiquantitative grading scheme (Table 3) (18). Ubiquitin-positive dystrophic neurites and NII were most abundant in the neocortex, and a high density of pathology was consistently detected in the superior/middle temporal

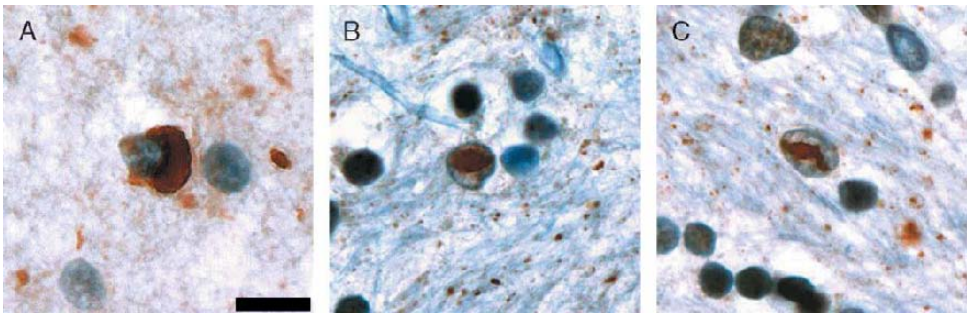

FIGURE 3. Ubiquitin pathology in inclusion body myopathy and Paget disease of bone. Neocortex from patient 5 **(A)** and basal ganglia from patient 4 **(B, C)** were immunostained with antibodies to ubiquitin. Only rare cytoplasmic inclusions were identified **(A)** that are characteristic features of both sporadic and familial frontotemporal lobar degeneration with ubiquitin-positive inclusions. Occasional small lentiform inclusions were present in white matter bundles of the basal ganglia in cells morphologically consistent with astrocytes **(B, C)**. Unfortunately, the low density of these inclusions in white matter precludes more definitive characterization. Scale bar = 10 μm.

TABLE 4. Comparison of Ubiquitin Pathology in Inclusion Body Myopathy and Paget Disease of Bone (IBMPFD) with Sporadic and Familial Frontotemporal Lobar Degeneration With Ubiquitin-Positive Inclusions (FTLD-U)

	Sporadic FTLD-U (n = 9)			Autosomal-Dominant FTLD-U (n = 10)			IBMPFD (n = 6)		
Region	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**	**NCI**	**DN**	**NII**
Neocortex frontal lobe	++	++	0	+++	+++	++	+	+++	+++
Neocortex temporal lobe	++	++	0	+++	+++	++	+	++++	++++
Neocortex parietal lobe	+	++	0	++	+++	+	+	+++	+++
Neocortex occipital lobe	+	+	0	+	+	0	+	++	+++
Dentate fascia	+++	0	0	++	0	+	0	0	0
Hippocampus CA1–4	0	0	0	0	0	0	0	0	+
Caudate	++	+++	0	++	+++	++	+	++	++
Putamen	++	++	0	++	+++	++	0	+	+
Globus pallidus	0	+	0	+	+	0	0	+	+
Substantia nigra	+	0	0	++	0	0	0	++*	0
Basis pontis	0	0	0	0	0	0	0	0	0
Inferior olives	0	0	0	+	+	0	0	0	0
Cerebellum	0	0	0	0	0	0	0	0	0

The semiquantitative scoring represents the average value in the indicated region for the number (n) cases indicated.
*, Dystrophic neurites are common in finding in the substantia nigra in aging.
NCI, neuronal cytoplasmic inclusions; DN, dystrophic neurites; NII, neuronal intranuclear inclusions; ND, not done.
0, None; +, rare; ++, low density; +++, moderate density; ++++, high density.

gyri, including in patient 3 without clinically evident dementia. Frontal, parietal, and occipital lobes were also affected to varying degrees in all 6 patients with FTD with 3 different *VCP* gene mutations. Both NII and dystrophic neurites were most numerous in the upper cortical layers, but NII were also present in neurons through the entire cortical thickness. The hippocampus, including the dentate gyrus, was relatively spared with only rare NII seen in pyramidal neurons (2 cases) and in small granular neurons of the dentate gyrus (one case). No neuronal cytoplasmic inclusions were present in dentate granule cells. Subcortical regions showed less severe pathology than the neocortex with the regions most frequently involved being the amygdala, basal ganglia, thalamus, and substantia nigra (Table 3). Ubiquitin-immunoreactive pathology was only rarely identified in the brainstem and cerebellum and there was no evidence of lower motor neuron disease in the hypoglossal or dorsal motor nucleus.

The morphology, distribution, and density of ubiquitin pathology in the IBMPFD cases was compared with a well-characterized cohort of patients with sporadic and familial FTLD-U (Table 4) (18). All 3 groups showed a similar anatomic distribution of dystrophic neurites. However, cortical involvement was more extensive in the IBMPFD cases than in FTLD-U, especially when compared with the sporadic FTLD-U cohort. Notably, in IBMPFD the occipital lobe consistently showed more pathology than either sporadic or autosomal-dominant FTLD-U in which ubiquitin-immunoreactive pathology was rare. In contrast, the striatum tended to be less severely involved in IBMPFD. Important differences also included the presence of more numerous neuronal cytoplasmic inclusions in the non-IBMPFD cases and the consistent absence of cytoplasmic inclusions in the dentate granule cells of the hippocampus in IBMPFD, a finding that is considered characteristic of most cases of FTLD-U (18). The lentiform morphology of NII and their presence in small neurons in IBMPFD was similar to that seen in cases of familial FTLD-U (7, 16, 17), but, neurons containing NII were much more numerous in all cases of IBMPFD than in any of the familial FTLD-U cases. Moreover, the presence of rare intranuclear inclusions in what appear to be glial cells is a finding that was unique to the IBMPFD cases.

Characterization of *Valosin-Containing Protein* in Inclusion Body Myopathy and Paget Disease of Bone

To determine if *VCP* gene mutations lead to *VCP* inclusions and/or aggregation, we immunostained sections from affected brain regions with a 3 different antibodies to *VCP* (2 monoclonal and one polyclonal antibodies) using a variety of antigen retrieval methods (see "Materials and Methods"). In contrast to a previous case report (19), only rare NII (<1%) were detected with antibodies to *VCP*, and these inclusions lacked the characteristic lentiform morphology observed in many of the ubiquitin-positive NII (Fig. 4). This finding suggests that ubiquitin and *VCP* immunohistochemistry may not recognize the same intranuclear structures. This interpretation is difficult to confirm, however, as a result of the extremely low frequency of *VCP*-positive inclusions. The neuritic pathology was not detected with any of the antibodies to *VCP*.

To determine if mutations in *VCP* leads to altered protein solubility or aggregation, we extracted protein from gray and white matter of frontal cortex from patients with IBMPFD as well as sporadic FTLD-U and age-matched controls. Although immunoblotting showed variability in the amount of VCP detected in all brains analyzed, there was no consistent alteration in VCP solubility in IBMPFD (Fig. 4C). In addition, we detected only a single protein band of approximately 97 kDa, consistent with the predicted molecular weight. We did not identify higher molecular weight

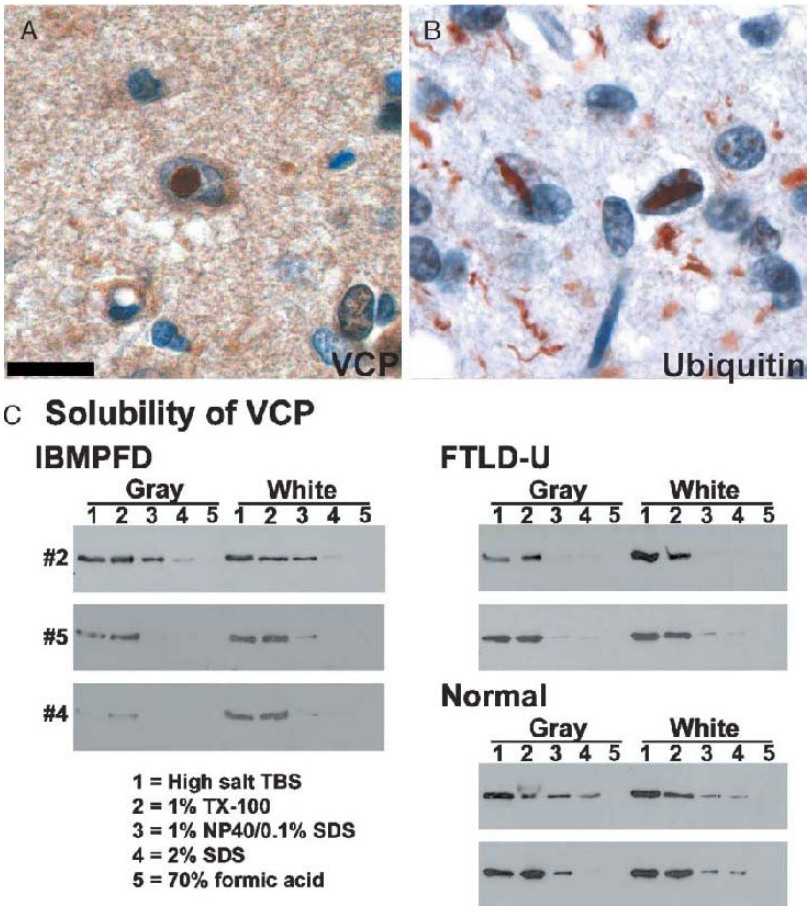

FIGURE 4. Characterization of *valosin-containing protein (VCP)* in inclusion body myopathy and Paget disease of bone (IBMPFD). Temporal neocortex from patient 7 was immunostained with antibodies to antibodies to *VCP* **(A)** and ubiquitin **(B)**. Only rare NII that lack the characteristic lentiform morphology were detected with a panel of antibodies to *VCP* representing a very small subset of the pathology detected with ubiquitin. Dystrophic neurites were not detected with any of the anti-*VCP* antibodies. Scale bar = 10 μm. **(C)** Gray and white matter from frontal cortex of IBMPFD, frontotemporal lobar degeneration with ubiquitin-positive inclusions, and control patients was sequentially extracted with buffers of increasing extraction strength. Extract corresponding to 20 mg of initial wet tissue weight were resolved by SDS-PAGE and immunoblotted with antibodies to *VCP*. There was no consistent alteration in *VCP* solubility or evidence of *VCP* protein aggregation in patients with IBMPFD.

species, indicative of protein aggregation and/or ubiquitination. This suggests that *VCP* is not the major ubiquitinated component of either the NII or neuritic pathology, and it is likely that the ubiquitin-positive pathology is composed of some other, yet unidentified, protein(s).

DISCUSSION

IBMPFD is a rare autosomal-dominant progressive disorder caused by mutations in the *VCP* gene (2). To date, there is only limited information on the pathology associated with each of the clinical features of this disorder. The IBM is characterized by adult-onset proximal and distal muscle weakness, clinically resembling limb girdle myopathy (1). Muscle biopsies from affected individuals show nonspecific myopathic changes, including variation in muscle fiber size, mildly increased endomysial connective tissue, and focal regions with "myopathic grouping" and rimmed vacuoles in a subset of patients (2). Immunohistochemical analysis has revealed small and large aggregates of *VCP* in scattered muscle fibers, including those without rimmed vacuoles or other morphologic changes (2). Intranuclear inclusions in muscle have not been reported in IBMPFD. The PDB associated with IBMPFD is characterized by an early age at onset (42 years compared with 50–55 years for sporadic PDB), but with the characteristic distribution of pathology in the spine, pelvis, and skull, and elevated alkaline phosphatase that is similar to sporadic disease (1). Detailed descriptions of the bone pathology in IBMPFD have not yet been published.

The dementia in IBMPFD typically presents later than the other clinical features, with a mean age at onset of 54 (the mean age at onset in the 6 patients in our study was 51 years). The cognitive alterations are typical of FTD, characterized by early and progressive changes in language, behavior, and personality with relative preservation of memory (1). There is frequent impairment of social functioning, disinhibition, apathy, and lack of insight. The first publication on the neuropathology of IBMPFD indicated only nonspecific changes (1). Recently, Schröder et al described the postmortem neuropathology in a 55-year-old German patient with an $^{R}155^{C}$ mutation and clinical IBM and FTD (19). There was severe atrophy of the frontal and temporal lobes as well as the striatum. Histopathologic examination revealed neuron loss and gliosis throughout the neocortex with intranuclear inclusions in some surviving neurons that were immunoreactive for both ubiquitin and *VCP*. A detailed description of the morphology, frequency, and anatomic distribution of the ubiquitin pathology was not provided in that case report; however, there appear to be several differences from what we have found. NII were only seen in "a few" neurons that were "predominantly pyramidal" (19). The shape of the NII was not described, but the figures

show what appear to be round structures, not lentiform. Finally, although it was indicated that there were no cytoplasmic inclusions, there is no comment as to the presence or absence of neuritic pathology.

In the current study, we provide the first detailed description of the neuropathology in a series of patients with IBMPFD, including a detailed assessment of the morphology, anatomic distribution, and areal density of the neuropathologic changes. All 6 subjects with clinical FTD or dementia had some degree of brain atrophy with associated neuron loss, spongiosis, and gliosis. Immunohistochemical analysis revealed a unique pattern of FTLD-U pathology with extensive ubiquitin-positive intranuclear inclusions and dystrophic neurites throughout the neocortex that was most severe in the temporal lobe. The inclusions were negative for tau, α-synuclein, and expanded polyglutamine repeats. This pattern of pathology was consistent in all subjects with FTD, regardless of the specific *VCP* gene mutation. The presence of similar but less robust pathology in one individual without dementia is interpreted as representing a preclinical stage of disease. This finding suggests the possibility that the relatively low frequency of clinical FTD in IBMPFD (approximately 30%) may be a reflection of the early age at death in most patients that occurs before the clinical manifestation of brain pathology.

We have also shown that the central nervous system pathology of IBMPFD is distinctive from that observed in other cases of FTD, including both sporadic and familial FTLD-U (17, 18, 30). The consistent presence of numerous ubiquitin-immunoreactive NII is the key diagnostic feature of FTD in IBMPFD. NII tend to be absent in sporadic FTLD-U, although 2 recent studies reported NII in up to 44% of sporadic cases. However, in these studies, the density of the NII is either low or not reported and the anatomic distribution may have been much more limited (i.e. striatum only) (31, 32). Several other studies have shown NII, with a similar morphology, to be a characteristic finding in a subset of familial FTLD-U; however, they are never as numerous as we observed in IBMPFD (16, 17). Furthermore, in contrast to FTLD-U, IBMPFD has only mild pathology in the hippocampus and in subcortical nuclei. The dentate gyrus tends to be spared in IBMPFD, whereas this is a characteristic area of involvement in FTLD-U. The low frequency of neuronal cytoplasmic inclusions in IBMPFD is also in contrast with FTLD-U in which they are considered the characteristic feature. Finally, the neuritic pathology often involves a wider anatomic distribution in the cerebral cortex in IBMPFD, including involvement of the occipital lobe that is typically spared in FTLD-U.

Schröder et al showed colocalization of *VCP* and ubiquitin in NII in their description of a demented patient with IBMPFD (19). With a panel of 3 different antibodies to *VCP*, including the same polyclonal antibody used in that previous report, we detected *VCP* in only rare NII in our cases and these inclusions did not exhibit the characteristic lentiform morphology observed in many of the NII with the ubiquitin immunohistochemistry. Furthermore, the dystrophic neurites were consistently negative. The differences in immunohistochemical staining between our results and those reported by Schröder et al may represent technical differences, particularly because our finding is that of an absence of staining. However, in Schröder et al, it is unclear what fraction of the NII show colocalization of both *VCP* and ubiquitin. Alternatively, there may be 2 types of NII, lentiform and round, and only the round NII are detected with antibodies to both *VCP* and ubiquitin. We were also unable to detect any biochemical alterations in *VCP* solubility or *VCP* aggregation in IBMPFD. This finding is in contrast to other neurodegenerative diseases characterized by protein aggregation such as the tauopathies and synucleinopathies that show prominent changes in protein solubility and posttranslational modification, including ubiquitination and crosslinking (33).

VCP is a 97-kDa ubiquitous member of the AAA-ATPase supergene family (34). The enzyme consists of 2 ATPase domains and an N-terminal domain that provide substrate specificity and are separated by flexible linkers (3). *VCP* functions as a molecular chaperone in a plethora of distinct cellular activities, including cell-cycle regulation, homotypic membrane fusion, nuclear envelope reconstruction, postmitotic Golgi reassembly, stress responses, programmed cell death, and ubiquitin-dependent protein degradation (3, 4). Almost all of these activities are directly or indirectly regulated by the UPS. *VCP* forms a homohexamer that binds to multiple ancillary proteins associated with UPS activity through the N-terminal domain. This *VCP* complex binds to polyubiquitin chains and untethers ubiquitinated proteins from their binding partners, thereby facilitating transport to the UPS. The loss of *VCP* function leads to accumulation of polyubiquitinated proteins (23, 35). Moreover, several studies have directly implicated *VCP* in endoplasmic reticulum-associated degradation whereby defective/abnormally folded and short-lived ER proteins are rapidly degraded. This function of *VCP* in endoplasmic reticulum-associated degradation is dependent on its binding partners Ufd1 and Np14 (36–39).

Before the identification of *VCP* gene mutations in IBMPFD, *VCP* was only indirectly implicated in the pathogenesis of neurodegenerative diseases. Specifically, *VCP* was found in a small proportion of the pathologic lesions in Alzheimer disease (senile plaques), Parkinson disease (Lewy bodies), amyotrophic lateral sclerosis (MND inclusions), and polyglutamine repeat diseases (intranuclear inclusions) (40–42). Experimentally, *TER94*, the *Drosophila* homolog of *VCP*, has been shown to modulate polyglutamine-induced neurodegeneration (43). Finally, investigations into endoplasmic reticulum-associated degradation indicate that dysfunction of *VCP* resulted in vacuole and inclusion body formation, thereby leading to cell death (40, 44, 45). Our data showing accumulation of ubiquitin inclusions that are not composed of *VCP* support the hypothesis that *VCP* gene mutations in IBMPFD lead to a dominant negative loss or alteration of *VCP* function such as impairment of UPS activity. This hypothesis is supported by the finding that assembly of *VCP* into the hexameric state is a prerequisite for biological function and that mutations in the ATPase domain have a dominant negative effect on *VCP* activity (39, 46, 47). More recently, Weihl et al demonstrated that

several of the *VCP* gene mutations directly impair endoplasmic reticulum-associated degradation in a cell culture model (48). Furthermore, most mutations in IBMPFD cluster in the N-terminal domain, the region that is involved in substrate binding including ubiquitin, Ufd1 and Np14 (2). However, an explanation for the intranuclear localization of many of the inclusions is not apparent. Interestingly, mutations of the ubiquitin binding domain of *sequestosome-1* (*SQSTM1*) cause autosomal-dominant PDB similar to *VCP* gene mutations (49, 50). Both *VCP* and sequestsome-1 regulate NF-κB function and thus, mutations may cause defects in similar UPS-dependent signaling pathways (51, 52).

The discovery that *VCP* is critical to UPS activity may provide insight into the role of mutations in the *VCP* gene in aggregate formation in other neurodegenerative diseases. Alterations in UPS function have been implicated in the pathogenesis of a variety of sporadic and familial neurodegenerative diseases, including Parkinson disease, Alzheimer disease, polyglutamine repeat diseases, and amyotrophic lateral sclerosis (53). In some instances, a direct linkage between UPS dysfunction and resulting pathology has been established. For instance, in approximately 50% of autosomal-recessive juvenile-onset Parkinson disease, there are mutations in *parkin*, an E3 ligase involved in ubiquitin conjugation to target substrate (54). In some patients with Alzheimer disease, frameshift mutations in the *ubiquitin B* gene leads to translation of nonsense ubiquitin peptide sequences that block proteasomal degradation (55). However, because aggregated proteins have been demonstrated to directly impair the UPS, it remains unclear whether UPS dysfunction is a primary cause or secondary consequence of protein aggregation.

The hypothesis that *VCP* gene mutations affect UPS function and protein degradation is indirectly supported by the finding of additional pathologies, albeit at low levels, in many of the brains of affected individuals. For example, despite a relatively early age at death in our patients (mean, 58 years; range, 47–70 years), Alzheimer disease pathology (senile plaques and tau inclusions) were relatively common findings (Table 2). In addition, patient 2, who died at age 52 without a history of dementia or a movement disorder, had extensive Lewy body and tau pathology in limbic and brainstem regions. Thus, the consequence of *VCP* mutations may be a generalized defect in the regulation of protein turnover leading to the accumulation of ubiquitinated proteins and FTLD-U pathology in the central nervous system. However, the disease phenotype is likely modulated by additional genetic and environmental factors leading to heterogeneity in clinical and pathologic features as observed between families carrying the same *VCP* gene mutation as well as within the same kindred.

At least 2 additional genetic loci, distinct from *VCP*, have been identified in familial FTLD-U (9q21–22 [56] and 17q21 [15, 57]). Furthermore, mutations in *CHMP2B*, encoding a component of the endosomal ESCRTIII complex, were recently identified in large Danish pedigree with autosomal-dominant FTD; however, the pathology in this family is most consistent with dementia lacking distinctive histopathology (58). Whether mutations in these as yet unidentified genes cause disease by 1) the accumulation of their abnormal protein product similar to many other neurodegenerative diseases such as Alzheimer disease, the tauopathies, and synucleinopathies; 2) through a generalized defect in protein ubiquitination and degradation; or 3) through another undisclosed perturbation remains unknown. Furthermore, the role, if any, of *VCP* in the pathogenesis of sporadic FTLD-U remains to be determined.

ACKNOWLEDGMENTS

The authors are indebted to the altruism and support of the patients and their caregivers who have facilitated the research described in this study. The authors thank Ms. Theresa Schuck for her technical assistance.

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