Phenotypic Variability Within the Inclusion Body Spectrum of Basophilic Inclusion Body Disease and Neuronal Intermediate Filament Inclusion Disease in Frontotemporal Lobar Degenerations With FUS-Positive Inclusions

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Abstract
Basophilic inclusion body disease and neuronal intermediate filament inclusion disease (NIFID) are rare diseases included among frontotemporal lobar degenerations with FUS-positive inclusions (FTLD-FUS). We report clinical and pathologic features of 2 new patients and reevaluate neuropathologic characteristics of 2 previously described cases, including an early-onset case of basophilic inclusion body disease (aged 38 years) with a 5-year disease course and abundant FUS-positive inclusion bodies and 3 NIFID cases. One NIFID case (aged 37 years) presented with early-onset psychiatric disturbances and rapidly progressive cognitive decline. Two NIFID cases had later onset (aged 64 years and 70 years) and complex neurologic deficits. Postmortem neuropathologic studies in late-onset NIFID cases disclosed α-intermixin-positive “hyaline conglomerate”-type inclusions that were positive with 1 commercial anti-FUS antibody directed to residues 200 and 250, but these were negative to amino acids 90 and 220 of human FUS. Early-onset NIFID had similar inclusions that were positive with both commercial anti-FUS antibodies. Genetic testing performed on all cases revealed no FUS gene mutations. These findings indicate that phenotypic variability in NIFID, including clinical manifestations and particular neuropathologic findings, may be related to the age at onset and individual differences in the evolution of lesions.

Key Words: α-Intermixin, Basophilic inclusion body disease, Frontotemporal lobar degeneration, FUS, Hyaline conglomerates, Immunohistochemistry, Neuronal intermediate filament inclusion disease.

INTRODUCTION
Recent consensus recommendations concerning frontotemporal lobar degeneration (FTLD) have grouped 3 disorders among the FTLD-FUS subgroup, all characterized by frontotemporal and variable subcortical neuronal degeneration, gliosis, superficial spongiosis, and the presence of FUS protein aggregates on histopathologic examination (1). These diseases comprise basophilic inclusion body disease (BIBD), neuronal intermediate filament inclusion disease (NIFID), and atypical FTLD with ubiquitin-positive inclusions (FTLD-U).

“Basophilic inclusion body disease” is the descriptive term for a disease characterized histologically by the presence of widespread round intraneuronal cytoplasmic basophilic inclusion bodies. These are mainly composed of abnormal FUS protein aggregates that are detectable by immunohistochemistry. “Neuronal intermediate filament inclusion disease” has been defined by the presence of intraneuronal inclusion bodies composed of neuronal intermediate filaments immunoreactive for α-intermixin and variably for FUS protein (1–3).

As in other neurodegenerative diseases, clinical manifestations depend on the distribution of the pathologic alterations in the CNS. Accordingly, BIBD and NIFID can manifest with behavioral and/or cognitive alterations, but also with motor dysfunction, such as parkinsonism and other abnormal movements, pyramidal signs, and a variety of other neurologic manifestations during the disease course when other cortical and/or subcortical structures such as caudate nucleus, substantia nigra, or motor neurons are involved (4–6).

To date, only a few cases of BIBD have been described (7–9). In recent comparative studies of 8 patients, 4 had the diagnosis of behavioral variant of frontotemporal dementia (bvFTD), which was associated with motor neuron disease in 2 patients and with parkinsonism in 1 patient. Two additional
patients were diagnosed with amyotrophic lateral sclerosis (ALS); 1 with progressive supranuclear palsy, and 1 with dementia with parkinsonism (8, 9). Reported mean age of onset for BIBD was 46.4 years (range, 29–56 years); mean age at death was 54.1 years (range, 39–68 years); and mean disease duration was 7.7 years (range, 5–12 years). None of the cases had a positive family history. In addition, in NIFID cases, disease onset is usually early, generally younger than 60 years, although later disease onset has also been described (6). Some of the reported cases had a family history of poorly defined neurologic disorder. In previous detailed reports of sporadic NIFID cases, all had an early age of onset (23–58 years), short disease duration (3–7 years; 1 with a duration of 13 years), and presented with combinations of bvFTD, memory and language deficits, motor neuron involvement, and parkinsonism during the disease course (3, 8, 10). Despite the clinical and pathologic variability of previously reported cases, common clinical and morphologic features have led NIFID to be considered as a distinct entity, although authors argue over the possible subgroups.

Here, we focus on BIBD and NIFID and report clinical and pathologic aspects of 2 new patients. We also reevaluated neuropathologic characteristics of 2 previously described cases that are currently classified as FTLD-FUS but showed different features. Particular focus is placed on inclusion body morphology and the results of FUS immunohistochemistry.

**MATERIALS AND METHODS**

**Patient Data**

Clinical data of the 4 patients were obtained from the treating neurologists at several centers that specialize in neurodegenerative diseases.

**Neuropathology**

Neuropathologic workup was performed at the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS according to standardized procedures. The Neurological Tissue Bank is based on a brain donor program in which patients and/or next of kin gives his or her informed consent for brain removal after death for diagnostic and research purposes. The left brain hemisphere and hemicerebellum of the 4 cases were sliced in the fresh state in 0.5- to 1-cm-thick coronal sections and frozen at −80°C. The right hemisphere, hemicerebellum, and alternate sections of the brainstem were fixed in 10% buffered formaldehyde solution for 4 weeks. For histopathologic evaluation, 5-μm-thick sections were cut from formalin-fixed and paraffin-embedded tissue from multiple brain areas of the right hemisphere, including frontal, temporal, parietal, and occipital cortices; motor cortex; anterior cingulate; anterior and posterior basal ganglia; anterior, medial, and posterior thalamic nuclei; hippocampus and parahippocampal gyrus; amygdala; nucleus basalis Meynert; midbrain;pons; medulla oblongata; olfactory bulb; cerebellar vermis; and dentate nucleus; as well as cervical segments of spinal cord in 3 cases and thoracic and lumbar spinal cord in 1 case.

Sections were stained with hematoxylin and eosin, Luxol fast blue, and for immunohistochemistry using the following monoclonal (mc) and polyclonal (pc) primary antibodies on an automated immunostainer (DAKO Autostainer Plus; DAKO, Glostrup, Denmark) after heat- or chemically induced epitope retrieval with formic acid: anti-β-amyloid (dilution 1:400, mc, clone 6F/3D; DAKO), anti-phosphorylated tau (dilution 1:200, mc, clone AT8; Thermo Scientific, Rockford, IL), ubiquitin (dilution 1:400, pc; DAKO), α-synuclein (dilution 1:500, mc, clone KM51; Novocastra, Newcastle, UK), TDP-43 (dilution 1:500, mc, clone 2E2-D3; Abnova, Taipei, Taiwan), neurofilaments (dilution 1:800, clone RT97; Novoceastra), anti-RD3 (dilution 1:1000, mc, clone 8E6/C11; Millipore, Temecula, CA), anti-RD4 (dilution 1:50, mc, clone 1E1/A6; Millipore), anti-α-intermixin (dilution 1:800, mc, clone 2E3; Invitrogen, CA), anti-α-B-crystallin (dilution 1:100, mc, clone G2JF; Novocastra), and anti-p62 (dilution 1:500, mc, clone 3/62Ick ligand; BD Transduction Laboratories TM, Franklin Lakes, NJ).

Anti-FUS immunohistochemistry was performed using anti-FUS A (dilution 1:1000, pc; Sigma Aldrich HPA008784, St. Louis, MO; incubated overnight at 4°C after heat-induced epitope retrieval in EDTA at pH 9) and anti-FUS B (dilution 1:500, pc, Lifespan Biosciences, Seattle, WA; incubated overnight at 4°C after heat-induced epitope retrieval in citrate buffer at pH 6).

The reported antigenic peptide of the Sigma antibody maps to a region between residues 90 and 220. The reported antigenic peptide of the Lifespan antibody maps to a region between residues 200 and 250 of human fusion (involved in t(12;16) in malignant liposarcoma) using the numbering given in SwissProt entry P35637 (GeneID 2521).

To compare immunoreactivity patterns of the 2 antibodies in different diseases, we selected 1 case of each of the following: hippocampus of Alzheimer disease with hyperphosphorylated tau-positive neurofibrillary tangles and neuritic plaques/mature β-amyloid deposits, hippocampus of Pick disease with 3-repeat tau-positive Pick bodies, frontal cortex of corticobasal degeneration with 4-repeat tau-positive astrocytic plaques, amygdala of argyrophilic grain disease with 4-repeat tau-positive grains, frontal cortex of FTLD-TDP43 of 1 sporadic and 1 genetic case with progranulin mutation with neuronal cytoplasmic and nuclear cat-eye inclusions, spinal cord of ALS with TDP43-positive skeinlike inclusions in motor neurons of anterior horn, medulla oblongata of Parkinson disease with α-synuclein immunoreactive Lewy bodies and Lewy neurites in dorsal nucleus of vagal nerve, striatum of multiple system atrophy with α-synuclein–positive glial cytoplasmic inclusions (Papp-Lantos inclusions), frontal cortex of Huntington disease with ubiquitin/p62 and anti-polyQ–positive neuronal nuclear and some granular cytoplasmic inclusions, cerebellum of sporadic Creutzfeldt-Jakob disease (CJD) with Kurin plaques (MV2K subtype), and frontal cortex of a sporadic CJD case with confluent vacuoles and patchy perivascular prion protein deposits (MM2C subtype), and a case without neurologic disease but with mild diffuse cortical gliosis.

**Genetic Studies of FUS**

Total genomic DNA was obtained from frozen brain tissue using the Qiagen DNiese Kit (Valencia, CA) according to the manufacturer’s instructions. Mutation screening was
**TABLE. Summary of Patient Clinical and Neuropathologic Data**

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age of onset, years</strong></td>
<td>38</td>
<td>37</td>
<td>64</td>
<td>70</td>
</tr>
<tr>
<td><strong>Age at death, years</strong></td>
<td>43</td>
<td>44</td>
<td>64</td>
<td>75</td>
</tr>
<tr>
<td><strong>Disease duration</strong></td>
<td>5 years</td>
<td>7 years</td>
<td>5 months</td>
<td>5 years</td>
</tr>
<tr>
<td><strong>Family history of neurologic disease</strong></td>
<td>Yes, mental retardation</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Symptoms at onset</strong></td>
<td>Apathy, aggressiveness, altered eating behavior</td>
<td>Apathy, indifference</td>
<td>Behavioral alterations, psychiatric symptoms</td>
<td>Asymmetric parkinsonism</td>
</tr>
<tr>
<td><strong>Final clinical diagnosis</strong></td>
<td>bvFTD-Pick disease</td>
<td>bvFTD</td>
<td>Diffuse encephalopathy, suspected autoimmune encephalitis</td>
<td>Atypical parkinsonism</td>
</tr>
<tr>
<td><strong>Unfixed brain weight</strong></td>
<td>960 g</td>
<td>1200 g</td>
<td>1200 g</td>
<td>1040 g</td>
</tr>
<tr>
<td><strong>Gross atrophy distribution</strong></td>
<td>Severe frontal, temporal, parietal atrophy with marked involvement of basal ganglia, limbic system, and marked nigral pallor</td>
<td>Severe frontotemporal atrophy with involvement of basal ganglia and mild nigral pallor</td>
<td>No evident gross atrophy</td>
<td>Moderate global atrophy with a prominent parietal involvement and mild involvement of basal ganglia; mild nigral pallor</td>
</tr>
<tr>
<td><strong>Histology: neuronal loss and gliosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Cingulum</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus CA1</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Striatum</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Medial thalamus</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>+++</td>
<td>++</td>
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<td>++</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Basis pontis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Inferior olives</td>
<td>+</td>
<td>++</td>
<td>+</td>
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</tr>
<tr>
<td>Dentate nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anterior horn spinal cord</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Hippocampal findings</strong></td>
<td>Hippocampal sclerosis</td>
<td>Hippocampal sclerosis</td>
<td>Hippocampus preserved</td>
<td>Hippocampus preserved</td>
</tr>
<tr>
<td><strong>Type of neuronal cytoplasmic inclusion</strong></td>
<td>Basophilic inclusions only</td>
<td>Basophilic, eosinophilic, cherry spots, and hyaline conglomerates</td>
<td>Hyaline conglomerates only</td>
<td>Hyaline conglomerates only</td>
</tr>
<tr>
<td><strong>Ubiquitin/p62 IHC</strong></td>
<td>Variable</td>
<td>Variable</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td><strong>Alpha-internexin IHC</strong></td>
<td>Negative</td>
<td>Variably positive depending on inclusion type</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>FUS A IHC pre-treatment with EDTA at pH9</strong></td>
<td>Positive</td>
<td>Variably positive depending on inclusion type</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>FUS B IHC pre-treatment with citrate buffer at pH6</strong></td>
<td>Positive</td>
<td>Variably positive depending on inclusion type</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td><strong>FUS gene mutations</strong></td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Final diagnosis</strong></td>
<td>BIBD</td>
<td>Intermediate form NIFID-BIBD</td>
<td>NIFID</td>
<td>NIFID</td>
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<tr>
<td><strong>FTLD classification</strong></td>
<td>FTLD-FUS</td>
<td>FTLD-FUS</td>
<td>FTLD-FUS</td>
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</tr>
</tbody>
</table>

0, absent; +, mild; ++, moderate; +++, severe.

BIBD, basophilic inclusion body disease; bv, behavioral variant; FTD, frontotemporal dementia; FTLD, frontotemporal lobar degeneration; IHC, immunohistochemistry; NIFID, neuronal intermediate filament inclusion disease.
performed through DNA amplification of the coding region and flanking intronic sequences of the 15 FUS exons (primer sequences provided under request). Cycle sequencing was performed using the Dye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA) and run on an ABI 3730xl genetic analyzer. Sequence chromatograms were analyzed using the Sequencher software (Genecodes, Ann Arbor, MI).

RESULTS

Clinical Summary

Case 1

A 38-year-old man experienced progressive apathy, loss of interest in recreational activities, reduction of libido, slight irritability that turned to aggressiveness toward his children, and a frank alteration of eating behavior. Two family members on his mother’s side (mother’s cousin and 1 brother) had mental retardation. No history of neurologic disorders could be obtained from the father’s family. The patient had had a successful career in computer engineering that progressively declined. After an initial diagnosis of depression, no improvement was observed after treatment with antidepressant drugs. He started having memory complaints, disorientation, alteration in daily living activities, and severe worsening of behavioral symptoms. At first visit to a neurologist, the patient had a relatively good general cognitive performance with a Mini-Mental State Examination score of 29 of 30 but with mild short- and long-term memory alteration, alteration of executive functions, attention deficit, utilization behavior, and alteration of interpretation. Physical examination results were normal. General and specific laboratory analyses (autoimmunity, antineuronal antibodies, infectious agents, tumor markers) in peripheral blood and specific laboratory analyzes (autoimmunity, antineuronal antibodies, infectious agents, tumor markers) in peripheral blood and cerebrospinal fluid did not reveal any remarkable findings. The unfixed brain weighed 960 g (Table). The brain showed mild posterior frontal hypometabolism (Fig. 1A). The diagnosis of frontotemporal dementia was made after excluding Huntington disease by genetic analysis. The MAPT mutations were not detected.

Two years later, after a severe worsening of the patient’s state and behavioral alterations, urinary and fecal incontinence appeared and he developed complete dependence for daily activities at the age of 40 years. Repeated brain magnetic resonance imaging showed much more pronounced cortical and subcortical atrophy and general bilateral hypometabolism, preserving the occipital lobes in single-photon emission computed tomography (Fig. 1B). He spent the last 3 years of his life in a nursing home and died of aspiration pneumonia 5 years after disease onset at the age of 43 years. The final clinical diagnosis was frontotemporal dementia, most likely Pick disease (Table).

Case 2

The clinical description of this patient was previously reported in detail (6). Briefly, a 37-year-old man presented with blunted affect; he developed progressive language and behavioral alterations with frequent falls and ended up in akinetic mutism. He died at the age of 44 years with a clinical diagnosis of bvFTD (Table).

Case 3

A 64-year-old man was admitted to the psychiatric clinic because of acute worsening of a 3-month history of behavioral alterations and hypomania. Symptoms started with hyperactivity and inappropriate euphoria and reduced sleep. Acute behavioral alterations with inappropriate laughing and puerile behavior were followed by language alterations, apraxia, and disorientation. Manic symptoms responded rapidly to neuroleptic drugs. During the disease course, there were fluctuations of the alterations. Neuropsychological tests disclosed moderate cortical and subcortical cognitive dysfunction, with important alteration of attention, apraxias, memory, language, and executive functions. Magnetic resonance imaging showed mild diffuse brain atrophy. Laboratory test results were within normal limits. Cerebrospinal fluid cell count and glucose and protein levels were also normal. The 14-3-3 protein, oligoclonal bands, angiotensin-converting enzyme, lactate dehydrogenase, viral and luetic serologies, and Treponema whippelii cultures were all negative.

At the time of the testing, cardiac arrhythmia (flutter) was detected and the patient was treated with anticoagulant therapy. He developed repeated partial seizures that were treated with valproate. Furthermore, he showed autonomic dysfunction and decompensation of his flutter with bradycardia, hypotension, and hemodynamic instability that required vasoad-ve drugs. In addition, tremor, fasciculations, and myokymia appeared. These manifestations raised the clinical suspicion of Morvan syndrome or autoimmune encephalitis, but all of the following autoantibodies tested were negative: voltage-dependent potassium antibodies, Hu, Ma2, CV2-CRMP5, Yo, Ri, amphiphysin, NMDAR, LGII, CASPR2, AMPAR, and GABA(B)R. There was no improvement after corticosteroid therapy or intravenous immunoglobulin. He developed a retroperitoneal hematoma associated with hemodynamic decompensation and died of hypovolemic shock and respiratory infection despite intubation, multiple blood transfusions, and intensive care 5 months from the onset of neurologic symptoms. The clinical diagnosis was diffuse encephalopathy probably caused by autoimmune encephalitis with negative onconeural antibodies (Table).

Case 4

The clinical description of this patient was previously reported in detail (6). Briefly, a 70-year-old woman presented with asymmetric atypical parkinsonism with gait disturbances and frequent falls during the disease evolution. Cognitive decline of the frontal type appeared about 2 years later. The disease progressed rapidly, and she died 5 years after the disease onset at the age of 75 years (Table).

Neuropathologic Examination

Case 1

The unfixed brain weighed 960 g (Table). The brain showed severe atrophy of frontal, temporal, and parietal lobes; basal ganglia; and limbic system. There was marked enlargement
of the ventricular system (Fig. 2A). There was diffuse narrowing of the cortical ribbon, with relative preservation of posterior parietal and occipital regions. Histologically, severe neuronal loss and reactive astrogliosis and microgliosis were observed in frontal and temporal cortices (with status spongiosis), caudate and putamen, amygdala, hippocampus (with segmental hippocampal sclerosis) and parahippocampus, with relative preservation of precentral and postcentral gyrus, occipital lobe, and thalamic nuclei. Adjacent white matter and corpus callosum showed severe Wallerian degeneration with abundant macrophages. In the brainstem, there was severe depletion of pigmented neurons of the substantia nigra. In areas with preserved neurons, round faint basophilic inclusion bodies were detected in neuronal somata in thalamic nuclei, brainstem nuclei, basis pontis, substantia nigra neurons, periaqueductal gray matter, hippocampus, and motor neurons of the anterior horn of the spinal cord at all levels (Fig. 3a1, a2). There was also a reduction in motor neurons; some of them with chromatolytic appearance (Fig. 3a2). By immunohistochemistry, inclusions were negative for hyperphosphorylated tau and 3- and 4-repeat tau isoforms, α-synuclein, α-internexin, and TDP43; and they were variably and weakly immunoreactive for ubiquitin and p62 while strongly positive for FUS (Fig. 3a3, a4). Because this antibody stains the nucleus and also, faintly, the perikaryon of neuronal elements, the presence of the inclusion (mainly in the basis pontis) gave the appearance of a “double nucleus” (Fig. 4b1). No neuronal nuclear inclusions or glial inclusion bodies were detected. Only a few delicate neurites were detected in affected cortical areas, caudate nucleus, or thalamus. The neuropathologic diagnosis was basophilic inclusion body disease or FTLD-FUS according to the current consensus classification (1).

Case 2
This case was previously described in detail (6). Briefly, there was frontotemporally accentuated gross atrophy with marked neuronal loss and superficial cortical spongiosis on histologic examination. There was also severe involvement
of striatum and hippocampal sclerosis, as in Case 1. Neuronal inclusion bodies included pale and fibrillar inclusions that were stained with α-internexin, but not with FUS antibodies, and round basophilic (Fig. 3c1) or slightly eosinophilic inclusions that were immunoreactive for FUS (Fig. 3c2, c4), some with a dense central eosinophilic dot (“cherry spot”) (Fig. 3c3). The neuropathologic diagnosis was NIFID or FTLD-FUS, according to the current consensus classification (1).

Case 3

The unfixed brain weight was 1,390 g. No marked atrophy was observed on gross examination (Fig. 2B). Histologic examination showed only mild neuronal loss, astrogliosis, and microglial activation in cortical areas and the limbic system. The hippocampus was relatively well preserved. There was only mild and focal neuronal loss with extracellular pigment in the substantia nigra. The most striking feature was the presence of abundant intracytoplasmic neuronal inclusion bodies. These were characterized by brightly eosinophilic branching fibrillar structures embedded in a round well-delineated glassy vacuole (Fig. 3b1). Inclusion bodies were widespread, nearly identical in all brain areas, and they were found in pyramidal neurons of frontal, temporal, and parietal cortices, cingulum, hippocampus (dentate gyrus and CA1), entorhinal and transentorhinal cortices, amygdala, nucleus basalis of Meynert, thalamic and hypothalamic neurons, large striatal neurons and neurons of basis pontis, inferior olives, dentate nucleus of cerebellum, isolated motor neurons of oculomotor nucleus and anterior horns, and olfactory bulb. They were not detected in pigmented neurons of locus coeruleus, substantia nigra, or Purkinje cells. All of these inclusions were strongly immunoreactive for the class IV intermediate filament α-internexin (Fig. 3b3). Ubiquitin and p62 immunoreactivities were rarely observed and, when present, they were isolated dots. There was no labeling of inclusions by anti-FUS immunohistochemistry using the Sigma antibody, which resulted in a punched out-appearing area in the cell body with a stained nucleus (Fig. 3b4). There were only occasional fine dots overlying the inclusion, similar to the granular immunoreactivity observed in the surrounding cytoplasm. In contrast, the inclusions were intensely stained with the anti-FUS antibody from Lifespan, where additional strong labeling of astrocytes was noted (Fig. 4a2 and inset, e2). Inclusions were also negative for hyperphosphorylated tau, α-synuclein, and TDP43 protein. Mild focal diffuse β-amyloid deposits were present in frontal, temporal, and occipital cortex and fine tau-immunoreactive neuropil threads and isolated pretangles were detected in the entorhinal and transentorhinal cortex, locus coeruleus, and substantia nigra. There was additional mild acute hypoxic-ischemic neuronal damage with red neurons in the CA1 sector of the

**FIGURE 2.** Macroscopic brain appearance of cases 1 and 3. (A) Case 1. Lateral view of right hemisphere (upper part of the figure) and coronal sections (lower part of the figure) show marked narrowing of the cortical ribbon with enlargement of sulci and of the body and temporal horn of the lateral ventricle. There is diffuse reduction of white matter volume and severe atrophy of the corpus callosum. Note the marked atrophy of the caudate nucleus, putamen, and posterior hippocampus. (B) In contrast, lateral view of right hemisphere (upper panel) and coronal sections of Case 3 with a disease evolution of 5 months shows no major brain atrophy. There is only mild enlargement of the lateral ventricle without basal ganglia, hippocampal, or cortical atrophy.
hippocampus and a segmental loss of cerebellar Purkinje cells. The neuropathologic diagnosis was NIFD or FTLD-FUS according to the current consensus classification (1).

Case 4
This case was previously described in detail (6). It was similar to case 3 but showed a more advanced disease stage. There was a moderate global atrophy and pallor of substantia nigra with loss of pigmented neurons on histologic examination. Neurons of all cortical areas, basal ganglia, brainstem nuclei, and isolated motor neurons contained abundant α-internexin-positive bright eosinophilic filamentous neuronal inclusions embedded in a round, well-delineated, glassy vacuole (b1 with few abundant fibrillar structures; b2 with abundant fibrillar structures). These inclusions are strongly immunoreactive for the Class IV intermediate filament α-internexin (b3). There was no labeling of inclusions by anti-FUS immunohistochemistry using the antibody from Sigma. This results in a punched out area in the cell body with a stained nucleus (b4). (c1–c4) Intermediate form of NIFD (Case 2). Neuronal inclusion bodies include round basophilic (b2) or slightly eosinophilic inclusions, which are immunoreactive for FUS (c2, c4) and some inclusions with a dense and central eosinophilic dot (cherry spot) (c3, arrow). Scale bars = (a1, b1, b2, b4, c1–c3) 10 μm; (a2, a3, a4, b3, c4) 20 μm.

FIGURE 3. Spectrum of inclusion bodies and immunoreactivities among basophilic inclusion body disease (BIBD) and neuronal intermediate filament inclusion disease (NIFID) (FTLD-FUS). (a1–a4) BIBD (Case 1). Basophilic inclusion bodies in the perikaryon of brainstem neurons (a1) and motor neurons of the anterior horn of spinal cord (a2, arrows), some of which have a chromatolytic appearance. Inclusions show strong immunoreactivity for FUS (a3, a4, Sigma anti-FUS antibody). (b1–b4) Hyaline conglomerate type of inclusions characterized by brightly eosinophilic branching fibrillar structures embedded in a round, well-delineated, glassy vacuole (b1 with few abundant fibrillar structures; b2 with abundant fibrillar structures). These inclusions are strongly immunoreactive for the Class IV intermediate filament α-internexin (b3). There was no labeling of inclusions by anti-FUS immunohistochemistry using the antibody from Sigma. This results in a punched out area in the cell body with a stained nucleus (b4). (c1–c4) Intermediate form of NIFID (Case 2). Neuronal inclusion bodies include round basophilic (b2) or slightly eosinophilic inclusions, which are immunoreactive for FUS (c2, c4) and some inclusions with a dense and central eosinophilic dot (cherry spot) (c3, arrow). Scale bars = (a1, b1, b2, b4, c1–c3) 10 μm; (a2, a3, a4, b3, c4) 20 μm.

Comparative Study of Anti-FUS Antibodies
The lack of FUS immunoreactivity of hyaline conglomerates using the widely used commercial antibody from Sigma (10) and the intense labeling of the same inclusions by another anti-FUS antibody from Lifespan prompted us to compare immunoreactivity patterns of the 2 antibodies in different diseases. Physiologically, antibody A (Sigma) stained nuclei in all cases and also faintly stained the cytoplasm, as previously described. In contrast, antibody B (Lifespan) did not stain neuronal or glial nuclei but strongly labeled processes of moderate senile changes with mature β-amyloid plaques and isolated neurofibrillary tangles and tau-immunoreactive neuropil threads in brainstem nuclei. The neuropathologic diagnosis was NIFD or FTLD-FUS, according to the current consensus classification (1).
normal fibrous, subpial, perivascular, and reactive astrocytes (Fig. 4e2). In white matter, there was a diffuse fibrillary background (Fig. 4e1). In addition, some ringlike immunoreactivity around small round nuclei suggestive of oligodendrocytes was observed in all cases (Fig. 4e1). Interestingly, this “glial” pattern of antibody B was only observed if the tissue sections had been pretreated with citrate buffer at pH 6 but not when using EDTA at pH 9. When that was done, immunostaining with antibody B was the same as that with antibody A (data not shown). Hyaline conglomerates of both NIFID cases were strongly stained with the Lifespan antibody, whereas we were negative using the Sigma antibody (Fig. 4a1, a2). Basophilic inclusion bodies of BIBD were stained with both but were more frequently and more intensely stained with the Sigma antibody (Fig. 4b1, b2).

Both antibodies also stained nuclear inclusions of Huntington disease (Fig. 4c1, c2), as described in polyglutamine expansion disorders (11). In addition, antibody B (Lifespan) showed intense immunoreactivity around cerebellar Kuru plaques in a sporadic CJD case (Fig. 4d2), but not in plaquelike or patchy perivascular deposits in the frontal cortex. This was not seen with antibody A (Fig. 4d1). Antibody A stained some of the granules of granulovacuolar degeneration in Alzheimer disease; this was not observed with antibody B. In FTLD, isolated small cells suggestive of oligodendrocytes displayed small ill-defined rounded cytoplasmic inclusions (not shown). None of these antibodies stained intraneuronal tangles. Pick bodies, Lewy bodies, grains, skeinlike inclusions, neuronal cytoplasmic or nuclear inclusions of FTLD, or glial cytoplasmic inclusions of multisystems atrophy cases.

**Genetic Data**

Genetic analysis of FUS coding region and its intron-exon boundaries was performed in the 4 cases but did not identify any mutation.

**DISCUSSION**

Basophilic inclusion bodies were described in the early 1990s in relation to sporadic juvenile- and adult-onset ALS (12, 13, 14). Immunohistochemical and ultrastructural features of these inclusion bodies were reported in 2000 as disorganized fibrils of 12 to 25 nm in diameter associated with granular material probably derived from ribosomes (15, 16). Their basophilia was thought to be caused by the presence of RNA and mRNA-associated proteins. A few years later, overlap of ALS and FTLD became evident in NIFID (17). In 2006 and 2008, both NIFID and BIBD were shown to have much in common, but they are difficult to differentiate on clinical grounds (7, 9). Both may present with a rapid evolution of bvFTD with psychiatric manifestations, early falls, involvement of basal ganglia, clinical or subclinical involvement of motor neurons, and akinetic mutism. The lack of a disease marker in vivo makes postmortem brain studies essential for diagnostic confirmation. Because inclusions in NIFID in conventional stains are similar to those of BIBD, the 2 diseases might be difficult to differentiate histologically without specific immunohistochemical stains. Yokota et al (18) reported that 2 of 6 cases, which they had previously classified as BIBD because of the presence of basophilic inclusions, had inclusions that were intensely immunoreactive for α-intermixin, thus changing the diagnosis to NIFID. However, because not all inclusions in NIFID were immunoreactive for α-intermixin, the authors stated that an unknown protein besides α-intermixin and neurofilament might play a pivotal pathogenic role in at least some NIFID cases.

The concepts changed rapidly in 2009 because of the discovery of FUS mutations as the cause of familial ALS type 6 (19, 20). Immunohistochemistry was soon applied, and cases of FTLD with or without motor neuron diseases could be classified according to the consensus classification of 2010 among the group of FUS proteinopathies (1). This group now includes atypical forms of FTLD-U, NIFID, and BIBD (1, 4–6, 8–10, 18, 21). The NIFID and BIBD share the topographic distribution of pathology, that is, involvement of frontal and temporal cortex associated with subcortical gliosis, moderate to severe involvement of basal ganglia and thalamus, severe reduction of hippocampal neurons as in hippocampal sclerosis, and marked loss of pigmented neurons of substantia nigra, as well as involvement of periaqueductal gray matter, inferior olives, cerebellar dentate nucleus, and upper and/or lower motor neurons in varying degrees. Although it has been suggested that FUS protein might be a key element in the pathogenesis of the diseases, it is still unclear whether FUS aggregation is really the primary protein defect in NIFID.

Histologically, inclusions of BIBD are uniform, well characterized, and universally FUS positive (9). In contrast, inclusion bodies in NIFID are more heterogeneous and may vary in morphology, histochemical staining, immunoreactivity, ultrastructure, and anatomic distribution (8, 10). Immunohistochemically, they are by definition TDP43 negative, tau negative, and α-synuclein negative, and variably positive for ubiquitin and p62. A fraction of them are stained with anti-α-intermixin antibodies and some NF subunits. FUS immunoreactivity varies depending on the type of inclusion. By routine

**FIGURE 4.** Comparison of immunoreactivity patterns of 2 anti-FUS antibodies. The left column shows immunoreactivity patterns observed using the Sigma anti-FUS antibody (A), and the right column shows the patterns observed using the Lifespan anti-FUS antibody (B). a1, a2] Hyaline conglomerates are negative for A and positive for B antibody. Note that antibody A shows the physiologic nuclear staining not observed for antibody B (inset). In addition, large ramified astrocytic processes are stained with antibody B (a2). b1, b2 Basophilic inclusions are stained with both antibodies but more intensely and distinctly with antibody A. Note that no nuclei are stained, and there is a fibrillar background (b2). c1, c2 Neuronal intranuclear inclusions (NNIs) of Huntington disease are stained by both antibodies. Antibody A also stains fine granular cytoplasmic aggregates, as usually observed when using anti-polyQ antibodies (not shown). d1, d2 Antibody B shows enhanced immunoreactivity around cerebellar Kuru plaques in a sporadic Creutzfeldt–Jakob disease (sCJD) case (d2), a feature that is not observed with antibody A (d1, arrow). e1, e2 Antibody B shows a diffuse fibrillar background in white matter and some ringlike immunoreactivity around small round nuclei, suggestive of oligodendrocytes (e1). In addition, in all cases, astrocytic processes of normal, subpial, perivascular, and reactive astrocytes are stained (e2). Scale bars = (a1, b2, c1, c2, e1) 10 μm; (a2, b1, d1, d2) 20 μm; (e2) 50 μm.

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histology, they may be reminiscent of Pick bodies, and they appear slightly eosinophilic or basophilic (10, 18). This type of inclusion is rarely argyrophilic, strongly FUS positive, weakly stained by ubiquitin and p62 antibodies, and only occasionally or not stained with anti-intermediate filament antibodies (10). It is also the predominant inclusion type in BIBD and was also observed in our Case 2, along with other inclusion types. Strong immunoreactivity for FUS is the basis for its classification among FUS proteinopathies.

In addition to other more tangible or crescent annular rings, which are not well seen in hematoxylin and eosin and which are weakly positive for ubiquitin and occasionally stained with anti-intermediate filament antibodies (10), intraneuronal inclusions with distinct eosinophilic cores or cherry spots or compound intraneuronal inclusion bodies have been described by several authors (4, 5, 18, 22) and were observed in our Case 2.

In addition, there is still a very characteristic type of inclusion that has been defined by Bigio et al (17), McKenzie and Feldman (23), and Neumann et al (10) as hyaline conglomerates. They are irregular eosinophilic multilobulated masses with 2 components: a large pale amorphophilic hyaline spherical inclusion (~15 μm) and a minor centrally or eccentrically located, somewhat hyalinized, component ranging from dotlike, spherical, or irregularly serpiginous to a delicate tapering curvilinear form (17) (Fig. 3b1 and b2). Bigio et al (17) described a distinct ultrastructural morphology, that is, the pale hyaline component consisting of densely packed and highly ordered interwoven fascicles of long wavy filaments with a diameter of 10 to 12 nm. The central amorphophilic component was composed of condensed electron-dense granular and amorphous material containing occasional vesicles with a density similar to that of cytoplasmic elements, including rough endoplasmic reticulum, probably representing invaginations and entrapment of cytoplasmic elements within condensing neurofilaments (17). These inclusions are strongly argentophilic in Bielschowsky silver impregnation and are not strongly immunoreactive for ubiquitin. Among all types of neuronal cytoplasmic inclusions, these hyaline conglomerates were the only type that was better demonstrated by intermediate filament immunostains by Neumann et al (10). These inclusions (reported to be the least frequent inclusion type among their 5 cases) were the only inclusion type present in our 2 later onset cases (cases 3 and 4).

The greater prominence and compaction of ramified, filamentous, and brightly eosinophilic processes in the round, pale, glassy inclusion in the patient with longer disease duration (5 years), compared with the very short duration case (6 months), suggest that there might be a maturation process for them. Moreover, the widespread presence of hyaline conglomerates even in the absence of marked neuronal loss and gross brain atrophy in the short disease duration case suggests that the formation of inclusion bodies might be an early event in the pathogenesis of the disease emerging in preclinical disease phases, similar to what has been described for other neurodegenerative diseases.

Immunohistochemically, hyaline conglomerate–type inclusions are universally and strongly immunoreactive for the intermediate filament α-internexin. Using the Sigma anti-FUS antibody, only occasional dotlike and peripheral granular FUS immunoreactivity has been described using conventional immunohistochemistry (10), double immunofluorescence, and immunogold electron microscopy (24).

A large number of commercially available antibodies are useful for immunohistochemical analyses of routinely processed postmortem brain tissue, but cautious interpretation and sound knowledge of the limits and potential pitfalls of the method and the reagents are in order. Applying the widely used Sigma anti-FUS antibody, we were not able to stain (and thus to classify at first) cases 3 and 4 with hyaline conglomerates among FUSopathies. The results of FUS immunohistochemistry are highly dependent on fixation time, but all of our cases were fixed in a standard manner in 4% buffered aqueous formaldehyde solution (10% formalin) for 4 weeks, and all showed physiologic nuclear and faint cytoplasmic staining. Thus, fixation time would not likely account for the negativity of these inclusion bodies in our 2 cases. Neumann et al (10) also described only occasional dotlike FUS immunoreactivity of this type of inclusion body. In contrast, hyaline conglomerates were clearly visible when stained with Lifespan anti-FUS antibody, but at the expense of strongly immunoreactive astrocytes in the background and a lack of nuclear staining. When comparing the 2 antibodies, the Lifespan antibody always stained glial processes but not inclusion bodies in other neurodegenerative diseases, except for nuclear inclusions in Huntington disease. Thus, the Lifespan antibody seems to cross-stain glial antigens.

These differences in immunohistochemical reactions might be caused by the different epitopes recognized by each of the anti-FUS antibodies. Antibody A from Sigma recognizes the peptide region between residues 90 and 220, whereas antibody B from Lifespan recognizes a region between residues 200 and 250. Furthermore, FUS may have different transcript variants caused by alternative splicing. Thus, different antibodies may recognize different isoforms of the protein predominantly expressed in some neuronal and/or glial cell populations. Alternatively, the exposition of the reactive domain of FUS to particular antibodies varies within the spectrum of NIFID. This is further influenced by pretreatment of the tissue sections with either citrate buffer at pH 6 or with the metal ion chelator EDTA at pH 9, which probably exposes different epitope sites. The former pretreatment used for FUS B antibody might have enhanced exposure of glial and neuronal cytoskeletal epitopes, thus immunoreacting with intermediate filament-rich hyaline conglomerates but also with components of the glial cytoskeleton. Therefore, when a diagnosis or even a classification system is supported by the results of immunohistochemistry, fixation type and time, tissue storage time, antigen retrieval method, detection systems, among other factors (25), have to be taken into account when interpreting results.

We did not find any pathogenic mutation in FUS gene in any of our cases. As far as we know, the other 8 BIBD patients described in the literature (9) were not screened for FUS mutations, and previously reported NIFID cases (10) and an atypical FTLD-U case (21) disclosed no mutations. Therefore, although results in these cases suggest that mutations in FUS do not cause BIBD or NIFID, even with a family history in some cases, we cannot rule out this possibility in others.
To summarize, we report 2 NIFID cases with later disease onset that showed exclusively filamentous and brightly eosinophilic ramified cytoplasmic inclusions embedded in a pale glassy hyaline spherical inclusion—hyaline conglomerates—that were FUS negative using 1 antibody but FUS positive using another commercially available antibody (cases 3 and 4). This initially led to difficulties in classifying these cases among FTLD-FUS.

In conclusion, NIFID is still appropriately considered a heterogeneous disease. The present observations show that phenotypic variability in NIFID and, in particular, neuropathologic findings such as hyaline conglomerate–type inclusions may be related to the age at onset and individual differences in the evolution of lesions.

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