Juvenile ALS with basophilic inclusions is a FUS proteinopathy with *FUS* mutations

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ABSTRACT

Background: Juvenile amyotrophic lateral sclerosis (ALS) with basophilic inclusions is a form of ALS characterized by protein deposits in motor neurons that are morphologically and tinctorially distinct from those of classic sporadic ALS. The nosologic position of this type of ALS in the molecular pathologic and genetic classification of ALS is unknown.

Methods: We identified neuropathologically 4 patients with juvenile ALS with basophilic inclusions and tested the hypothesis that specific RNA binding protein pathology may define this type of ALS. Immunohistochemical findings prompted us to sequence the fused in sarcoma (*FUS*) gene.

Results: Motor symptoms began between ages 17 and 22. Disease progression was rapid without dementia. No family history was identified. Basophilic inclusions were strongly positive for FUS protein but negative for TAR DNA binding protein 43 (TDP-43). Granular and compact FUS deposits were identified in glia and neuronal cytoplasm and nuclei. Ultrastructure of aggregates was in keeping with origin from fragmented rough endoplasmic reticulum. Sequencing of all 15 exons of the *FUS* gene in 3 patients revealed a novel deletion mutation (c.1554_1557delACAG) in 1 individual and the c.1574C>T (P525L) mutation in 2 others.

Conclusion: Juvenile ALS with basophilic inclusions is a FUS proteinopathy and should be classified as ALS-FUS. The *FUS* c.1574C>T (P525L) and c.1554_1557delACAG mutations are associated with this distinct phenotype. The molecular genetic relationship with frontotemporal lobar degeneration with FUS pathology remains to be clarified. *Neurology*® 2010;75:611-618

GLOSSARY

ALS = amyotrophic lateral sclerosis; **FTLD** = frontotemporal lobar degeneration; **FTLD-FUS** = frontotemporal lobar degeneration with FUS pathology; **NIFID** = neuronal intermediate filament inclusion disease.

Amyotrophic lateral sclerosis (ALS) is an adult-onset motor neuron disease with an incidence of 1.5 to 2.5 per 100,000 that peaks between 75 and 79 years of age.¹ While more than 90% of cases occur without a family history,² sporadic ALS is thought to have a significant genetic component.³ Indeed, mutations in genes known to cause familial ALS (*SOD1*,⁴ *TARDBP*,⁵ *ANG*,⁶ and *FUS*⁷) may be found in apparently sporadic cases.^{6,8-10} Sporadic ALS with onset below 25 years of age is rare¹ and has been proposed to constitute a distinct clinical entity.^{11,12} A number of these cases have been defined by the presence of basophilic intraneuronal inclusions in motor and nonmotor neurons.^{11,13-16} These protein aggregates are distinct from those seen in typical sporadic ALS. However, some investigators have suggested that the basophilia of the aggregates may relate to prolonged artificial ventilation of patients with otherwise classic sporadic ALS.¹⁷ RNA binding proteins have been identified in basophilic inclusions¹⁸; however, the relevance of this finding for the specific molecular and genetic classification of juvenile ALS with basophilic inclusions remains to be clarified. Here, we describe a small series of these patients and test the hypothesis that a specific RNA

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binding protein may be pathogenic. We demonstrate that this form of juvenile ALS is a rapidly progressive FUS proteinopathy with *FUS* mutations.

METHODS Subjects. Four cases of juvenile ALS came to autopsy and, following identification of basophilic inclusions, were referred to the Thomas Willis Brain Bank in Oxford for further study. Case 3 was reported as an educational case of the month.¹⁹ Good quality DNA was available from 3 of these individuals. DNA from a cohort of patients with adult-onset ALS from the Oxford Motor Neuron Disease Clinic was used for comparison ($n = 128$, 76 male, mean age 61 years, 52 female, mean age 70 years, 15 had non-SOD1 familial ALS).²⁰ Spinal cord sections from an individual without neurologic disease and a case of classic sporadic ALS with TDP-43 pathology were also used.

Standard protocol approvals and patient consents. The Oxfordshire Research Ethics committee approved use of autopsy material and DNA. Informed consent was obtained from patients or next of kin.

Histology and immunohistochemistry. Six-micrometer-thick sections were cut from paraffin-embedded formalin-fixed autopsy material and processed for hematoxylin-eosin, cresyl violet, and Luxol fast blue stains. For electron microscopy, samples were fixed in 2% glutaraldehyde, postfixed in osmium tetroxide, and embedded in resin. Sections were examined on a JEOL JEM1200X electron microscope. For immunohistochemistry, sections were dewaxed in xylene, and rehydrated through graded ethanol baths. For DAB labeling, sections were treated with 3% hydrogen peroxide in PBS for 30 minutes to quench endogenous peroxidase activity. Antigen retrieval was performed by microwaving for 10 minutes in 10 mM citrate, pH 6.0 at 800 W prior to blocking sections with 10% fetal calf serum in TBS-T for 30 minutes. Primary antibodies were applied overnight at 4°C (Sigma rabbit anti-FUS 1:50, 1:250, 1:500, 1:1,000, Proteintech rabbit anti-TDP-43 1:500, BD Bioscience mouse anti-SMN 1:320, mouse anti-p62 [sequestosome-1] 1:800, Abcam rabbit anti-PABP 1:800). Labeling was visualized with the Dako Envision kit. Digital bright field images were taken on a Leica DM4000 microscope and assembled in Photoshop. For immunofluorescent studies, pretreatment with hydrogen peroxide was omitted. Staining was visualized by incubation with Alexa Fluor 488 or 594 goat antirabbit and Alexa Fluor 488 or 594 goat anti-mouse secondary antibodies (Invitrogen) at 1:500 for 2 hours at room temperature. Sections with strong autofluorescence were counterstained with 0.1% Sudan Black B in 70% ethanol for 5 minutes. Sections were mounted in Vectashield Hardset medium with DAPI (Vector Labs) and examined using a Zeiss fluorescent microscope. Images were compiled using NIH Image J software.

Molecular genetic analysis. Genomic DNA was extracted from white blood cells, frozen brain, or muscle tissue (cases 1, 2, 4) with DNA extraction kits (Nucleon BACC [Amersham Biosciences] and DNeasy blood and tissue kit [Qiagen]). *FUS* exons 1–15 including the 3'UTR were PCR amplified with Taq DNA polymerase (exons 1–14) (Sigma) or Expand High-Fidelity DNA polymerase (exon 15) (Roche) using oligonucleotide primers designed to include approximately 120 base pairs of adjacent introns. PCR products were purified by ethanol precipitation and sequenced using BigDye 3.1 dideoxy terminator methods (Applied Biosystems) using the amplification primers. *FUS* exons 14 and 15 only were sequenced in the cohort of patients with adult-onset ALS. Samples showing abnormal chromatograms were reamplified and

resequenced for confirmation. The effect of the identified variant was analyzed using SIFT prediction.²¹ To confirm the suspected 4 base pair deletion in exon 15, PCR product was subcloned into pCR 2.1 TOPO vector (Invitrogen), and 10 clones were sequence analyzed. Sequence variants were described in relation to the translation initiation codon of NCBI reference sequence NM_004960.2. The novel protein sequence resulting from the deletion mutation was predicted using the ExPASy Proteomics server (<http://www.expasy.ch/tools/dna.html>), and the novel nucleotide sequence was examined for the presence of potential exonic splice enhancer sites using ESE finder release 3.0.²²

RESULTS Clinical summaries. Patient 1 was a 22-year-old woman with a 6-month history of widespread, progressive muscle weakness of asymmetric onset initially affecting left hip flexion. She had no bulbar, sensory, cognitive, or sphincter symptoms. Frank upper motor neuron signs were absent. EMG showed widespread acute denervation. She died from respiratory failure approximately 10 months after onset of symptoms. There was no history of neurologic disease in her family.

Patient 2 was an 18-year-old woman who developed painful left upper arm weakness, which progressed to more distal left arm weakness within 6 weeks, before rapidly spreading to the right arm and both legs. She had no sensory, sphincter, cognitive, or bulbar symptoms, but showed evidence of tongue wasting and a brisk jaw jerk. She died 11 months after symptom onset. She had a healthy sibling and no family history.

Patient 3 was a 25-year-old woman who developed a pure motor syndrome affecting the arms following a Caesarean section. Within a few months weakness progressed to affect the legs. Ten months after symptom onset, she developed bulbar dysfunction and respiratory failure requiring mechanical ventilation, which was performed for 27 months before death. There were no sensory, sphincter, or cognitive symptoms or signs. She had a healthy sibling and no family history.

Patient 4 was an 18-year-old man with mild learning difficulties and congenital deformities of both feet that were interpreted as a forme fruste of arthrogryposis. No other joints were affected. Presenting symptoms included bilateral arm weakness rapidly spreading to involve neck muscles, followed by swallowing difficulties. Tongue, shoulder girdle, and hand muscles were wasted and fasciculating. Reflexes were brisk and plantars were extensor. Bulbar disease was rapidly progressive and the patient died 6 months after onset of symptoms. He had 2 healthy siblings and no family history.

Neuropathology. There was no gross atrophy in any case (figure 1A). Anterior roots of the spinal cord were shrunken at all levels. Corticospinal tract degeneration varied; dorsal columns were spared (figure 1, B and C). By definition, cytoplasmic basophilic neuronal inclusions were present in all cases. These were

Figure 1 Neuropathology of amyotrophic lateral sclerosis (ALS) with basophilic inclusions and *FUS* mutations

(A) No evidence of frontotemporal atrophy (case 1, P525L mutation). Severe (B, case 1) and mild (C, case 2) degeneration of the corticospinal tract in the P525L *FUS* mutation. (D–F) Compact basophilic neuronal cytoplasmic inclusions (arrows) were present in upper and lower motor neurons of all cases with *FUS* mutations. (D) Case 2, Betz cell, (E) case 4, Betz cell, (F) case 1, nucleus hypoglossus. (G) Fragments of straight filaments associated with electron-dense granules characterize a spinal cord neuronal cytoplasmic inclusion in case 1 with the *FUS* P525L mutation ($\times 15,000$ magnification). Luxol fast blue cresyl violet (B, C), hematoxylin-eosin (D–F).

most prominent in the motor cortex and lower motor neurons (figure 1, D–F). Rare basophilic inclusions were present in the substantia nigra, nuclei raphe, inferior olives, and dentate nucleus of the cerebellum. In case 3, numerous inclusions were present in neocortical regions outside the motor cortex and in the substantia nigra. Inclusions in granule cells of the hippocampus were not seen in any case.

Basophilic aggregates showed some features similar to Nissl substance, as has been described previously (figure 1, D–F, and figure 2, A–C).^{11,15} Indeed, many neurons showed only minimal coarsening and clustering of Nissl substance, while others contained the large confluent or multilobular inclusions known as the defining feature of this form of ALS (figure 2I, inset). Ultrastructurally, neuronal cytoplasmic aggregates consisted of 12–15 nm tubulofilamentous structures associated with electron-dense granules, in keeping with origin from endoplasmic reticulum with ribosomes or related organelles (figure 1G), as described before.^{11,15}

Immunohistochemistry. p62 (sequestosome-1), a scaffold protein at the interface of proteasome and autophagy pathways, labeled compact aggregates and coarsened Nissl substance (figure 2D), a feature that is not seen in normal neurons.²³ Immunohistochemistry with anti-*FUS* antibody revealed extensive pathology. In addition to pathology in neuronal perikarya (figure

2, E–I), we observed neuronal nuclear inclusions, glial cytoplasmic inclusions, and dystrophic neurites (figure 2, J and K). As has been observed before,^{24,25} currently available *FUS* antibodies require case-by-case adjustment of the protocol depending on fixation time. Dilutions can be titrated in a way that staining only reveals presumed aggregated *FUS* but not presumed physiologic *FUS*; images from this approach are shown in figure 2, E–L. In our hands, *FUS* is present in normal cells in both nuclei and cytoplasm to a degree that varies between cell types (data not shown), in agreement with previous reports.²⁶

Immunofluorescence. While p62-positive inclusions in classic sporadic ALS are TDP-43 positive (figure 3A), this is not the case for p62-positive basophilic inclusions (figure 3B). In keeping with the idea that basophilic inclusions contain elements of the RNA processing machinery, poly-A binding protein (PABP) was consistently redistributed to inclusions (figure 3C). Bright field microscopy suggested that *FUS* antibody was the most sensitive marker of pathology. This was confirmed by combining p62 with *FUS* staining, which demonstrated that *FUS* detects more inclusions than p62 (figure 3, D and E).

Molecular genetic analysis. Direct sequencing of all 15 exons of *FUS* showed the heterozygous c.1574C>T

(A) A normal lower motor neuron from a control case. Note the thin crisp nuclear membrane and basophilic Nissl substance at the periphery of the cytoplasm. A consistent feature of FUS mutation cases on routine hematoxylin-eosin and cresyl violet stains was disruption, clumping, and redistribution of Nissl substance in motor neurons (arrows, B, C). p62 (sequestosome-1) antibody does not normally label Nissl substance but does so in a neuron in a FUS P525L case, which also shows a small aggregation focus (arrow, D). Numerous presumed pathologic FUS protein deposits can be seen when antibodies are titrated in a way that does not stain normal diffuse nuclear FUS (E-L). (E) Overview of the hypoglossal nucleus in FUS P525L case 1. (F-I) Granular (F) to increasingly compact cytoplasmic FUS deposition (G-H); P525L mutation. The latter correlates with the large basophilic inclusions that define this type of amyotrophic lateral sclerosis (I, inset). Nuclear (arrowheads, J) and glial (arrows, J) as well as neuritic (arrows, K) FUS pathology is also present. Note granular perinuclear neuronal FUS deposit (arrowhead, K). (L) Only case 3 had extensive neuronal and glial (arrow) FUS pathology outside the corticospinal system, and the substantia nigra is shown here.

(P525L) mutation in cases 1 and 2. The P525L mutation is located in an evolutionary conserved residue at the very C-terminal end of the protein in close vicinity to most other reported mutations (figure 4). It was previously described in patients with autosomal dominant ALS of both North American and Italian origin.^{7,27}

In case 4, we identified a novel 4-base pair deletion in exon 15 (c.1554_1557delACAG), which was confirmed by analyzing mutant and wild-type alleles separately through subcloning PCR product from genomic patient DNA into a sequencing vector. Wild-type and mutant alleles were found to be present in a 6:4 ratio in 10 sequenced clones, in keeping with a heterozygous deletion. The deletion was predicted to lead to a frame-shift and substantially alter the last 8 amino acids of

FUS (figure 4). In silico analysis using ESE finder suggested that the deletion potentially disrupts binding of SR proteins of the SF2/ASF family by altering an exonic splicing enhancer site, but no patient RNA was available to test this possibility.

None of our patients had a family history of neurologic disease, and at the time of the study no family member DNA was available for testing (see Note Added in Proof). To assess the frequency of these mutations in patients with adult-onset ALS, exons 14 and 15 were directly sequenced in 128 patients. No sequence variants were identified in this cohort.

DISCUSSION In this report, we have demonstrated that juvenile ALS with basophilic inclusions, pre-

Figure 3 Characterization of FUS pathology by immunofluorescence

(A) Compact cytoplasmic inclusions in classic sporadic amyotrophic lateral sclerosis are labeled with p62 and TDP-43. (B) Compact basophilic inclusions are also labeled by p62, but are negative for TDP-43, which retains its normal nuclear distribution. (C) Most basophilic inclusions are immunostained with PABP antibody, indicating the presence of polyadenylated RNA. (D) Basophilic inclusions that are p62 positive are also FUS positive (D), but not all FUS inclusions contain p62 (E, arrow). FUS nuclear staining is sometimes not completely abolished in cells with cytoplasmic FUS inclusions (E, arrowhead).

dicted to be a specific subtype of ALS purely on morphologic grounds long ago,¹¹ is indeed pathologically and genetically distinct from the classic form of sporadic ALS. TDP-43 pathology and rare *TARDBP* mutations are found in classic sporadic ALS; however, causality of this association remains to be proved.^{5,28} All our cases of juvenile ALS with basophilic inclusions show extensive FUS pathology by immunohistochemistry, and all 3 cases with available DNA for complete sequencing show mutations in the *FUS* gene. We therefore propose that juvenile ALS with basophilic inclusions should be classified as ALS-FUS. Recently, frontotemporal lobar degeneration (FTLD) with FUS pathology (FTLD-FUS) has

been reported.^{24,29} However, no *FUS* mutations were identified in any individual.^{24,25} ALS and FTLD are commonly associated and likely represent different neuroanatomic manifestations of a shared biologic pathway; this has been convincingly demonstrated at both the molecular pathologic and genetic level for ALS-TDP and FTLD-TDP.^{5,28,30} Whether the same applies to cases of juvenile ALS-FUS and all types of FTLD-FUS remains to be seen. It is noteworthy that cytopathologically only FTLD-FUS with basophilic inclusions²⁹ is similar to our series, particularly case 3, which had extensive cortical involvement outside the motor cortex and widespread nigral pathology. DNA of sufficient quality for full *FUS* sequencing was not available for this case and it remains possible that this case does not carry a *FUS* mutation. There are no *FUS* sequencing data in the series of FTLD-FUS with basophilic inclusions.²⁹ Disease onset of FTLD-FUS with basophilic inclusions occurs significantly later (average of 46 years) and progression is slower (average of 8 years) than in our patients.²⁹ FTLD-FUS previously described as neuronal intermediate filament inclusion disease (NIFID)²⁵ and atypical FTLD with ubiquitin-only inclusions²⁴ are not characterized by the distinct neuropathology described here, although occasional basophilia of aggregates may be present in NIFID.²⁵ However, the range of FUS pathology in FTLD-FUS shows overlap with our observations and it is conceivable that *FUS* mutations may be found eventually in these subgroups and that different mutations may be associated with distinct neuropathologic phenotypes.

Cytoplasmic mislocalization of FUS has been described in 4 cases of dominantly inherited ALS-FUS with the R521G, R521C, and R521H mutations.^{7,31} However, these reports do not describe any basophilic inclusions or any other neuronal or glial cytopathology. The *FUS* mutations were different from those observed in our patients, and therefore it remains to be seen if the range of cytopathologic features described by us is associated with specific ALS *FUS* mutations or depends on other as yet unidentified factors.

We have shown how neuropathology suggested *FUS* as the candidate gene in our individuals; indeed, despite a negative family history, all 3 cases with available DNA had exon 15 mutations. The c.1554_1557delACAG mutation has not been identified previously in patients with ALS or the large number of control subjects in whom *FUS* was sequenced,^{7,10,27,31,32} while the P525L mutation has been described twice in cases of autosomal dominant ALS with young onset and rapidly progressive disease.^{7,27} In our retrospective neuropathologic study, no parental DNA was available for any of the cases

Figure 4 Genetics of juvenile amyotrophic lateral sclerosis (ALS) with basophilic inclusions

(A) Sequence traces of control and patient DNA showing a 4-base pair deletion in *FUS* exon 15 (c.1554_1557delACAG) confirmed by sequence analysis of cloned wild-type (insert top, bases deleted in the mutant allele are marked by the box) and mutant allele (insert bottom). The deletion is predicted to cause a frameshift with resulting alteration of the terminal 8 amino acids of *FUS* (bottom). (B) Two patients were heterozygous for the c.1574C>T (P525L) mutation (top, control, bottom, case), altering the penultimate amino acid of *FUS* in an evolutionary conserved residue (C). The *FUS* protein structure according to reference sequence PRO_0000081591 (<http://www.uniprot.org/uniprot/>) is shown in (D). An asterisk marks residues previously found to be mutated in patients with familial or sporadic ALS. aa = amino acid.

(see Note Added in Proof); however, the absence of a family history was very well-documented. This is surprising given the aggressive nature of disease in all described P525L mutation carriers and the new deletion mutation. In addition to the possibility of non-paternity, this implies that other genetic or epigenetic factors are required for penetrance, or our patients had de novo mutations. Although not rare in neurologic diseases, de novo mutations have so far only been described in a case of *SOD1*-related ALS.³³

Like TDP-43, the protein at the core of pathology in most cases of adult-onset ALS, *FUS* is a nuclear-cytoplasmic shuttling protein with DNA and RNA binding properties and various functions in RNA metabolism from transcription regulation³⁴ and mRNA splicing³⁵ to regulation of mRNA transport and local translation regulation in neurites³⁶ as well

as stress granule formation.²⁶ The TDP-43 C-terminal is crucial for cellular localization,³⁷ and the fact that *FUS* proteins with C-terminal mutations mislocalize in cell culture models⁷ suggests that this is also the case for *FUS*; indeed, the C-terminal domain of *FUS* contains a nonclassic nuclear localization signal.³⁸ It is possible that altered distribution of *FUS* could sequester RNA and other RNA binding proteins with subsequent disturbance of RNA metabolism. The presence of poly-A and other¹⁸ RNA binding proteins in basophilic inclusions would support this idea. The range of cytopathology in our *FUS* mutation cases suggests that structural disruption of nuclear and cytoplasmic RNA processing complexes are a feature of ALS-*FUS*. We propose that granular *FUS* deposits associated with progressive disruption of Nissl substance (figure 2) are morphologic precursors

sor lesions of the large compact basophilic aggregates. An analogy is found in ALS-TDP in which granular cytoplasmic reactivity, previously undetected by routine neuropathology, may also represent a precursor lesion of compact inclusions.³⁹ The FUS C-terminal domain is also important for direct protein–protein interactions and RNA target sequence recognition,⁴⁰ which might be disrupted by mutations, potentially leading to pre-mRNA splicing abnormalities even before frank protein aggregation occurs.

The novel c.1554_1557delACAG mutation is predicted to substantially alter the C-terminal domain at protein level by substituting 6 of the last 8 amino acids, suggesting a mechanism of pathogenesis similar to, but possibly more severe than, that of point mutations. On the other hand, the alteration at nucleotide level alone could potentially alter terminal exon recognition by disrupting exonic-splicing-enhancer binding sites, as well as alter mRNA expression by modification of the 3'UTR sequence through the frameshift. Functional studies are needed to clarify how the reported *FUS* mutations cause disease.

NOTE ADDED IN PROOF

DNA from the parents of index patient 1 became available after this manuscript was accepted for publication. Sequencing of the whole *FUS* gene revealed no mutations.

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