Published in final edited form as:

Acta Neuropathol. 2011 July ; 122(1): 87–98. doi:10.1007/s00401-011-0838-7.

Pathological heterogeneity in amyotrophic lateral sclerosis with *FUS* mutations: two distinct patterns correlating with disease severity and mutation

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Abstract

Mutations in the gene encoding the fused in sarcoma (*FUS*) protein are responsible for ~3% of familial amyotrophic lateral sclerosis (*ALS*) and <1% of sporadic *ALS* (*ALS-FUS*). Descriptions of the associated neuropathology are few and largely restricted to individual case reports. To better define the neuropathology associated with *FUS* mutations, we have undertaken a detailed comparative analysis of six cases of *ALS-FUS* that include sporadic and familial cases, with both juvenile and adult onset, and with four different *FUS* mutations. We found significant pathological heterogeneity among our cases, with two distinct patterns that correlated with the disease severity and the specific mutation. Frequent basophilic inclusions and round *FUS*-immunoreactive (*FUS*-

ir) neuronal cytoplasmic inclusions (NCI) were a consistent feature of our early-onset cases, including two with the p.P525L mutation. In contrast, our late-onset cases, that included two with the p.R521C mutation, had tangle-like NCI and numerous FUS-ir glial cytoplasmic inclusions. Double-labeling experiments demonstrated that many of the glial inclusions were in oligodendrocytes. Comparison with the neuropathology of cases of frontotemporal lobar degeneration with FUS-ir pathology showed significant differences and suggests that *FUS* mutations are associated with a distinct pathobiology.

Keywords

fused in sarcoma; FUS; amyotrophic lateral sclerosis; ALS; basophilic inclusions

Introduction

Mutations in the gene encoding the *fused in sarcoma* protein (FUS; also known as *translocated in liposarcoma*, TLS), were recently identified as a cause of familial amyotrophic lateral sclerosis (fALS) [18, 38]. Subsequently, genetic screening of ALS cohorts from many centres in North America, Europe and Asia have confirmed that *FUS* mutations are responsible for approximately 3% of fALS and <1% sporadic ALS (sALS) (ALS-*FUS*) [22]. The associated neuropathology has been described in only a small number of cases from these genetic series; however, neuronal cytoplasmic inclusions (NCI) that are immunoreactive for FUS (FUS-ir) but negative for the transactive response DNA binding protein with M_r 43 kD (TDP-43) seem to be a consistent feature [4, 8, 10, 18 29, 38]. More detailed descriptions of the anatomical distribution and morphology of FUS-ir pathology have only been provided in a few individual case reports [29, 30].

Following the discovery of *FUS* mutations in ALS, the role of FUS was investigated in the related neurodegenerative condition, frontotemporal lobar degeneration (FTLD) [25, 27, 28]. In several uncommon FTLD subtypes, where the major molecular defect was not yet known (not tau or TDP-43), the characteristic pathological inclusions were found to be FUS-ir. These included cases previously designated as atypical FTLD with ubiquitinated inclusions (aFTLD-U) [19, 27, 31], neuronal intermediate filament inclusion disease (NIFID) [28] and basophilic inclusion body disease (BIBD) [25]. As a result, these three conditions have now been grouped together within the broad molecular category of FTLD-FUS [21]. To date, genetic analysis has only been reported for a small number of pathologically confirmed FTLD-FUS cases, none of which has been found to have any abnormality of the *FUS* gene [27, 28, 32, 37].

A number of case reports, mostly from Japan, have described patients with clinical ALS and the unusual pathological feature of basophilic inclusions (BI). These include sporadic and familial cases with both juvenile and adult-onset [1, 9, 12, 16 17, 33, 36]. Following the report that the BIBD subtype of FTLD was characterized by FUS-ir pathology [25], several cases of ALS with BI were re-evaluated [3, 6, 11, 15, 23, 34, 35, 39]. These studies demonstrated that the BI in cases of ALS are also consistently FUS-ir and that most [3, 11, 15, 34, 35, 39], but not all cases [11, 6, 23], are associated with a *FUS* mutation.

These findings raise several questions regarding the correlation between FUS molecular genetics and neuropathology. Specifically, (i) do cases of ALS-*FUS* have a consistent and recognizable pathology that includes BI, (ii) do all cases of ALS with BI have underlying *FUS* mutations, and (iii) what is the relationship between ALS-*FUS*, BIBD and the other FTLD-FUS subtypes? To help address these questions, we have undertaken a detailed comparative analysis of the neuropathology of six cases of ALS with pathogenic *FUS*

mutations. Although relatively small, this none-the-less represents the largest such pathological series reported to date, and includes sporadic and familial cases, with both juvenile and adult onset, and with four different mutations. Our findings are compared with those of previously published case reports of ALS-*FUS* and pathological series of FTL-*FUS*.

Materials and Methods

Cases

Post mortem pathological material was obtained from six cases with clinically pure ALS (no FTD), in which *FUS* mutations had previously been identified (Table 1). Clinical, pathological and molecular genetics data of five of the cases has been reported previously in individual case reports or small series [3, 29, 30]. In the unpublished case (case 2), the c. 1561C>T (p.R521C) *FUS* mutation was identified using previously published molecular genetics methods [29].

Histopathology

BI were evaluated on sections stained with hematoxylin and eosin (HE), representing a wide range of anatomical regions (Table 2).

Immunohistochemistry and immunofluorescence

FUS immunohistochemistry (IHC) was performed on 5 µm thick sections of formalin fixed, paraffin embedded tissue using the Ventana BenchMark® XT automated staining system (Ventana, Tuscon, AZ) and developed with either aminoethylcarbazole (AEC, in Vancouver) or diaminobenzoic acid (DAB, in Zurich). The primary *FUS* antibodies included Sigma-Aldrich anti-*FUS* (1:25 – 1:200)* and Bethyl Laboratories A300–302A (1:500), both with initial overnight incubation at room temperature, following microwave antigen retrieval. Based on the amount of normal physiological staining, it was apparent that the anti-*FUS* sensitivity was greatly influenced by the degree of tissue fixation and that this was only partially reversed by antigen retrieval. Therefore, the dilution of the primary antibody was adjusted in each case to allow for faint physiological staining that ensured sensitivity (internal positive control) but did not compromise visualization of the pathology. All of the quantitation was based on sections stained using the *FUS* antibody from Sigma, with the Bethyl antibody being used primarily for confirmation. Individual tissue sections were exchanged between the two research centres (Vancouver and Zurich) to ensure consistency in immunostaining and standardization in quantitation. * *We have found that newer batches of Sigma anti-FUS, obtained following completion of this study, provide similar results when used at concentrations of 1:500– 1:2000.*

Double-label immunofluorescence was performed on selected cases, using the rabbit polyclonal anti-*FUS* (Sigma, 1:1000) and a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Dako, 1:100) as marker for astrocytes, a mouse anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) antibody (Sternberger Monoclonals, Lutherville, MD, clone SMI91, 1:800) as marker for oligodendrocytes and a mouse monoclonal anti-CD68 (Dako, 1:200) and anti-HLA-DR antibodies (DAKO, clone CR3/43, 1:500) as markers for microglia. The secondary antibodies were Alexa Fluor 594 conjugated anti-mouse and Alexa Fluor 488 conjugated anti-rabbit (Molecular Probes; 1:500). 4'-6-diamidino-2-phenylindol (DAPI) was used for nuclear counterstaining.

Semiquantitative evaluation of cellular inclusions

The number of each type of inclusion was scored in different anatomical regions using a semiquantitative grading system, employed in previous studies; absent (–), single (+/–), rare

(+), occasional (++) , moderate (+++) or numerous (++++) [20]. Inclusions were considered “single” when only one example of the particular type of inclusion was identified in all available sections from that anatomical region, “rare” if only a few examples could be found in each section of the region, “occasional” if they were relatively easy to find but not present in every medium power (20x) microscopic field, “moderate” if at least a few examples were present in most microscopic fields, and “numerous” when many were present in every microscopic field. Different morphological subtypes of FUS-ir NCI were also recorded in each region as being common (one of the predominant subtypes in a majority of the cases) or uncommon.

Results

Molecular genetics and clinical features

The cases fell into two distinct groups, based on the course of their disease. Cases 1 – 3 had ALS onset in mid- to late-adult life (late-onset; age, 44 – 62 years) with survival for several years (2 – 4 years) (Table 1). In contrast, cases 4 – 6 had a much more aggressive course, with onset in early-adult life (early-onset; age, 18 – 22 years) and rapid progression to death in less than a year. The clinical phenotypes were otherwise similar with predominant lower motor neuron (LMN) involvement, usually presenting as proximal limb weakness, mild or late-onset bulbar features and no significant cognitive or extrapyramidal motor dysfunction. All mutations involved the final exon (15). Although the number of cases with each specific mutation was too small to determine firm correlations, it is worth noting that both cases with the p.P525L mutation had early-onset aggressive disease while the two with the p.R521C mutation were late-onset with a more benign course.

Neuropathology

Basophilic inclusions—Classical BI were sharply-defined, single or multiple, round, oval or multilobulated bodies, often similar in size to the neuronal nucleus, with a pale blue-grey colour on HE stain (Fig. 1a, b). At least some BI were identified in all cases; however, their frequency and anatomical distribution varied significantly. In the early-onset group (cases 4 – 6), BI were a very obvious pathological feature, with multiple examples identified in every section of the spinal cord and hypoglossal nucleus examined (average, 3 to 5 BI per tissue section) (Table 2). Moderate numbers of BI were also present in large neurons of the primary motor cortex, presumed to be upper motor neurons (UMN). Two of the early-onset cases also had occasional BI in the nuclei of the basis pontis; whereas, other neuroanatomic sites were inconsistently affected and none had more than rare BI. In contrast, BI were difficult to find in the late-onset cases (cases 1 – 3). Although they were most common in spinal cord LMN, no tissue section had more than a single BI (Table 2).

In addition to classical BI, some UMN and LMN contained smaller, sometimes multiple, densely basophilic deposits (Fig. 1c). These were always less numerous than classical BI but were a consistent finding in the early-onset cases. Only one of the late-onset cases was found to have rare examples of this type of inclusion, restricted to LMN of the spinal cord.

FUS-ir NCI—Significant numbers of FUS-ir NCI were found in LMN of the brainstem and spinal cord in all cases; however, these tended to be more numerous in the early-onset group (Table 3). NCI were also present in many other neuroanatomical regions, but generally in smaller numbers. Although the anatomical distribution was similar, the early-onset cases tended to have more consistent and severe involvement of the primary motor cortex, nuclei of the basis pontis and cerebellar dentate nucleus, whereas the basal ganglia was more affected in the late-onset cases. FUS-ir NCI were present in all anatomical regions where there was significant neurodegeneration, as evidenced by neuronal loss and reactive gliosis

(not quantified), but also in some regions that appeared otherwise intact (i.e. pontine nuclei in early-onset cases).

The morphology of the FUS-ir NCI differed significantly among the cases (Table 4). The most common type in the late-onset cases appeared as a collection of thick filaments within the perikaryon, some of which had a globose arrangement while others appeared as a flame-shaped collection with one or more filaments extending into the proximal axon (Fig. 2a-c). These tangle-like NCI were only rarely found in the early-onset cases. In contrast, the early-onset cases had a predominance of compact, round NCI (Fig. 3). Based on their size and anatomical distribution, larger examples of round NCI were thought to correspond with classical BI seen with HE stain. Round NCI were not a common finding in any of the late-onset cases. Non-compact collections of FUS-ir cytoplasmic granules were also a common finding in many anatomical regions of all cases (Fig. 2d).

FUS-ir neuronal intranuclear inclusions—In two of the early-onset cases (cases 5 and 6), two or three neurons in the hypoglossal nucleus contained multiple small round FUS-ir inclusions in the nucleus that were smaller than the adjacent nucleolus (Fig. 3d). Similar neuronal intranuclear inclusions (NII) were not found in the other cases or in any other anatomical regions. None of the cases had any “vermiform” NII that have previously been reported as a characteristic finding of some subtypes of FTLDFUS [20, 27, 28].

FUS-ir glial pathology—FUS-ir glial cytoplasmic inclusions (GCI) were numerous and anatomically widespread in all the late-onset cases but only rarely found in the early-onset group (Table 5). In the late-onset cases, there was a strong correlation between the number of GCI and NCI in each neuroanatomical region (Tables 3 and 5); however, glial inclusions were always more numerous. GCI were also present in the corticospinal tracts and many other white matter regions.

The morphology of the GCI was variable and included small round, crescentic or flame-shaped perinuclear bodies, as well as some that extended into single or multiple ramified processes (Fig. 4). Short FUS-ir thread-like structures were also common in areas with GCI and were likely of glial origin. The nuclei adjacent to the GCI were most often round, typical of oligodendroglia, but some were more oval or elongated. Double-labeling experiments clearly demonstrated FUS-positive inclusions within the cytoplasm of CNPase-positive oligodendrocytes, but there was no colocalization of FUS with GFAP or the microglial markers CR3/43 and CD68 (Fig. 5).

Discussion

The neuropathology of ALS-*FUS* has been described in detail in only a few previous reports [29, 30], with the largest series consisting of three of the patients included in the present study [3]. Although the presence of FUS-ir cytoplasmic inclusions in LMN seems to be a consistent feature, the spectrum of neuropathology has not been clearly defined and pathological correlates for different clinical phenotypes and specific *FUS* mutations have not yet been determined. In this study, we performed a detailed comparative analysis of the neuropathology in six cases of ALS-*FUS* and found two distinct patterns of pathology that correlated with the clinical course and the mutation.

In cases with early-onset ALS and an aggressive course, BI in UMN and LMN were a prominent feature, readily identified in all sections of primary motor cortex, medulla and spinal cord. With FUS IHC, most NCI had a compact, round morphology and were numerous in the motor cortex, hypoglossal nucleus and ventral grey matter of the spinal cord and less abundant, but consistently present, in the spinal cord dorsal grey matter, nuclei basis

pontis, substantia nigra and cerebellar dentate nucleus. In contrast, FUS-ir glial pathology was remarkably sparse in these cases, even in anatomical regions with severe neuronal involvement. These early-onset cases included two with the p.P525L mutation.

Our cases with onset in mid-adult life, with longer disease duration, had very rare BI that were only identified after thorough examination of multiple tissue sections. The predominant type of FUS-ir NCI had a filamentous, tangle-like morphology. Compared with the early-onset cases, NCI were less numerous in UMN, LMN and the pons but more abundant in the basal ganglia. A particularly striking feature was the abundance of FUS-ir GCI that were present in virtually all neuroanatomical regions examined. These late-onset cases included two with the p.R521C mutation.

Although differences in methodology and the amount of detail provided make comparison difficult, previous reports of other patients with *FUS* mutations have described a similar range of pathological variation and generally match the correlations we have identified. Only one other case with the p.P525L mutation has been reported and it also presented as juvenile onset sporadic ALS [11]. The pathology was similar to our early-onset cases, with many BI in sensorimotor cortex and spinal cord, and FUS IHC demonstrating NCI but no GCI. Cases with adult-onset (range 28–69 years) have included several with the common p.R521C mutation [4, 15, 34–36, 38, 39] and others with p.R521G [18], p.R521H [8, 38], p.R524W [10] and p.G507N mutations [10]. With the exception of the p.G507N mutation case, all have had a family history of ALS. In these, BI in LMN were either not commented upon [4, 8, 10, 18, 38] or described using terms such as “few” or “some” [34, 36, 39], implying they were not a prominent feature. FUS-ir GCI have been a consistent feature [10, 15, 35, 39] and the morphology of NCI is most often filamentous [4, 8, 10]. The anatomical distribution of FUS-ir pathology in the case described by Kobayashi *et al.* was also quite similar to that found in our late-onset cases [15].

Although most of the previously reported cases have pathological features similar to one of the two patterns we identified, some additional variations have been reported. In the case described by Hewitt *et al.*, with p.G507N mutation, FUS IHC demonstrated GCI but no NCI; however, only sections of spinal cord were examined and there were few LMN remaining [10]. Kobayashi *et al.* reported a late-onset case (p.R521C) in which the NCI were described as being round, crescentic or mushroom shaped, with no mention of filamentous forms [15]. Finally, the late-onset case (p.R521C) described by Tateishi *et al.* had BI in several neuroanatomical regions (excluding LMN) and an unusual distribution of FUS-ir pathology, with severe involvement of neocortex, hippocampus and cerebellar white matter [35]. However, this case also had an unusual clinical course, with disease duration being prolonged to 18 years with artificial ventilation. These additional variations confirm that *FUS* mutations are associated with a range of neuropathology and suggest that the two distinct patterns we have identified may represent opposing ends of this spectrum. Determining whether this variation directly reflects differences in pathogenesis, as determined by the specific mutations, or is a secondary manifestation of the course of the disease (specifically, disease duration), must await evaluation of additional cases.

The sensitivity and specificity of BI as a pathological marker for underlying *FUS* mutations is difficult to determine from previous publications, due to various biases in the study designs and approaches. Most of the initial studies identified *FUS* mutations by screening large cohorts of clinically defined ALS patients. In the few cases from these series with pathological evaluation, the presence or absence of BI is never mentioned [4, 8, 10, 18, 29, 38]. It is not clear whether this reflects a true absence of such pathology or perhaps BI were simply overlooked, due to inadequate tissue sampling or greater focus on the results of FUS IHC. Following the discovery that BIBD is characterized by FUS-ir pathology [25], a

number of cases that had previously been reported as ALS with BI were re-evaluated and many were found to have associated *FUS* mutations [3, 6, 11, 15, 23, 34, 35, 39]. Since these cases were selected on the basis of BI having been previously identified, one might anticipate more consistent pathology. However, review of these reports suggests the frequency of BI to be highly variable, with some described as having only a “few” BI in brainstem and spinal cord LMN [34, 36]. Moreover, the description of the morphology and tinctorial characteristics raises questions as to whether the designation of “BI” has been used in a consistent fashion. In the report by Kobayashi *et al.*, BI are described as being “near to pink, rather than blue” on HE stain [15] and the case of Tateishi *et al.* had inclusions with an eosinophilic core and pale basophilic halo, reminiscent of hyaline conglomerate inclusions of NIFID [35]. Other reports describe basophilic aggregates with features similar to Nissl substance [3] while argyrophilia seems to be an inconsistent feature [25, 35]. Although we identified BI in LMN of all of our patients with *FUS* mutations, their number in the late-onset cases was so few, that they were initially overlooked and only recognized after careful review of multiple tissue sections. In contrast, several reports have described cases of ALS with frequent FUS-ir BI in which no *FUS* mutation was detected [6, 11, 23]. Taken together, these results indicate that the frequency of BI is highly variable and they cannot be considered a reliable indicator of an associated *FUS* mutation.

The variability in the frequency of BI may be explained by recent studies that have examined the functional consequences of different *FUS* mutations [5, 13, 14]. *FUS* is a multifunctional DNA/RNA binding protein [40] that continually shuttles between the nucleus and cytoplasm [41]; but in neurons and glia, it is predominantly localized to the nucleus [2]. Most ALS-associated *FUS* mutations affect highly conserved regions of exon 15 that encodes the extreme C-terminus [22], including the non-canonical nuclear localization signal (PY-NLS) [5, 13, 14]. *In vitro* studies have shown that these mutations disrupt the PY-NLS and result in relative redistribution of *FUS* to the cytoplasm where it is recruited into stress granules (SG), cytosolic structures that temporarily store mRNA during cellular stress [5, 13, 14]. With persistent high cytosolic levels of *FUS* and prolonged stress, these SG grow and coalesce, and eventually form large cytoplasmic inclusions. Previous studies have shown that BI contain both mRNA and markers for SG and likely represent the endstage of this process [7, 24, 26]. The fact that different *FUS* mutations affect nuclear import to differing degrees (i.e the p.P525L mutation results in severe disruption while p.R521C causes relatively little impairment) [5, 13, 14], likely explains the variations in the associated clinical course and the extent of BI and FUS-ir pathology in the cases examined in this study.

FUS-ir pathology is also a characteristic feature of several clinicopathological subtypes of FTLN (FTLN-FUS), including aFTLN-U, NIFID and BIBD [20]. The majority of FTLN-FUS cases are sporadic and none has yet been associated with a *FUS* mutation [27, 28, 32, 37]. Although there are some similar features, comparison of the neuropathology suggests that ALS-FUS represents a distinct entity rather than an extension of the FTLN-FUS spectrum. None of our ALS cases showed involvement of the broad range of neuroanatomical regions that is affected in all cases of FTLN-FUS [20]. Vermiform FUS-ir NII are a consistent feature of both aFTLN-U and NIFID [20] but are not present in ALS-FUS. Cases of FTLN-FUS (particularly NIFID and BIBD) also display greater variation in NCI morphology, including crescentic, annular and complex forms that were not present in any of our ALS-FUS cases. These differences suggest that the pathobiological pathway triggered by *FUS* mutations may be distinct from that involved in most cases of FTLN-FUS.

Finally, although FUS-ir GCI have been described in previous reports of ALS-FUS [3, 10, 15, 29, 30, 35, 39] and FTLN-FUS [20, 25, 27, 28], this study represents the most detailed analysis of their anatomical distribution and the first attempt to characterize their cellular

identity. Most of the GCI in our cases were adjacent to round nuclei, characteristic of oligodendrocytes (Fig. 4). The oligodendroglial localization of many FUS-ir GCI was confirmed with double-labeling for CNPase; an enzyme that, in brain, is exclusively expressed by myelin-forming oligodendrocytes (Fig. 5). Although the complex ramified morphology of some GCI and their association with more irregular oval nuclei (Fig. 4) suggested possible astrocytic or microglial origin, this could not be confirmed by double-labeling experiments using sensitive and specific markers of these cell types. In late-onset cases, GCI were more abundant and anatomically widespread than NCI (Tables 3 and 5). In contrast, the early-onset cases had very few GCI, even in regions with severe neuronal involvement. These findings suggest that (i) glial and neuronal FUS pathology develop independently, (ii) GCI probably reflect a more chronic disease process, and (iii) glial involvement is less likely to be central to the disease pathogenesis.

In summary, this study represents the largest pathological series of ALS-*FUS* reported to date and the first to directly compare the neuropathology of cases with different mutations and clinical phenotypes. We found significant heterogeneity among our cases, with two distinct patterns of neuropathology that correlated with the clinical course and the type of *FUS* mutation with respect to degree of nuclear import impairment. Frequent BI and round FUS-ir NCI were a consistent feature of our early-onset cases, while abundant tangle-like NCI and GCI in oligodendrocytes were characteristic of our late-onset cases. Differences in the neuropathology suggest that ALS-*FUS* has a pathobiology that is distinct from that of FTL-*FUS*.

Acknowledgments

We thank Margaret Luk and Mareike Schroff for their excellent technical assistance. This work was supported by grants from Canadian Institutes of Health Research (grant number 74580, IM); the Pacific Alzheimer Research Foundation (IM); the NIHR Oxford Biomedical Research Centre (OA); the NIH/NIA R01 AG26251 (RR); the ALS Association (RR); the Swiss National Science Foundation (grant number 31003A-132864, MN); the Stavros-Niarchos Foundation (MN); and the Synapsis Foundation (MN).

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Fig. 1. Basophilic inclusions (BI). Most BI were well-defined, single or multiple, round or multilobulated structures with a pale blue-grey color (**a**). In early-onset cases, multiple BI were present in all sections of spinal cord, medulla and motor cortex (**b**). Some UMN and LMN contained smaller, densely basophilic deposits that resembled abnormally clumped Nissl substance (**c**). Hematoxylin and eosin. *Scale bar* 30 µm (**a**); 50 µm (**b**); 20 µm (**c**)

Fig. 2. FUS-immunoreactive (FUS-ir) neuronal cytoplasmic inclusions in late-onset ALS-*FUS* cases most often appeared as a collection of thick filaments with either a globose or flame shape (**a – c**; lower motor neuron (**a**), upper motor neuron (**b**), basal ganglia (**c**)). Non-compact collections of FUS-ir cytoplasmic granules were also common in both late- and early-onset cases (**d**). FUS immunohistochemistry, developed with AEC. Scale bar 15 μ m (**a**); 25 μ m (**b**); 75 μ m (**c**); 20 μ m (**d**)

Fig. 3. FUS-immunoreactive (FUS-ir) neuronal cytoplasmic inclusions in early-onset ALS-*FUS* cases usually had a compact, round morphology and were often of similar size and shape to BI (**a – d**; spinal cord lower motor neuron (**a**), primary motor cortex (**b**), basis pontis (**c**), hypoglossal nucleus (**d**)). In some early-onset cases, neurons of the hypoglossal nucleus contained small round FUS-ir intranuclear inclusions (**d**). FUS immunohistochemistry, developed with DAB. *Scale bar* 20 μ m (**a, d**); 100 μ m (**b, c**)

Fig. 4. FUS-immunoreactive glial cytoplasmic inclusions (GCI) had variable morphology, including small round or crescentic bodies (**a**) that often extended into a few (**b**) or multiple ramified processes (**c**, **d**). GCI were only numerous in the late-onset cases where they had a wide neuroanatomical distribution (basal ganglia (**e**), cerebral white matter (**f**)). FUS immunohistochemistry, developed with AEC. *Scale bar* 10 μm (**a** – **c**); 15 μm (**d**); 20 μm (μ); 40 μm (**f**)

Fig. 5. Double-labeling immunofluorescence of FUS-immunoreactive (FUS-ir) glial inclusions. Glial cells with FUS-ir inclusions were often labeled by the oligodendrocyte marker CNPase (**a**, **b**). FUS-ir inclusions were not found in GFAP-positive astrocytes (**c**) or CR3/43 positive microglia cells (**d**). Double-labeling immunofluorescence; glial markers red, FUS green. Scale bar 10 μ m (**a-c**); 40 μ m

Table 1

Demographic and clinical features of cases.

case	<i>FUS</i> cDNA mutation (<i>FUS</i> protein mutation)	sex	ethnicity	family history	onset (years)	duration (years)	onset	ALS features		bulbar	dementia	park.	ref.
								LMN	UMN				
1	c.1561C>T (p.R521C)	F	Caucasian	yes	62	4	symmetric shoulders, hips	++	+	-	no	no	[29]
2	c.1561C>T (p.R521C)	F	Caucasian	yes	44	2	left leg	++	++	+	no	no	-
3	c.[1542G>T;1547A>T] p.[R514S;/E516V]	M	Chinese	no	44	3	bilateral arms	++	+	+	no	no	[30]
4	c.1574C>T (p.P525L)	F	Caucasian	no	22	<1	left hip	++	-	-	no	no	[3]
5	c.1574C>T (p.P525L)	F	mixed (Afro- Caucasian)	no	18	<1	left upper arm	++	+	+	no	no	[3]
6	c.1554_1557delACAG (p.Q519fsX9)	M	Caucasian	no	18	<1	bilateral arms	++	+	+	mild learning difficulty	no	[3]

++, presenting/predominant; +, late-onset/minor; -, absent.

ALS, amyotrophic lateral sclerosis; LMN, lower motor neuron; park., parkinsonism; ref., reference; UMN, upper motor neuron.

Table 2

Semiquantitative scoring of basophilic inclusions.

case	motor cortex	front. cortex	HC	striat.	GP	thal.	SN	PAG	pont. nuc.	ION	CN XII	spinal LMN	cer. cortex	cer. dent.
1	-	-	-	-	na	-	-	-	-	-	+/-	+	-	-
2	-	-	-	-	+/-	-	+/-	-	-	-	+/-	+	na	na
3	-	-	-	-	-	-	+/-	-	+	-	-	+	-	-
4	+++	-	-	-	-	na	+	-	++	-	+++	+++	-	-
5	+++	-	-	-	-	na	+/-	-	-	-	++	+++	-	+
6	+++	-	-	-	-	na	na	-	++	-	+++	++	na	na

cer. cortex, cerebellar cortex; cer. dent, cerebellar dentate nucleus; front. cortex, frontal cortex; CN XII, cranial nerve twelve; GP, globus pallidus; HC, hippocampus; ION, inferior olivary nucleus; LMN, lower motor neurons; PAG, periaqueductal grey; pont. nuc., pontine nuclei; SN, substantia nigra; striat., striatum; thal., thalamus.

Grading: -, none; +/-, single, one inclusion in all sections of region; +, rare; ++, occasional; +++, moderate; +++++, numerous; na, not available.

Table 3

Semiquantitative scoring of FUS-immunoreactive neuronal cytoplasmic inclusions.

case	motor cortex	front. cortex	HC	striat.	GP	thal.	SN	PAG	pont. nuc.	ION	CN XII	spinal LMN	dorsal grey	cer. ctx	cer. dent.
1	-	-	-	+	na	+	++	+/-	+/-	+	+++	+++	++	-	-
2	+/-	-	-	+	+++	++	+++	+/-	-	-	+++	+++	++	na	na
3	++	+	-	++	++	++	++	++	+	-	+++	+++	++	-	++
4	+++	-	-	+	++	na	++	+	+++	+	++++	+++	++	-	++
5	+++	-	-	-	-	na	++	-	+	+	++++	++++	++	-	+++
6	+++	-	-	-	na	na	na	na	+++	-	++++	++++	++	na	na

cer. cortex, cerebellar cortex; cer. dent., cerebellar dentate nucleus; front. cortex, frontal cortex; CN XII, cranial nerve twelve; GP, globus pallidus; HC, hippocampus; ION, inferior olivary nucleus; LMN, lower motor neurons; PAG, periaqueductal grey; pont. nuc., pontine nuclei; SN, substantia nigra; striat., striatum; thal., thalamus.

Grading: -, none; +/-, single, one inclusion in all sections of region; +, rare; ++, occasional; +++, moderate; +++++, numerous; na, not available.

Table 4

Morphological subtypes of FUS-immunoreactive neuronal cytoplasmic inclusions.

	Motor cortex	Front. cortex	Striat.	GP	Thal.	SN	PAG	Pont. nuc.	ION	CN XII neu.	Spinal LMIN	Dorsal gray	Cer. dent.
<i>Cases 1–3</i>													
Common			t	t	tg	tg				gt	tg	tg	
Uncommon	t	t	g	g		R	tgR	gt	t				gtR
<i>Cases 4–6</i>													
Common	Rr					r		Rrg		Rrg	Rrg	rRg	gr
Uncommon	g		tg	gr		Rtg	g	rg	t	t			R

Table 5

Semiquantitative scoring of FUS-immunoreactive glial cytoplasmic inclusions.

case	motor cortex	front. cortex	front. white	striat.	GP	thal.	SN	PAG	pont. nuc.	ION	CN XII	spinal LMN	dorsal grey	CST	post. col.	cer. ctx	cer. dent.
1	+	-	+	++	na	++	+++	+	++	+	+++	++++	+++	++	+	-	-
2	+	+	++	+++	++++	+++	++++	++	-	+	+++	++++	++	++	+	na	na
3	+++	++	+++	+++	++++	++++	++++	+++	++	+++	+++	++++	+++	+++	++	-	++
4	-	-	-	+	++	na	-	-	-	-	-	-	-	-	-	-	+
5	-	-	-	-	-	na	-	-	-	-	-	-	-	+	-	-	+
6	-	-	-	-	na	na	na	na	-	-	+	+	-	+	-	na	na

cer. cortex, cerebellar cortex; cer. dent., cerebellar dentate nucleus; front. cortex, frontal cortex; CN XII, cranial nerve twelve; CST, corticospinal tract; GP, globus pallidus; HC, hippocampus; ION, inferior olivary nucleus; LMN, lower motor neurons; PAG, periaqueductal grey; pont. nuc., pontine nuclei; post. col., posterior column; SN, substantia nigra; striat., striatum; thal., thalamus.

Grading: -, none; +, rare; ++, occasional; +++, moderate; +++++, numerous; na, not available.