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## De Novo Truncating *FUS* Gene Mutation as a Cause of Sporadic Amyotrophic Lateral Sclerosis

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### Abstract

Mutations in the gene encoding fused in sarcoma (*FUS*) were recently identified as a novel cause of amyotrophic lateral sclerosis (ALS), emphasizing the genetic heterogeneity of ALS. We sequenced the genes encoding superoxide dismutase (*SOD1*), TAR DNA-binding protein 43 (*TARDBP*) and *FUS* in 99 sporadic and 17 familial ALS patients ascertained at Mayo Clinic. We identified two novel mutations in *FUS* in two out of 99 (2.0%) sporadic ALS patients and established the *de novo* occurrence of one *FUS* mutation. In familial patients, we identified three (17.6%) *SOD1* mutations, while *FUS* and *TARDBP* mutations were excluded. The *de novo FUS* mutation (g.10747A>G; IVS13-2A>G) affects the splice-acceptor site of *FUS* intron 13 and was shown to induce skipping of *FUS* exon 14 leading to the C-terminal truncation of *FUS* (p.G466VfsX14). Subcellular localization studies showed a dramatic increase in the cytoplasmic localization of *FUS* and a reduction of normal nuclear expression in cells transfected with truncated compared to wild-type *FUS*. We further identified a novel in-frame insertion/deletion mutation in *FUS* exon 12 (p.S402 P411delinsGGGG) which is predicted to expand a conserved poly-glycine motif. Our findings extend the mutation spectrum in *FUS* leading to ALS and describe the first *de novo* mutation in *FUS*.

### Keywords

*FUS/TLS*; fused in sarcoma; amyotrophic lateral sclerosis; *de novo* mutation; *FUS* splice-site mutation; *FUS* truncating mutation

## INTRODUCTION

Major advances have recently been made in our understanding of the genetic causes underlying amyotrophic lateral sclerosis (ALS) (Dion, et al., 2009; Van Damme and Robberecht, 2009). ALS is a neurodegenerative disorder characterized by the loss of motor neurons in the spinal cord, brain stem, and motor cortex, resulting in progressive muscle weakness and atrophy which is typically fatal within 3–5 years (Boillee, et al., 2006). Most

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patients with ALS are sporadic, however, a positive family history of ALS has been found in 5–10% of all reported cases (Mitchell and Borasio, 2007). ALS is a genetically complex disorder with multiple causal genes and genetic risk factors contributing to the disease. Mutations in the copper/zinc superoxide dismutase 1 gene (*SOD1*; MIM# 147450) are the most common cause of ALS and account for about 15–20% of familial adult-onset patients with ALS and 1–2% of sporadic patients (Dion, et al., 2009; Rosen, et al., 1993). More recently, mutations in the genes encoding the TAR DNA-binding protein 43 (*TARDBP*; MIM# 605078) and fused in sarcoma/translocated in liposarcoma (*FUS/TLS*; MIM# 137070), two multifunctional DNA/RNA binding proteins, were identified in familial ALS with subsequent reports of mutations in sporadic patients (Kabashi, et al., 2008; Kwiatkowski, et al., 2009; Lagier-Tourenne and Cleveland, 2009; Sreedharan, et al., 2008; Vance, et al., 2009). Several other less common gene mutations linked to familial adult-onset ALS with or without frontotemporal dementia (FTD; MIM# 600274) have been reviewed elsewhere (Dion, et al., 2009). An important locus of the combined phenotype of ALS and FTD has been mapped to a region on chromosome 9p (Le Ber, et al., 2009; Morita, et al., 2006; Vance, et al., 2006); however, the causative gene has not yet been identified.

*FUS/TLS* is a member of the TET family of RNA binding proteins, which also includes Ewing's sarcoma (*EWSR1*; MIM# 133450) and TATA-binding associated factor (*TAF15*; MIM# 601574) (Tan and Manley, 2009). The *FUS* gene is located on chromosome 16p11.2 and consists of 15 exons encoding a protein of 526 amino acid residues (Morohoshi, et al., 1998). Like other TET proteins, *FUS* has an N-terminal transcriptional activation domain enriched in glutamine, glycine, serine, and tyrosine (QGSY) residues, a glycine-rich region and several structural motifs involved in RNA-binding, including an RNA-recognition motif (RRM), multiple arginine-glycine-glycine (RGG) repeats and a putative zinc finger domain, at the C-terminus (Iko, et al., 2004). *FUS* binds RNA in vitro and in vivo (Croizat, et al., 1993; Zinszner, et al., 1997) and is involved in several mRNA-processing steps, such as transcription regulation (Uranishi, et al., 2001), RNA splicing (Meissner, et al., 2003; Yang, et al., 1998), and RNA transport including nucleo-cytoplasmic shuttling (Zinszner, et al., 1997). *FUS* also binds DNA and plays a role in DNA repair through homologous DNA pairing and recombination (Baechtold, et al., 1999) and acts as a transcriptional regulatory sensor of DNA damage signals to assure genomic integrity (Wang, et al., 2008). *FUS* deficient mice indeed show high levels of chromosomal instability and perinatal mortality (Hicks, et al., 2000) and an increased sensitivity to ionizing radiation (Kuroda, et al., 2000). Through chromosomal translocations, the N-terminus of *FUS* functions as an essential transforming domain for a number of fusion oncoproteins implicated in the development of human liposarcomas and leukemias (Law, et al., 2006). *FUS* shows an almost ubiquitous nuclear expression but is also present in the cytoplasm of most cell types (Andersson, et al., 2008). In neurons, *FUS* is predominantly localized to the nucleus (Andersson, et al., 2008). Interestingly, neuropathological analysis of brain and spinal cord of ALS patients carrying *FUS* mutations showed cytoplasmic retention and the formation of *FUS* and ubiquitin-positive neuronal aggregates, although at least some preservation of normal physiological nuclear *FUS* immunoreactivity was observed (Kwiatkowski, et al., 2009; Vance, et al., 2009).

In less than one year, 23 different mutations have been identified in *FUS* in 38 families and 12 sporadic ALS patients (Belzil, et al., 2009; Blair, et al., 2009; Chio, et al., 2009; Corrado, et al., 2009; Drepper, et al., 2009; Kwiatkowski, et al., 2009; Rademakers, et al., in press; Ticozzi, et al., 2009; Van Damme, et al., 2009; Vance, et al., 2009). The majority of the mutations are missense changes affecting the highly conserved most C-terminal part of *FUS*; however missense changes and one in-frame deletion have also been reported in the QGSY and glycine-rich region. The reported mutation frequencies of *FUS* in familial ALS range from 1–5%, with lower estimates (0–1%) in populations of sporadic ALS patients (Belzil, et

al., 2009; Blair, et al., 2009; Chio, et al., 2009; Corrado, et al., 2009; Drepper, et al., 2009; Kwiatkowski, et al., 2009; Rademakers, et al., in press; Ticozzi, et al., 2009; Van Damme, et al., 2009; Vance, et al., 2009). Here we aimed to determine the spectrum and contribution of *SOD1*, *TARDBP* and *FUS* mutations in a newly ascertained Mayo Clinic ALS series and identified three known *SOD1* mutations in familial ALS patients (17.6%) and two novel *FUS* mutations in sporadic ALS patients (2.0%), including one patient with a *de novo* *FUS* mutation. Importantly, we identified a splice-site mutation defining a new category of *FUS* mutations characterized by C-terminal truncation of FUS. Using cellular expression studies we show a remarkable increase in cytoplasmic localization of truncated FUS compared to full-length wild-type FUS, supporting the presence of a nuclear localization signal (NLS) within the C-terminus of FUS.

## MATERIALS AND METHODS

### Study Population

Our study cohort consisted of 108 unrelated ALS patients and 9 patients with other diagnosis (61 males, 56 females) from a consecutive clinical case series seen at Mayo Clinic Florida by the ALS Center in the period 2008–2009. All patients agreed to be in the study and biological samples were obtained after informed consent. Each patient underwent a full neurological evaluation including electromyography, clinical laboratory testing and imaging as appropriate to establish the clinical diagnosis of ALS. A total of 108 patients were diagnosed with ALS according to El Escorial criteria, 6 patients were diagnosed with primary lateral sclerosis (PLS), 1 patient with progressive muscular atrophy (PMA) and 2 patients with monomelic amyotrophy. In the ALS patient cohort, 15.7% of patients (17/108) showed a positive family history of ALS defined as having at least one affected relative within 3 generations. The disease onset was bulbar in 23.1% of ALS patients and spinal in 76.9%. Thirty-seven patients (34.3%) showed frontotemporal cognitive impairment and/or pseudobulbar affect. The average age of onset in our complete patient population was  $56.6 \pm 10.9$  years (range 20–78 years). DNA samples of 700 healthy control individuals (range 30–99 years) were also ascertained at the Department of Neurology at Mayo Clinic Florida.

### *FUS* sequencing and genotyping analyses

Genomic DNA (gDNA) was extracted from blood samples using standard automation protocols with the AutoGenprep 245T (Autogen, Holliston, MA, USA). For each patient, polymerase chain reaction (PCR) amplification was performed for all 15 exons of *FUS*, all 5 exons of *SOD1* and exon 6 of *TARDBP*, using primers designed to flank intronic sequences using Qiagen products (Qiagen, Valencia, CA, USA) (PCR conditions and primer sequences available on request). PCR products were purified using the Ampure system (Agencourt Bioscience Corporation, Beverly, MA, USA) and sequenced using Big dye terminator V.3.1 products (Applied Biosystems, Foster City, CA, USA). Sequencing products were purified using the CleanSEQ method (Agencourt) and analyzed on an ABI 3730 DNA analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher software (Gene Codes, Ann Arbor, MI, USA). We determined the presence of the g.10747A>G mutation in control individuals by PCR and sequencing analysis of exon 14 of *FUS*. The presence of the complex g.10096\_10124delinsGGAGGTGGAGG mutation in exon 12 of *FUS* in control individuals was performed by PCR amplification of exon 12 of *FUS* using one fluorescently labeled primer (Ex12F-FAM-GGAGCAGACCCATACTTG and Ex12R-ACACGCACATACCTCAGTAC) followed by fragment length analysis on an automated ABI3730 DNA-analyzer (Applied Biosystems). The 18-bp difference between the wild-type and mutant allele was scored using GENEMAPPER v4.0 (Applied Biosystems). Haplotype sharing analysis of six short tandem repeat (STR) markers on

chromosome 16 flanking *FUS* (D16S401, D16S3068, D16S769, D16S753, D16S411 and D16S3136) in family ALS046 was also analyzed by GENEMAPPER.

### FUS transcript analyses

RNA extraction for patient ALS046-01, 8 control individuals and 7 unrelated ALS patients without *FUS* mutations was performed from 2-ml frozen blood aliquots collected in EDTA tubes, using a modified version of the protocol described by Beekman *et al.* (Beekman, et al., 2009). In short, the blood was thawed on ice for 2–3h and transferred to a PAXgene RNA collection tube (PreAnalytix; Qiagen, Valencia, CA, USA). After an incubation of 16–21h at room temperature we extracted RNA using the PAXgene RNA extraction kit (PreAnalytix) using the protocol recommended by the manufacturer. RNA concentrations were measured using a NanoDrop (Thermoscientific, Wilmington, DE, USA) and ranged from 10 to 100 ng/μl. First-strand synthesis was carried out using the Superscript III system (Invitrogen, Carlsbad, CA, USA), with 70–400 ng of total RNA as the template and a combination of random hexamers and oligo(dT) primers. The resultant complementary DNA (cDNA) was used as the template for RT-PCR reactions (Qiagen) using the following primer pairs: cDNA12/13F-GTCCTAATCCACCTGTGAG and utrR-CTTGGGTGATCAGGAATTG. RT-PCR reactions were denatured for 3 minutes at 94 °C, then cycled at 60-50 °C touchdown (30", 30", 45") for 35 cycles. RT-PCR products were visualized by electrophoresis on 1.5 % agarose gels, gel-excised, then DNA extracted using the QIAquick Gel Extraction Kit (Qiagen) and sequenced in both directions as described above.

### Cloning

A human cDNA clone encoding full-length wild type *FUS* (Invitrogen clone #2822692) was used as a PCR template to generate V5-tagged wild-type *FUS* (V5-*FUS*<sub>WT</sub>) and p.R521C mutant *FUS* (V5-*FUS*<sub>R521C</sub>) cDNA using primers listed in Supp. Table S1. cDNA containing p.G466VfsX14 truncated *FUS*, predicted to result from the deletion of *FUS* exon 14 (V5-*FUS*<sub>Δ14</sub>), was generated by PCR using cDNA of patient ALS046-01 as a template and the same primer set used to generate V5-*FUS*<sub>WT</sub>. Inserts were cloned into the pAG3 mammalian expression vector using *Xho*I and *Bam*HI restriction sites. The sequences of all pAG3 *FUS* expression constructs were confirmed by direct sequencing of the complete cDNA inserts and flanking vector sequences.

### Immunocytochemistry and immunoblot analysis

N2A (mouse neuroblastoma cell line) cells were cultured in Dulbecco's Modified eagles medium (DMEM) supplemented with 10% fetal bovine serum. For immunofluorescence microscopy, N2A cells were plated on 13mm diameter glass coverslips in a 24-well plate and transfected using 0.2% Lipofectamine2000 (Invitrogen) with 600ng V5-*FUS*<sub>WT</sub>, V5-*FUS*<sub>R521C</sub>, or V5-*FUS*<sub>Δ14</sub> respectively. After 36 hours, cells were fixed with 4% paraformaldehyde for immunostaining. The fixed cells were blocked for 1 hour with 5% BSA and subsequently incubated with 1:500 of primary antibody (mouse anti-V5 antibody, Invitrogen) for 1 hour. Fluorescent signal was detected using 1:1000 dilution of Alexa Fluor 488-labeled donkey anti-mouse antibody (Invitrogen). The cells were observed using 100× magnification by confocal microscopy. For Western blot analysis, N2A cells were plated in 6-well dishes and transfected using 0.2% Lipofectamine2000 (Invitrogen) with 2μg V5-*FUS*<sub>WT</sub>, V5-*FUS*<sub>R521C</sub>, or V5-*FUS*<sub>Δ14</sub> respectively. Nuclear and cytoplasmic enriched protein fractions were isolated from the cells 48 hours post-transfection using the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, Ontario, Canada) following the manufacturer's protocol. Total protein concentrations of the cytosolic and nuclear fractions were quantified using the bicinchoninic acid (BCA) assay (Pierce). 30μg of protein from the nuclear fraction and 8μg of protein from the cytosolic fractions were

separated on 10% SDS-PAGE gels, transferred to PVDF membranes and blocked for 1 h at RT with 5% skim milk/TBST. V5-FUS proteins were detected with anti-V5 primary antibody (1:5000; Invitrogen) followed by anti-mouse HRP conjugated secondary antibody (1:5000; Jackson Immuno Research, West Grove, PA) and ECL-Plus (Perkin Elmer, Waltham, MA). Re-probing of the blots with anti-GAPDH (1:5000; Biodesign International, Saco, ME) and anti-H3 (1:1000; Cell Signaling, Danvers, MA) was performed to confirm enrichment and equal loading of the cytosolic and nuclear fractions.

## RESULTS

### Two novel *FUS* mutations in sporadic patients from the Mayo Clinic ALS series

We performed systematic mutation analyses of *SOD1*, *TARDBP* and *FUS* through direct sequencing in 117 patients diagnosed with ALS, PLS, PMA or monomelic amyotrophy from a consecutive clinical case series ascertained at Mayo Clinic Florida. Our sequence analysis identified two novel mutations in *FUS* in two sporadic ALS patients (Figure 1) and three known mutations in *SOD1* in three familial ALS patients (Table 1). *TARDBP* mutations were not identified in our patient series. The population frequency of *SOD1* mutations was thus 17.6% of familial ALS (3/17), while *FUS* mutations were absent from familial patients but accounted for 2.0% of sporadic ALS (2/99). A summary of synonymous coding changes and genetic variants identified in the 3' untranslated regions of *SOD1* and *FUS* is provided in Supp. Table S2.

A heterozygous splice-site mutation, g.10747A>G (IVS13-2A>G), at the splice acceptor site of *FUS* intron 13 was identified in one sporadic ALS patient (ALS046-01) (Figure 2A). This mutation was predicted to affect the correct splicing of *FUS* exon 14. Frozen blood of this patient was available as a source of mRNA to study the effect of this mutation on *FUS* splicing. Amplification of *FUS* cDNA showed evidence of an aberrant product corresponding to the skipping of exon 14 (148bp) in addition to the full-length wild-type *FUS* transcript, which was further confirmed by gel-excision and sequencing analysis (Figure 2B). No aberrant PCR products were identified by cDNA analysis of 8 control individuals or 7 ALS patients without *FUS* mutations. The exclusion of exon 14 (c.1394\_1541del) in patient ALS046-01 is expected to result in a frameshift and a premature termination codon (PTC) in exon 15 leading to a truncated *FUS* protein (p.G466VfsX14) lacking most of the RGG-domain enriched in arginine-glycine-glycine motifs. A complex heterozygous 29-bp deletion and 11-bp insertion mutation in *FUS* exon 12, g.10096\_10124delinsGGAGGTGGAGG (c.1204\_1232delinsGGAGGTGGAGG), was identified in another sporadic ALS patient (ALS058-01) (Figure 2C). This mutation is predicted to lead to the deletion of ten amino acids and the insertion of four novel glycine residues within an evolutionarily conserved glycine-rich region (p.S402\_P411delinsGGGG) (Figure 2D). Both novel *FUS* mutations were excluded from 700 healthy control individuals.

### Clinical characteristics of *FUS* mutation carriers

Patient ALS046-01 was a 20-year-old woman who developed progressive dysarthria and dysphagia accompanied by tongue atrophy and fasciculations. Cervical, upper and lower limb weakness bilaterally followed, with diffuse muscle atrophy and widespread fasciculations leading to mutism, dysphagia, head drop and severe difficulty handling oral secretions. There were no definite upper motor neuron signs. Clinical findings supported a diagnosis of El Escorial clinically suspected ALS. A feeding tube was placed 11 months after onset; noninvasive ventilation (NIV) began at 14 months. Limited airway protection led to tracheostomy at 15 months. She died of ventilatory failure 22 months after onset of weakness. Autopsy was not performed.

Patient ALS058-01 was a 65-year-old man who developed exertional dyspnea with atrophy and fasciculations in tongue, trunk, and limb muscles bilaterally. Bulbar poliomyelitis at age 6 years had left him with mild dysphagia but no other motor symptoms. He was recently retired from a job requiring routine physical labor. Increasing respiratory muscle weakness and dysphagia led to initiation of NIV 9 months after onset of weakness, and a feeding tube at 11 months. Clinical findings supported a diagnosis of El Escorial clinically probable laboratory supported ALS. Weakness progressed to ventilatory failure and death 13 months after onset of weakness. Autopsy was not performed. Weakness was more pronounced proximally in both patients. Neither patient exhibited overt cognitive impairment or pseudobulbar affect. In both patients, EMG findings were compatible with ALS, and brain and spinal MRI and laboratory evaluation identified no alternative cause.

### De novo occurrence of *FUS* mutation

Both *FUS* mutation carriers identified in this study presented without any known family history of motor neuron disease or dementia. The parents of ALS046-01 are currently 53 years of age and healthy. To determine whether ALS046-01 inherited the g.10747A>G mutation from one of her unaffected parents, or whether the mutation occurred *de novo*, we obtained DNA samples from her father (ALS046-02) and mother (ALS046-03) and performed sequencing analysis of *FUS* exon 14 and flanking intronic sequences. These analyses showed that the g.10747A>G mutation was not present in the DNA of either one of the parents (Figure 2E). Haplotype analysis using six short tandem repeat markers flanking *FUS* further supported their genetic kinship, suggesting that the g.10747A>G mutation occurred *de novo* in ALS046-01. Since both parents of patient ALS058-01 are deceased, we could not evaluate the origin of the *FUS* mutation in this patient.

### *FUS* truncation mutation results in cytoplasmic mislocalization *in vitro*

To assess the functional importance of the truncated *FUS* transcript identified in this study, we created mammalian expression vectors expressing N-terminal V5-tagged wild-type *FUS* (V5-*FUS*<sub>WT</sub>), the previously reported p.R521C mutant *FUS* (V5-*FUS*<sub>R521C</sub>), and p.G466VfsX14 truncated *FUS*, predicted to result from the deletion of *FUS* exon 14 (*FUS*<sub>Δ14</sub>). Transient transfection of V5-*FUS*<sub>WT</sub>, V5-*FUS*<sub>R521C</sub>, and V5-*FUS*<sub>Δ14</sub> in N2A cells showed a predominant nuclear localization of V5-*FUS*<sub>WT</sub> in all transfected cells, while mutant V5-*FUS*<sub>R521C</sub> and V5-*FUS*<sub>Δ14</sub> generally showed dense cytoplasmic fluorescent signal with a correspondingly dramatic loss of detection in the nucleus (Figure 3A). In line with previous reports, a small fraction of cells transfected with V5-*FUS*<sub>R521C</sub> retained the predominant nuclear localization of *FUS*. In contrast, predominant nuclear localization of V5-*FUS*<sub>Δ14</sub> was rarely observed. The dramatic increase in cytoplasmic localization of V5-*FUS*<sub>R521C</sub> and particularly V5-*FUS*<sub>Δ14</sub> compared to V5-*FUS*<sub>WT</sub> was further confirmed by immunoblot analysis of cytosolic and nuclear enriched fractions obtained by subcellular fractionation of N2A cells transfected with each construct (Figure 3B). This analysis also showed the predicted smaller molecular weight band for V5-*FUS*<sub>Δ14</sub> compared to V5-*FUS*<sub>WT</sub> and V5-*FUS*<sub>R521C</sub>, consistent with the C-terminal truncation of *FUS*.

## DISCUSSION

We report the identification of two novel *FUS* mutations as the cause of ALS in two sporadic patients from the Mayo Clinic Florida ALS series. Our findings broaden the mutational spectrum of *FUS* in ALS to include a complex in-frame insertion/deletion mutation affecting multiple amino acid residues (p.S402\_P411delinsGGGG) and a splice-site mutation leading to the C-terminal truncation of *FUS* (p.G466VfsX14). Mutation and segregation analysis performed in relatives of one mutation carrier further revealed the *de*

*nov*o occurrence of the *FUS* mutation, explaining the sporadic nature of the disease in this family.

Mutations in the gene encoding the multifunctional DNA/RNA-binding protein *FUS* were identified earlier this year as the cause of familial ALS type 6 and have been suggested to account for 1–5% of familial ALS (Belzil, et al., 2009; Blair, et al., 2009; Chio, et al., 2009; Corrado, et al., 2009; Drepper, et al., 2009; Kwiatkowski, et al., 2009; Rademakers, et al., in press; Ticozzi, et al., 2009; Van Damme, et al., 2009; Vance, et al., 2009). *FUS* mutations have also been reported in a total of 12 sporadic patients although several studies failed to identify *FUS* mutations in sporadic patients (Blair, et al., 2009; Kwiatkowski, et al., 2009) and mutation frequencies were <1% in all sporadic ALS populations studied (Belzil, et al., 2009; Corrado, et al., 2009; Drepper, et al., 2009). These mutation frequencies however may have been underestimated since analysis was restricted in all (Drepper, et al., 2009) or in a subset (Belzil, et al., 2009; Corrado, et al., 2009; Rademakers, et al., in press) of patients to exons previously found mutated in ALS. In our Mayo Clinic ALS series we performed mutation analysis of all *FUS* exons and identified mutations at a slightly higher frequency, 2 out of 99 sporadic patients or 2.0% of our population. Both mutations were located outside of exon 15, including one mutation in exon 12, an exon not previously implicated in ALS. In contrast, in 17 familial ALS patients we identified three *SOD1* mutation carriers (17.6%) but no mutations in *FUS*; however, these results may be biased due to the small sample size of this population. Despite published *TARDBP* mutation frequencies of ~3% in familial ALS and ~1.5% in sporadic ALS, we did not identify mutations in exon 6 of *TARDBP* in any of our ALS patients.

In contrast to the previously published *FUS* missense mutations and a single codon in-frame deletion (Figure 1), the present study describes a new type of *FUS* mutations characterized by the C-terminal truncation of *FUS*. Mutation g.10747A>G (IVS13-2A>G) identified in patient ALS046-01 results in skipping of exon 14 from the *FUS* transcript leading to a premature termination codon in exon 15 eliminating the C-terminus of *FUS* including most of the RGG-domain (p.G466VfsX14). Using transcript analysis we confirmed the effect of this mutation on mRNA splicing and determined that this transcript was not subject to nonsense-mediated decay. Our findings emphasize the importance of the *in-vivo* validation of the effect of mutations on mRNA splicing, especially for newly identified disease genes. It is conceivable that some of the previously published *FUS* mutations, including g.10895A>G (p.R514G), g.11185G>C (p.R514S) and the double point mutation g.11185G>T; g.11186G>T (p.R514S; p.G515C) may also induce aberrant *FUS* splicing and the production of truncated *FUS* proteins. Similarly, the current mutation frequency of *FUS* in familial and sporadic ALS may be underestimated because out-of frame exonic deletions or duplications resulting in truncated *FUS* proteins could have been missed in routine gDNA sequencing analyses.

We further identified a complex 29-bp deletion and 11-bp insertion mutation (g.10096\_10124delinGGAGGTGGAGG) in exon 12 in patient ALS058-01 predicted to result in the in-frame deletion of ten amino acids and the insertion of four novel glycine residues within a glycine-rich region (p.S402\_P411delinsGGGG). Although we were unable to demonstrate segregation of this mutation with disease, we believe this mutation is likely pathogenic since it was not identified in 700 control individuals and it affects evolutionary conserved residues within a functionally critical region of the *FUS* protein (Figure 2D). In fact, this is the sixth *FUS* mutation localized to a glycine-rich region identified in sporadic ALS patients, lending support for the recent hypothesis that mutations within glycine-rich regions of *FUS* may represent low-penetrance mutations (Corrado, et al., 2009).

The clinical presentation of the *FUS* mutation carriers identified in our series was consistent with classical ALS with a bulbar onset in both patients, except that ALS046-01 did not develop clinical upper motor neuron signs. Absence of upper motor neuron signs has been reported in other patients with *FUS* mutations but appears to be uncommon (Chio, et al., 2009; Kwiatkowski, et al., 2009; Ticozzi, et al., 2009; Vance, et al., 2009). The phenotype otherwise was similar to that reported in other series (Belzil, et al., 2009; Chio, et al., 2009; Kwiatkowski, et al., 2009; Ticozzi, et al., 2009; Vance, et al., 2009). Interestingly, the onset ages of our two patients differed significantly with an extreme early onset (20 years) in ALS046-01 carrying the *FUS* truncation mutation, which may be the result of its severe effect on *FUS* localization and function. However, a wide variability in onset ages has previously been observed in *FUS* mutation carriers (Belzil, et al., 2009; Blair, et al., 2009; Chio, et al., 2009; Kwiatkowski, et al., 2009; Ticozzi, et al., 2009; Van Damme, et al., 2009; Vance, et al., 2009), even within patients carrying the same mutation, suggesting that additional genetic and/or environmental factors may be important in defining the age-related disease penetrance of *FUS* mutations.

Both *FUS* mutation carriers identified in this study presented with sporadic ALS. For one patient, ALS046-01, we were able to obtain DNA samples from her unaffected parents which revealed a *de novo* occurrence of the *FUS* mutation in this family. Unfortunately, like all previously reported sporadic *FUS* mutation carriers, relatives of ALS058-01 were unavailable for study such that we could not distinguish between a *de novo* mutation and reduced penetrance in this family. Interestingly, a *de novo SOD1* mutation was previously reported as the cause of young-onset sporadic ALS (Alexander, et al., 2002). Together these studies suggest *de novo* mutations may be more important than previously recognized. Demonstration of a *de novo FUS* mutation in a sporadic ALS patient in this study emphasizes that mutational analysis should be considered in isolated instances of sporadic ALS patients, in addition to those with a positive family history.

A limited characterization of the pathology associated with mutations in *FUS* was previously reported showing a partial retention of *FUS* in the cytoplasm and the formation of *FUS* and ubiquitin-positive neuronal aggregates (Kwiatkowski, et al., 2009; Vance, et al., 2009). Also, in cell culture, mislocalization of mutant *FUS* was demonstrated for at least two C-terminal missense mutations (p.R521C, p.R521G) (Kwiatkowski, et al., 2009; Vance, et al., 2009). These findings, combined with previous data on the identification and characterization of the nuclear localization signal (NLS) in EWSR1, another TET family member highly homologous to *FUS* in the C-terminal region, suggests that the 18 most C-terminally located amino acids of *FUS* comprise a non-conventional NLS required for the nuclear import and retention of *FUS* (Zakaryan and Gehring, 2006). In this scenario, all previously published *FUS* missense mutations clustered at the extreme C-terminus of *FUS* would directly affect the NLS sequence, while the truncation mutation (p.G466VfsX14) identified in this study would completely eliminate the NLS. Immunofluorescence assays in N2A cells transfected with V5-FUS $\Delta$ 14, V5-FUS<sub>R521C</sub>, and V5-FUS<sub>WT</sub> indeed showed a dramatic increase in cytoplasmic *FUS* localization and a reduction of nuclear detection in cells transfected with V5-FUS<sub>R521C</sub> and V5-FUS $\Delta$ 14 compared to V5-FUS<sub>WT</sub>. Immunoblot analysis of nuclear and cytoplasmic enriched protein fractions further confirmed the cytoplasmic accumulation of mutant *FUS*, and suggested a more severe mislocalization in cells transfected with V5-FUS $\Delta$ 14 compared to V5-FUS<sub>R521C</sub>. Autopsy tissue was not available for the *FUS* truncation mutation carrier to confirm these effects *in vivo*. Whether the mislocalization of *FUS* is critical in the disease pathogenesis, either through the loss of one of the normal nuclear functions of *FUS* in mRNA processing and transport or from the toxic effect of the cytoplasmic aggregates, remains unclear.

The identification of a truncating *FUS* mutation and a complex insertion/deletion mutation in a conserved poly-glycine motif in two sporadic ALS patients from our series extends the spectrum of *FUS* mutations and underlines the importance of extensive population-based mutation screening of newly identified genes. Additional *FUS* mutation screenings, including cDNA transcript analysis, should lead to a better understanding of the complete spectrum and frequency of *FUS* mutations and the contribution of *de novo* mutations in *FUS* to sporadic ALS. Further in depth *in vitro* and *in vivo* studies are needed to elucidate the pathological mechanism underlying ALS and related *FUS* proteinopathies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

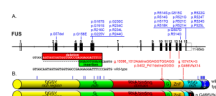
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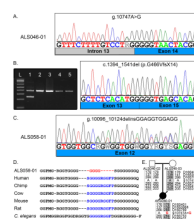
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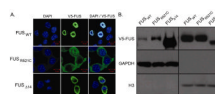
**Figure 1.**

Overview of *FUS* mutations identified in ALS. **(A)** Schematic overview of the 11.65kb genomic region of *FUS* showing all mutations identified to date in blue above the genomic structure. Black boxes represent coding exons, white boxes represent 5' and 3' untranslated regions. Mutations are shown using protein numbering according to GenBank Accession number CAG33028.1. The two novel *FUS* mutations identified in this study are shown in red below the genomic structure with protein numbering and gDNA numbering relative to GenBank Accession number NG\_012889.1 starting at Met1. The mutant allele of the insertion/deletion mutation in exon 12 is shown in detail with the wild-type sequence as a reference. **(B)** Schematic overview of the protein structure of *FUS* with its domain organization. QGSY-rich denotes a region rich in glutamine, glycine, serine, and tyrosine amino acids, G-rich denotes a region rich in glycines, RGG-rich represents a region enriched in arginine-glycine-glycine motifs, ZnF indicates a Cys<sub>2</sub>/Cys<sub>2</sub>-type zinc finger motif and NLS indicates the nuclear localization signal predicted to be located in the most C-terminal 18 amino acids. Horizontal bars above the protein structure indicate the positions of previously described (blue) and novel (red) *FUS* mutations.



**Figure 2.**

Novel mutations in *FUS* identified in this study. **(A)** gDNA sequence chromatogram of patient ALS046-01 with *FUS* g.10747A>G (p.G466VfsX14) showing the overlay of the wild-type and mutant allele. **(B)** Agarose gel electrophoresis of *FUS* cDNA amplified from patient ALS046-01 (lane 1) and 4 unrelated control individuals (lanes 2–5) showing the wild-type allele (393bp) and the mutant allele (245bp) with the cDNA sequence chromatogram of the mutant allele confirming the skipping of *FUS* exon 14. **(C)** gDNA sequence chromatogram of patient ALS058-01 with *FUS* g.10096\_10124delinsGGAGGTGGAGG (p.S402\_P411delinsGGGG) showing the overlay of the wild-type and mutant allele. **(D)** Evolutionary conservation of the p.S402\_P411delinsGGGG mutation in *FUS* generated by MUSCLE (Edgar, 2004). **(E)** Pedigree of family ALS046 showing that the *FUS* g.10747A>G mutation observed in ALS046-01 is not present in gDNA samples collected from her parents. Segregation analysis using 6 short tandem repeat markers flanking *FUS* revealed that the mutation occurred *de novo* in this family. The mutant G-allele of g.10747A>G was arbitrarily assigned to the haplotype inherited from the mother (ALS046-03).



**Figure 3.**

Functional analysis of *FUS* p.G466VfsX14 truncation mutation. **(A)** Immunofluorescent analysis of N2A cells transfected with V5-tagged wild-type *FUS* cDNA ( $FUS_{WT}$ ), V5-tagged p.R521C mutant *FUS* ( $FUS_{R521C}$ ) and V5-tagged p.G466VfsX14 truncated *FUS* predicted to result from the deletion of exon 14 ( $FUS_{\Delta 14}$ ). Left panel shows nuclear diamidino-2-phenylindole (DAPI) staining, middle panel shows green AlexaFluor488 V5-*FUS* immunofluorescence, and overlay images showing colocalization of AlexaFluor488 signal for the V5-*FUS* with the nuclear DAPI fluorescence are shown in the right panel. **(B)**. N2A cells transfected with V5-tagged wild-type *FUS* cDNA ( $FUS_{WT}$ ), V5-tagged p.R521C mutant *FUS* ( $FUS_{R521C}$ ), and V5-tagged p.G466VfsX14 truncated *FUS* predicted to result from the deletion of exon 14 ( $FUS_{\Delta 14}$ ) were separated into cytosolic and nuclear enriched fractions and analyzed by immunoblotting using an antibody to V5, with binding detected with chemiluminescence. A representative immunoblot of V5-*FUS* fusion proteins in cytosolic and nuclear fractions with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone 3 (H3) as loading control and purity markers for the cytosolic and nuclear fractions is shown. Note the smaller molecular weight of V5- $FUS_{\Delta 14}$  compared to V5- $FUS_{WT}$  and V5- $FUS_{R521C}$ , consistent with the predicted C-terminal truncation.

Table 1

Clinical characteristics of *SOD1* and *FUS* mutation carriers

Patient	ALS006-01	ALS021-01	ALS046-01	ALS058-01	ALS088-01
Gene	<i>SOD1</i>	<i>SOD1</i>	<i>FUS</i>	<i>FUS</i>	<i>SOD1</i>
Mutation <sup>1</sup>	p.I113T	p.G72S	p.G466VfsX14	p.S402_P411delinsGGGG	p.A4V
Gender	M	M	F	M	F
Family history of ALS	Yes	Yes	No	No	Yes
Age of onset (years)	45	44	20	65	54
Age at death (years)	N/A	N/A	22	66	55
Disease duration (months)	144	39	22	13	4
Site of onset	Upper limb	Lower limb	Bulbar	Bulbar	Upper & lower limb
Frontotemporal cognitive impairment	No	No	No	No	No
Pseudobulbar affect	No	No	No	No	No
UMN signs <sup>2</sup>	Yes	Yes	No	Yes	No
LMN signs <sup>3</sup>	Yes	Yes	Yes	Yes	Yes
El Escorial Level	Definite	Probable	Suspected	Probable	Suspected

<sup>1</sup> Numbering according to GenBank Accession number CAG33028.1.

<sup>2</sup> UMN: upper motor neuron.

<sup>3</sup> LMN: lower motor neuron.