Analysis of *FUS* gene mutation in familial amyotrophic lateral sclerosis within an Italian cohort

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ABSTRACT

Objective: Mutations in the *FUS* gene on chromosome 16 have been recently discovered as a cause of familial amyotrophic lateral sclerosis (FALS). This study determined the frequency and identities of *FUS* gene mutations in a cohort of Italian patients with FALS.

Methods: We screened all 15 coding exons of *FUS* for mutations in 94 Italian patients with FALS.

Results: We identified 4 distinct missense mutations in 5 patients; 2 were novel. The mutations were not present in 376 healthy Italian controls and thus are likely to be pathogenic.

Conclusions: Our results demonstrate that *FUS* mutations cause ~4% of familial amyotrophic lateral sclerosis cases in the Italian population. ***Neurology*® 2009;73:1180–1185**

GLOSSARY

ALS = amyotrophic lateral sclerosis; **FALS** = familial amyotrophic lateral sclerosis; **FTD** = frontotemporal dementia; **gDNA** = genomic DNA; **LMN** = lower motor neuron; **NLS** = nuclear localization signal; **UMN** = upper motor neuron.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of adult life characterized by a selective loss of upper motor neurons (UMN) and lower motor neurons (LMN). The cause of the disease is largely unknown. Approximately 10% of all ALS cases are familial (FALS), of which ~20% are caused by mutations in the *SOD1* gene.[1] Variants in *TARDBP*, which encodes the DNA/RNA binding protein TDP-43, account for an additional ~5% of cases.[2-4] Pathogenic mutations in 5 other genes (*ALS2, SETX, VAPB, DNCT1,* and *ANG*) have also been associated with atypical ALS phenotypes in isolated families.[5,6] Recently, mutations in the *FUS* gene, which also encodes a DNA/RNA binding protein, have been identified in ~5% of patients with FALS who tested negative for *SOD1* and *TARDBP* mutations.[7,8] The similarities between *TARDBP* and *FUS* suggest that alterations in RNA processing may play an important role in ALS pathogenesis. The *FUS* gene was initially isolated analyzing chromosomal translocations in myxoid liposarcoma[9] and subsequently found in a variety of cancer-associated fusion genes. The protein plays a role in transcription, splicing,[10] and shuttling of RNA from the nucleus to the cytoplasm.[11] FUS also acts as a transcriptional regulatory sensor of DNA damage signals and thus is required in maintaining the integrity of the genome.[12] *FUS* is diffusely expressed in the CNS where it regulates the morphology of hippocampal dendritic spines and stabilizes the structure of the synapse.[13] To further define the frequency and spectrum of *FUS* mutations, we have screened a cohort of Italian patients with FALS.

METHODS

Patients and controls. Peripheral blood samples were collected from 94 unrelated patients with FALS (53 men and 41 women) in the period between 2002 and 2007. All the patients were of Italian descent and were diagnosed with probable or definite ALS according to the El Escorial revised criteria.[14] Familial history was considered positive for ALS if the proband had at least 1 affected relative within 3 generations. The disease onset was bulbar in 24% of patients and spinal in 76%. Six patients (4 men and 2 women) developed frontotemporal dementia (FTD). In agreement with previous epidemiologic surveys on motor neuron disease in Italy,[15] an initial involvement of bulbar motor neurons was slightly more frequent in women (30%) than men (19%). The average age at onset in our cohort was 46.8 ± 15.2 years. Mutations in *SOD1*, *ANG*,[16] and *TARDBP*[17] were excluded in all samples in the present study. Control DNA was obtained from 376 unrelated, age-matched Italian subjects with a negative personal and familial history for neurodegenerative diseases.

Standard protocol approvals, registrations, and patient consents. We received approval for this study from the ethical standards committees on human experimentation of the IRCCS Istituto Auxologico Italiano and of the Fondazione IRCCS Istituto Neurologico "Carlo Besta." Written informed consent was obtained from all patients and healthy subjects participating in the study (consent for research).

Molecular analysis. Genomic DNA (gDNA) was extracted from peripheral blood leukocytes using standard procedures and was subsequently amplified using the Illustra™ GenomiPhi HY DNA Amplification Kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). For all FALS samples, the 15 exons of *FUS* were amplified by touchdown PCR using primers located in adjacent intronic or noncoding regions. PCR primers and conditions have been described elsewhere.[7] PCR products were subsequently purified by incubation with Exonuclease I and Shrimp Alkaline Phosphatase, sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and then resolved by capillary electrophoresis on an ABI 3730XL DNA Analyzer (Applied Biosystems). Sequence analysis was performed using the PHRED/PHRAP/Consed software suite (www.phrap.org/phredphrapconsed.html),[18-21] and variations in the sequences were identified with the Polyphred v6.15 software (http://droog.gs.washington.edu/polyphred).[22] All identified mutations were further confirmed by bidirectional sequencing of the original gDNA sample. The screening of the c.701G>T mutation in control samples was performed using a custom TaqMan SNP Genotyping Assay using the File Builder 3.1 software (Applied Biosystems) which consisted of a mix of unlabeled PCR forward and reverse primers (forward: 5′-GCGGCGGTGGT-GGTT-3′; reverse: 5′-CACGACCTCTGGGTTCATAGC-3′) and 2 dye-labeled probes (allele X: VIC-CACTGCTGCGGT-TGT-NFQ; allele Y: FAM-CACTGCTGAGGTTGT-NFQ). Since the TaqMan assay designed for the c.467G>A mutation failed to generate consistent results, exon 5 of the *FUS* gene was directly sequenced in all control samples.

Bioinformatic analysis. The effect on splicing of the noncoding and synonymous variants identified was assessed using the electronic tools ESEfinder 3.0 (http://rulai.cshl.edu/tools/ESE/),[23,24] NNSplice (http://www.fruitfly.org/seq_tools/splice.html),[25] and SpliceView (http://bioinfo.itb.cnr.it/oriel/spliceview.html).[26] In silico prediction of the putative functional effects of the missense mutations was obtained with the SNAP (http://cubic.bioc.columbia.edu/services/SNAP/)[27] and PMut (http://mmb2.pcb.ub.es:8080/PMut/)[28] software.

RESULTS

To identify novel *FUS* mutations and further define the frequency of all *FUS* gene variants in FALS, we sequenced the coding region (exons 1–15) and the intron/exon boundaries in 94 FALS samples of Italian descent. Our sequence analysis identified 2 previously described missense mutations in the *FUS* gene. The mutation c.1561C>T produces an arginine to cysteine change at amino acid 521 (p.R521C) and was observed in 2 patients. The c.1561C>G mutation was identified in a single patient and alters the same base pair but changes the arginine residue to a glycine (p.R521G). In addition, we have identified 2 novel missense mutations. The mutation c.467G>A was identified in a single patient; it changes a glycine to a glutamic acid at position 156 (p.G156E). The mutation c.701G>T, also identified in a single patient, changes an arginine residue to a leucine at position 234 (p.R234L). The pedigrees of the patients carrying *FUS* mutations are shown in figure 1. Chromatograms displaying the mutations are shown in figure e-1 on the *Neurology*® Web site at www.neurology.org. Unfortunately, since all the affected relatives of the probands are deceased, we could not evaluate the segregation of the mutations in families. In addition to the missense mutations, we also identified a novel synonymous substitution (c.1566G>A [p.R522R]) as well as 5 novel variants in intronic (c.832 + 36A>G, c.833 − 29C>T, c.1066 + 82C>G) and 3′ UTR (c.*10C>T, c.*41G>A) regions of *FUS* (table e-1). In silico analysis with the ESEfinder 3.0 software showed that the synonymous variant does not disrupt any exonic splicing enhancer motif, while NNSplice and SpliceView programs predicted that the intronic and 3′ UTR variants do not affect splicing mechanisms. These variants were not further studied.

To determine whether the 2 novel missense mutations are low allele frequency polymorphisms in the population, we genotyped the p.G156E and p.R234L mutations on a panel of 376 healthy control samples of Italian descent. Neither variant was identified in these normal controls. This finding strongly argues that these missense variants represent pathogenic mutations.

The p.G156E is located within the SYQG domain of the FUS protein, which acts as a transcriptional activation domain. Additionally, the p.R234L mutation is located within the G-rich domain, a region in which 3 mutations have previously been reported (figure 2). To further evaluate the pathogenicity of these novel mutations, we used 2 software

Figure 1   Pedigrees of familial amyotrophic lateral sclerosis cases harboring *FUS* mutations

G156E   R234L   R521G   R521C

Family 303   Family 535   Family 20   Family 51   Family 153
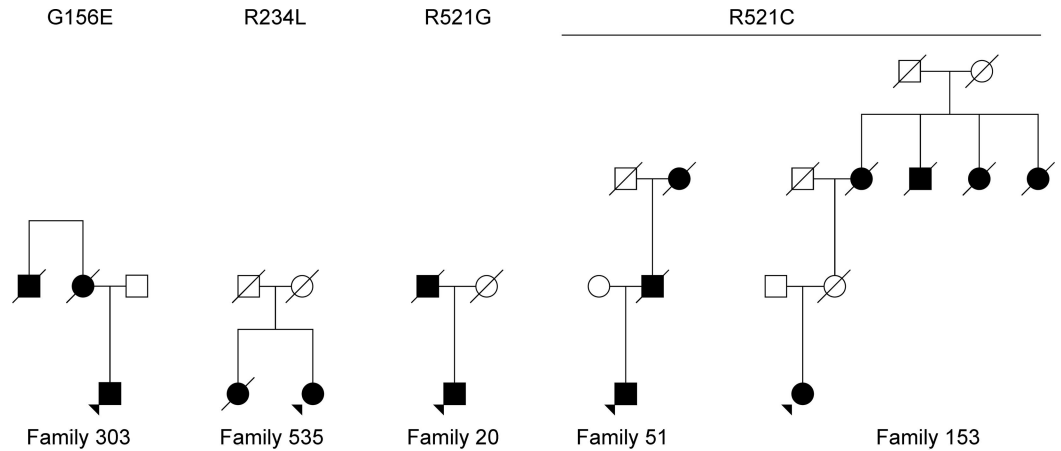
This illustrates the structure of the 5 unrelated Italian pedigrees to which the 5 cases studied here belong. Affected individuals are shown in black; unaffected individuals are shown in white. Arrows indicate the individual harboring the mutation.

applications, PMut and SNAP, which predict whether a missense mutation is pathogenic or benign. As expected, the previously described mutations (p.R521C and p.R521G) were predicted to be deleterious by both PMut and SNAP (table). The novel mutations p.G156E and p.R234L are similarly predicted to be deleterious by both PMut and SNAP (table). In support of this prediction, we note that the residues affected by these mutations (G156 and R234) are highly conserved, being present in mammals, reptiles, and zebra fish (figure 3). This observation also supports the view that the corresponding missense mutations are adverse and likely to be pathogenic.

Phenotypic information for the 5 patients carrying *FUS* mutations is provided in the table. Detailed clinical records were available for 3 (B303, A51, and B153) of the 5 patients and other affected family members, and are reported in appendix e-1. Patient B303 harbors the p.G156E mutation whereas both A51 and B153 have the p.R521C mutation. Notably, each affected individual in the 3 pedigrees developed early symptoms of symmetric weakness of the scapular or pelvic girdles and of the proximal muscles of the upper or lower limbs. Also prominent was the involvement of the axial muscles of the neck and trunk.

In total, we have identified 5 missense mutations within a panel of 94 Italian patients with FALS which were prescreened and found not to have mutations in the *SOD1* and *TARDBP* genes (which represent ~25% of all FALS cases). We therefore estimate

Figure 2   Location of *FUS* mutations in familial amyotrophic lateral sclerosis
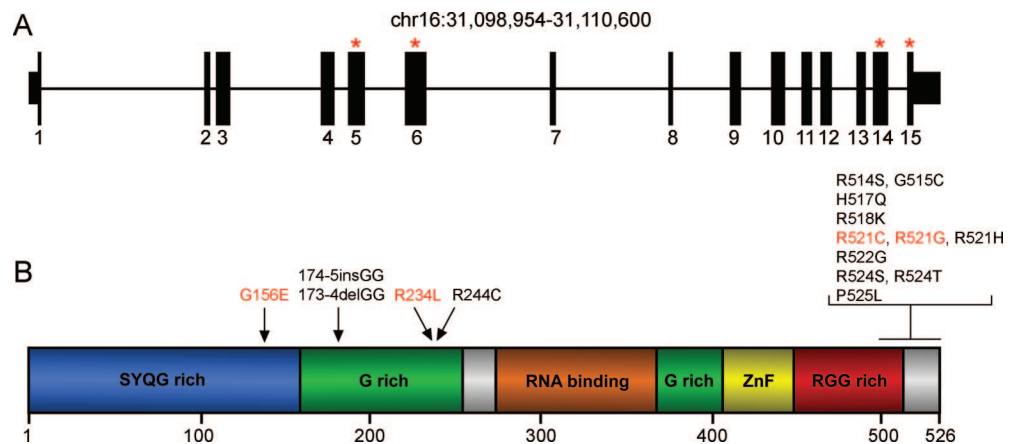
(A) Gene structure of *FUS* indicating coding exons (thick bars) and untranslated regions (medium-height bars). Red asterisks indicate the exons where mutations have been identified. (B) Linear protein structure of FUS indicating the different domains. Amino acid changes are shown above the protein with arrows indicating their position. Mutations found in Italian patients are shown in red.

Patient	Sex	Nucleotide change	Amino acid mutation	Exon	Age at onset, y	Site of onset	Survival, mo	Clinical phenotype	Dementia	PMut prediction	SNAP prediction
B303	M	c.467G>A	G156E	5	33	Bulbar	11	UMN/LMN	Yes	Pathogenic	Non-neutral
B535	F	c.701G>T	R234L	6	66	Spinal	N/A	N/A	No	Pathogenic	Non-neutral
A51	M	c.1561C>T	R521C	15	32	Bulbar	11	LMN	No	Pathogenic	Non-neutral
B153	F	c.1561C>T	R521C	15	25	Spinal	60	LMN	No	Pathogenic	Non-neutral
B20	F	c.1561C>G	R521G	15	n/a	Spinal	N/A	N/A	No	Pathogenic	Non-neutral

UMN/LMN = presence of both upper and lower motor neuron signs; LMN = prevalence of lower motor neuron signs; N/A = not available.

that ~4% of FALS cases within the Italian population are caused by mutation in the *FUS* gene. This frequency is similar to that reported in a recent screening in another FALS cohort.[29]

DISCUSSION *FUS* mutations were initially identified in 20 cases of FALS.[7,8] A vast majority of the alterations (16 out of 20) were missense mutations clustered in 5 arginine residues located in exon 15 of the *FUS* gene. Interestingly, a p.H517Q homozygous mutation within exon 15 was found in a family presenting a recessive inheritance pattern; all other identified families displayed a dominant inheritance pattern. Additional mutations were identified in exon 5 (p.174–175insGG, p.173–174delGG), exon 6 (p.R244C), and exon 14 (p.R514G). The predicted amino acid sequence of the FUS protein is highly evolutionarily conserved and consists of an N-terminal SYQG-rich region that acts as a transcriptional activation domain, two G-rich regions, an RNA binding domain, a Cys_2/Cys_2-zinc finger motif, and a C-terminal RGG-rich region, which contains the nuclear localization signal (NLS).[30,31]

In our cohort of 94 unrelated Italian patients with FALS, we identified *FUS* mutations in 5 patients.

The observed mutational frequency in our Italian cohort is similar to that observed in the 2 previous studies of *FUS* mutations in ALS.[7,8] Previous mutational screenings have suggested that the prevalence of *SOD1* mutations in Italian ALS families is about 15%.[32,33] *ANG* mutations account for ~2% of all familial cases,[16] while a recent study identified *TARDBP* mutations in 6 out of 125 Italian patients with FALS (4.8%).[17] To date, in the Italian population, mutations in the *ALS2* gene have been reported only in an handful of families affected by infantile ascending hereditary spastic paralysis[34] or juvenile primary lateral sclerosis,[35] while a screening for *VAPB* variants in patients with ALS was negative.[36] To our knowledge, there are no reports of *SETX* or *dynactin* mutations in Italian patients with ALS. Our findings thus suggest that *FUS* mutations represent the third most significant cause of FALS in Italy after *SOD1* and *TARDBP*.

In our cohort, we found 2 previously identified mutations, p.R521G and p.R521C. The substitution of the arginine at codon 521 disrupts the NLS and leads to an aberrant subcellular distribution of FUS, with retention and apparent aggregation of the mutant protein in the cytoplasm.[7] The 2 novel mutations observed in our cohort are located in the N-terminal SYQG-rich domain (p.G156E) and in the first G-rich region (p.R234L), where other mutations have been found[7] (figure 2B). No data are available on the functional role of these variants, since they are not located in the NLS and thus presumably do not alter the subcellular localization of FUS. Moreover, very little information is available on the activity of both the SYQG-rich and G-rich domain of FUS. We hypothesize, however, that p.G156E and p.R234L are pathogenic for several reasons. The amino acids at these positions are highly evolutionarily conserved (figure 3) and these variants were not detected in 376 Italian healthy controls (752 chromosomes). Finally, both in silico SNAP and PMut software analyses predicted that p.G156E and p.R234L affect protein function (table), although these data should be interpreted with caution.

Figure 3 Evolutionary conservation of *FUS* mutations

The evolutionary conservation of all 4 identified mutations is shown. For each, the mutated amino acid is shown in red.

We note that the clinical presentation of the Italian patients with *FUS* gene mutations (symmetric, proximal, and axial weakness at onset) was distinct from typical ALS, which often begins in a single limb distally.[37] Severe weakness of neck extensor muscles at onset, as seen in one Italian case, is present in only about 1% of patients with ALS,[38] and thus is also unusual. We also note that in his fourth decade, patient B303 (p.G156E mutation) developed FTD concurrently with motor neuron disease; patients with ALS in the 2 previous reports on *FUS* mutations were not described as having concomitant dementia. It will be of interest to determine whether *FUS* mutations consistently correlate with these distinctive clinical phenotypes in other patients with ALS.

Given the complexity of cellular functions and pathways in which FUS is involved, further studies will be required to delineate the explicit mechanisms whereby these *FUS* mutations cause motor neuron death. In particular, it will be of interest to assess if the pathogenic pathways involving *FUS* and *TARDBP* overlap. The 2 genes encode for DNA/RNA binding proteins involved in gene regulation and RNA processing. Mutations in *FUS* and *TARDBP* may alter the binding of their nucleic acid targets, resulting in altered mRNA splicing. Additionally, some mutations have been shown to impair the capability of both proteins to shuttle between the nucleus and the cytoplasm.[39] Widespread defects in RNA processing as a result of either, or both, of these mechanisms could lead to motor neuron death. The precise pathogenic mechanism notwithstanding, our study provides strong evidence that mutations in the *FUS* gene represent a significant cause of familial ALS in the Italian population. It also suggests that some patients carrying *FUS* mutations may have uncommon clinical phenotypes, a possibility that merits further analysis as more opportunities arise to correlate *FUS* genotypes with clinical phenotypes.

AUTHOR CONTRIBUTIONS

Writing team: N.T., V.S., A.L., P.K, R.H.B., J.L.; all others received and approved the manuscript. Patient selection, preliminary genetic screening, and record collection: N.T., V.S., A.R., C.G., F.T., P.S. Sequencing and genotyping: N.T., A.L., P.K. Data analysis: N.T., V.S., R.H.B., J.E.L. Scientific planning and direction: N.T., V.S., A.L., T.K., R.H.B., J.E.L.

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DISCLOSURE

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