Mutations in the 3′ untranslated region of FUS causing FUS overexpression are associated with amyotrophic lateral sclerosis

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Mutations in the gene encoding fused-in-sarcoma (FUS) have been identified in a subset of patients with sporadic and familial amyotrophic lateral sclerosis (ALS). Variants in the 3′ untranslated region (3′ UTR) of FUS have also been reported in ALS patients, but their pathogenic role has not been assessed. We sequenced the whole 3′ UTR of FUS in 420 ALS patients who were negative for mutations in the currently known ALS genes and in 480 ethnically matched controls. We detected four 3′ UTR variants (c.∗48 G>A, c.∗59 G>A, c.∗108 C>T and c.∗110 G>A) in four sporadic and in one familial ALS patients compared with none in controls (P=0.02). We investigated whether these variants impaired FUS expression in primary fibroblast cultures from three patients harbouring the c.∗59 G>A, c.∗108 C>T and c.∗110 G>A variants, respectively. The pattern of FUS expression was also investigated in fibroblasts from one ALS patient with FUS R521C mutation, in two ALS patients without mutations in the known ALS genes and in four control individuals. By immunostaining and immunoblotting, large amounts of FUS were observed in both the cytoplasm and nuclei of mutant 3′ UTR FUS fibroblasts. In FUS R521C mutant fibroblasts, we observed a slight increase of FUS in the cytoplasm associated with a remarkable loss of detection in nuclei. Our findings show that mutations in 3′ UTR of FUS are overrepresented in ALS patients and result into translation de-regulation of FUS. Overexpression and mislocalization of wild-type FUS likely contribute to ALS pathogenesis in these cases.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disorder involving upper and lower motor neurons. A breakthrough in understanding ALS pathogenesis was the discovery of TAR DNA binding 43 (TDP-43) and fused-in-sarcoma (FUS) as major components of the protein aggregates observed in both sporadic (SALS) and familial (FALS) cases (1–3). Notably, mutations in the genes encoding TDP-43 and FUS have been identified in a subset of ALS patients (4–7). Mutations in the 3′ UTR of FUS have also been reported in ALS patients but their pathogenic role is unknown (12–15).

In the present study, we sequenced the 3′ UTR region of the FUS gene in 420 ALS patients who were negative for mutations in major ALS genes and investigated functional consequences of the identified mutations by analysing FUS expression in primary fibroblast cultures.

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RESULTS

We analysed 420 patients with ALS admitted to the Neurological Institute of the Catholic University of Rome, which is the referral ALS Center of Lazio region. Forty patients were classified as FALS while in the remaining 380 the disease was sporadic. All the patients were negative for mutations in SOD1, C9ORF72, TARDBP, OPTN, ANG, ATXN2, PFN1, in all coding regions and in the 5′UTR region of FUS.

The mean age of onset was 61 years (range 22–86). A total of 237 patients were males and 183 females. The site of onset was bulbar in 26%, spinal in 73% and respiratory in 1% of patients.

Genetic study

Sequencing the whole 3′UTR region of FUS in our 420 patients revealed four rare heterozygous variants (c.*48 G>A, c.*59 G>A, c.*108 C>T and c.*110 G>A) in five individuals. DNA from relatives was not available. We also detected two known polymorphisms, namely the c.*41G>A variant (rs8031724) in 21 patients and the c.*214 C>T variant (rs140875749) in 4 patients (Table 1).

Sequencing the whole 3′UTR region in 480 ethnically matched controls revealed only the c.*41G>A common variant in 17 individuals and the c.*214 C>T common variant in 10 individuals (Table 1). If these two known polymorphic variants were excluded, the proportion of 3′UTR variants was 5/420 (1.2%) in patients compared with 0/480 in controls (P = 0.02).

Clinical findings

Four patients had a sporadic disease while one of the two patients harbouring the c.*48 G>A variant reported that her maternal aunt had died from ALS. Clinical characteristics of the five patients with 3′UTR variants of FUS are summarized in Table 2.

Cell cultures

To investigate functional consequences of the detected mutations in the 3′UTR region of FUS, we analysed FUS expression in primary cultures of skin fibroblasts. Skin biopsies could be obtained from three patients harbouring the c.*59 G>A, c.*108 C>T and c.*110 G>A variants, respectively. Results were compared with those of four controls and of two SALS patients without mutations in the currently known ALS genes.

One patient with definite FALS harbouring the FUS R521C variant was also analysed. Mutant FUS 3′UTR and R521C fibroblasts did not show different growth characteristics with respect to controls. By direct sequencing, the identified 3′UTR mutations of FUS were also detected at a heterozygous status in genomic DNA and cDNA obtained from fibroblasts in all patients.

Immunostaining

In control fibroblasts, immunostaining with anti-FUS antibody revealed intense and compact staining within nuclei, while the cytoplasm was negative or showed a smooth FUS staining in few cells (Fig. 1A–D). The mean percentage of FUS-immunoreactive nuclei was 94% (range 91–95). None of the cells with negative nuclear staining showed significant cytoplasmic FUS reactivity.

In all patients with mutations in the 3′UTR of FUS, a large amount of FUS was observed in the cytoplasm of near all fibroblasts. Mutant FUS c.*59 G>A fibroblasts showed large collections of FUS-immunoreactive material in the cytoplasm, frequently disclosing a compact pattern of staining (Fig. 1E–H). In patients with c.*108 C>T (Fig. 1I–N) and c.*110 G>A (Fig. 1O–R) variants the increased FUS staining appeared as diffusely distributed fine granules in the cytoplasm. Fibroblasts retained normal nuclear FUS staining, as the percentages of cells with positive nuclear immunostaining were 96, 95 and 90% in patients with the c.*59 G>A, c.*108 C>T and c.*110 G>A variant, respectively. Cells with negative nuclear staining always showed intense cytoplasmic FUS deposits.

The patient with the R521C mutation in FUS showed a slight increase of cytoplasmic FUS signal associated with a significant loss of detection in nuclei (Fig. 1S–V). The percentage of FUS-immunoreactive nuclei was 56%, significantly lower than that found in controls (P < 0.001) and in mutant 3′UTR FUS cells (P < 0.001).

FUS staining in the two ALS patients without mutations in ALS genes showed a near exclusive nuclear staining, as in controls (Supplementary Material, Fig. S1).

Immunoblotting

To further analyse the level of expression and nuclear–cytoplasmic localization of FUS, we performed a series of immunoblotting experiments. Total cell extracts (TCEs) of mutant 3′UTR and R521C fibroblasts showed significantly higher levels of FUS when compared with controls (Fig. 2A). FUS was increased by 2.2-4.4-fold in patients with 3′UTR variants, and only 1.4-fold in the patient with the R521C variant (Fig. 2B).

We investigated the subcellular localization of FUS by compartmental fractionation of fibroblasts and immunoblotting of these fractions. In patients with 3′UTR and R521C mutations, a large amount of the protein was observed in the cytosol (Fig. 3A). On the contrary, FUS was detected in nuclei but was absent in the cytoplasm of cells from controls and from patients without mutations in ALS genes (Supplementary Material, Fig. S1). Densitometric analysis showed that the level of

| Table 1. Variants in the 3′UTR region of FUS |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Variants** | **Rare variants** | **Patients (n = 420)** | **Controls (n = 480)** | **P-value** |
| Number | % | Number | % |
| c.*48 G>A | 2 | 0.4 | 0 | — |
| c.*59 G>A | 1 | 0.2 | 0 | — |
| c.*108 C>T | 1 | 0.2 | 0 | — |
| c.*110 G>A | 1 | 0.2 | 0 | — |
| Total | 5 | 1.2 | 0 | 0.02 |
| **Known polymorphisms** | | | | |
| c.*41G>A | 20 | 4.7 | 17 | 3.5 | 1 |
| c.*214 C>T | 6 | 1.4 | 10 | 2.1 | 1 |

*Reported in NHLBI 6500 Exome data sets.

*Not reported in NHLBI 6500 Exome data sets, 1000 genomes and dbSNP.
cytoplasmic FUS was much higher in patients with 3′UTR variants than in the patient with the R521C variant (Fig. 3B). The nuclear amount of FUS was increased in patients with 3′UTR variants with respect to controls (Fig. 3C).

Real-time PCR analysis
To establish whether the identified 3′UTR FUS variants altered the mRNA level, we determined the FUS transcript level by quantitative RT–PCR. The FUS transcript level was significantly increased by 2- and 2.4-fold in repeated samples from patients with the c.*108 C>T and the c.*59 G>A 3′ variants, respectively (Fig. 4). The mRNA level was slightly increased also in the patient with c.*110 G>A variant, while no significant difference was observed in the patient with R521C mutation.

**DISCUSSION**

In the present study, we found variants in the 3′UTR region of FUS in 5 of 420 (1.2%) ALS patients and several pieces of genetic and biochemical data support that they may contribute to the disease pathogenesis. First, the cumulative frequency of rare variants in 3′UTR was significantly higher in our cohort of 420 ALS patients than in 480 ethnically matched controls (5 versus 0; P = 0.02). The known polymorphic 3′UTR variants c.*41G>A (rs 80301724) and c.*214 C>T (rs140875749), which were found in the same proportion of patients and controls, were not considered in the present analysis. Of note, frequency of 3′UTR variants of FUS we report on in ALS patients is comparable with that previously reported in the coding regions of the gene (6,7,9,10). Secondly, three of the 3′UTR variants found in patients (c.*59 G>A, c.*108 C>T and c.*110 G>A) are not present in 1000 genomes (http://www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), nor NHLBI 6500 Exome data sets (http://evs.gs.washington.edu/EVS/) databases. The c.*48 G>A variant, which was detected in two of our patients, has been reported in only 1/8599 controls in NHLBI 6500 Exome data sets. Thirdly, nucleotides involved by mutations in the 3′UTR are semiconserved in different species (Supplementary Material, Fig. S2).

Finally, we observed marked FUS overexpression in both the cytoplasm and nuclei of primary fibroblast cultures from patients harbouring 3′UTR FUS variants.

The huge accumulation of FUS we found in both nuclei and cytoplasm of mutant 3′UTR FUS fibroblasts suggests a mechanism of translation de-regulation. This hypothesis is consistent with the notion that the 3′UTR region plays an essential role in post-transcriptional control of gene expression by regulating mRNA translation, subcellular targeting and stability (16,17). Control of translational machinery by 3′UTRs is mediated by trans-acting factors, including RNA-binding proteins and micro-RNAs, which interact with cis-regulatory elements within the 3′UTR. Generally, cis elements in the 3′UTR inhibit gene expression by repressing translation or facilitating mRNA degradation. Consistent with these notions, mutations in the 3′UTR region of FUS may alter mRNA stability or the binding of trans-acting factors, resulting into protein overexpression in the wild-type (WT) configuration. The increase of the FUS transcript level observed in repeated samples from our patients, suggests that these variants likely affect mRNA stability, resulting in up-regulation of translation (Fig. 4). Furthermore, it is known that in the case of TDP-43 modulation of protein levels occurs through a negative feedback loop triggered by binding of TDP-43 to its own RNA in the 3′UTR region (18). Whether or not 3′UTR of FUS contributes to autoregulatory mechanisms of protein levels remains to be investigated.

Interestingly, the pattern of FUS overexpression we observed in mutant 3′UTR FUS fibroblasts was different from that of mutant FUS R521C fibroblasts. By immunostaining and immunoblotting, levels of cytoplasmic FUS were significantly higher in cells from patients with 3′UTR variants than in those from the patient with the R521C mutation. Furthermore, the percentage of FUS positive nuclei was strikingly higher in mutant 3′UTR than in mutant R521C fibroblasts (P < 0.0001).

The R521C mutation is located in the C-terminus of the protein containing a non-classical nuclear localization sequence. Most FUS mutations found in ALS, are clustered in this region (6,7). Impaired transportin-mediated nuclear import of FUS, resulting from disruption of this motif, has been shown as a likely mechanism for cytoplasmic mislocalization of FUS in a number of studies (19,20). The finding of an increased cytoplasmic FUS level associated with loss of nuclear staining in our mutant R521C fibroblasts is in keeping with this hypothesis.

Mechanisms by which mutations in the coding regions of FUS cause motor neuron degeneration remain to be elucidated. It is still unclear whether the disease is caused by loss of the
normal protein function in the nucleus, gain of toxic function in the cytosol, or both (8,21,22).

Our data led us to hypothesize that FUS deposits in the WT configuration may have toxic properties in mutated 3′UTR FUS patients. Studies in different animal models show that increased expression of WT FUS induces neurodegeneration though it has been suggested that mutant FUS is more toxic to neurons than WT FUS (21). Of importance, it was recently reported that overexpression of WT human FUS in transgenic mice causes ALS with an aggressive phenotype (23). In this model, toxicity of FUS has been shown to correlate with cytoplasmic accumulation in a dose-dependent fashion. Though the number of our cases is too small to draw any definite conclusion, it is worth noting that in our study clinical phenotypes correlated with FUS levels. In fact patients with a higher FUS cytoplasmic level, namely those harbouring the c.*59 G > A and c.*108 C > T variants, had a more severe clinical course with respect to the patient with the c.*110 G > A variant, in whom a lower amount of FUS was observed.

Although in our cases FUS pathology was studied in skin fibroblasts, the same mechanism can be considered to occur in neurons.
as well. In fact FUS is ubiquitously expressed throughout human tissues, including skin fibroblasts, in which the protein has a nearly exclusive nuclear localization as in neuronal cells (24). Sequencing of 3′ regulatory regions of FUS should be performed in patients with FUS pathology, including ALS and frontotemporal dementia, when exonic mutations are not identified. Further studies are needed to clarify the role of WT FUS overexpression in the pathogenesis of ALS.

PATIENTS AND METHODS

Patients recruitment

This study was approved by the local Ethic Committee. A written informed consent was provided by all subjects. Both patients and controls were from the Centre or the South of Italy. Diagnosis of ALS was made according to revised El Escorial/Airlie House Criteria (25). Patients with one or more affected relatives were diagnosed as FALS. Based on clinical patterns in the fully developed state of the disease, each patient was classified as having one of the following phenotypes: classic, upper motor neuron dominant, flail arm or pure lower motor neuron, as we have previously described (26). Survival was defined as the period from disease onset to last follow-up, or death or tracheostomy. Sequence analysis of SOD1, C9ORF72, TARDBP, OPTN, ANG, ATXN2, PFN1, all coding regions and the 5′UTR region of FUS was performed in a cohort of 533 ALS patients, of which 480 were sporadic and 53 were familial. We detected mutation in 53 of sporadic cases (11%) and in 22 (41%) of FALS (10,27). Of the remaining 458 patients in which no mutations were found, 420 were screened for mutations in the 3′UTR region of FUS.

Sequencing analysis

Genomic DNA was extracted from leucocytes using the Wizard Genomic DNA Purification Kit (Promega). Coding exons and flanking intronic regions of SOD1 (MIM:147450), ANG (MIM: 105850), TARDBP (MIM: 605078), FUS (MIM: 137070) and OPTN (MIM:602432) were amplified and screened by direct sequencing, on an ABI3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) according to standard protocols. Sequence analysis was performed using the DNA Sequencing Analysis Software v.5.1 and the SeqScape Software v.2.5 (Applied Biosystems). ATXN2 (MIM: 601517) was studied to determine the polyQ-repeat size in exon 1, using a fluorescent PCR. Fragment length analysis was performed on an ABI3130 Genetic Analyzer (Applied Biosystems) and data were analysed using the GeneMapper 4.0 software (Applied Biosystems). A subject with a 22/22 homozygous genotype was used as a control. A repeat-primed PCR was used to identify patient carriers of the hexanucleotide GGGGCC expansion in the first intron of the C9ORF72 gene (MIM: 614260). The whole 3′UTR of FUS was amplified and screened by direct sequencing on an ABI3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) according to standard protocols. Primers are listed on Supplementary Material, Table S1). Sequence analysis was performed using the DNA Sequencing Analysis Software v.5.1 (Applied Biosystems).

Western blot analyses

TCEs were prepared in RIPA buffer [50 mM Tris–HCl (pH 8), 600 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40 and 1 mM EDTA], supplemented with protease-inhibitor mix (Roche). TCEs were resolved on precast NuPAGE 4–12% gels (Invitrogen), transferred into nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and analysed by western blotting with the following antibodies: rabbit anti-FUS (Sigma AV40278-50UG), anti-tubulin monoclonal Ab (MAB 10285, Immunologic Sciences), rabbit anti-Sp1 polyclonal Ab (sc-59, Santa Cruz Biotechnology). Immunoreactivity was determined using the ECL chemiluminescence (Amersham Corp) following manufacturer’s instructions.

Nuclear–cytoplasmic fractionation

Nuclear–cytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit.
Cytoplasmic and nuclear extract was resolved on precast NuPAGE 4–20% and analysed by western blotting with the following antibodies: rabbit anti-FUS (Sigma), anti-α-tubulin monoclonal Ab (moAb) (Immunological Sciences), rabbit anti-Sp1 polyclonal Ab (Santa Cruz Biotechnologies). Immunoreactivity was determined using the ECL chemiluminescence (Amersham Corp) following manufacturer’s instructions. Anti-α-tubulin or anti-Sp1 antibodies were used as a protein loading control. Densitometry of western blots was performed using the Image J software.

**Immunofluorescence and microscopy**

For immunofluorescence experiments, cells were seeded in 35 mm dishes (81156, ibiTreat, Ibidi) and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were washed three times in phosphate buffered saline (PBS), permeabilized in 0.3% Triton X-100 in PBS, and then blocked in 5% bovine serum albumin in PBS for 1 h at room temperature. Cells were incubated with primary antibody anti-FUS (SIGMA, 1:500) over night at 4°C, then washed and incubated with Alexa Fluor 555-conjugated goat anti-rabbit (Invitrogen 1:1000) and Phalloidin (Sigma 1:400). DNA was counterstained with Hoechst 33258 (Molecular Probes, Inc., Eugene, OR, dilution 1:1000). Samples were analysed using a TCS SP5 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).
RNA extraction and real-time RT–PCR
RNA was extracted by fibroblast cultures of patients and control subjects and retro-transcribed with ‘High Capacity cDNA Reverse Transcription Kit’ (Applied Biosystems). Semi-quantitative real-time PCR was performed on cDNA by using the FUS-specific TaqMan® Gene Expression Assay (Hs01100224_m1, Applied Biosystems) and run on an ABI PRISM 7900 Genetic Analyzer (Applied Biosystems). Experimental data were obtained with the SDS v2.2.2 Software (Applied Biosystems) and to analyse the expression of FUS in affected individuals and in control subjects the comparative threshold cycle (Ct) method (with the calculation of the 2−ΔΔCt method) was used, comparing FUS versus glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1, Applied Biosystems). Real-time PCR reactions of each sample were repeated for three times.

Fibroblast cultures
Patients underwent a 4-mm punch skin biopsy at the distal leg. Skin samples were cut into pieces of ~1 mm in diameter on Petri dishes containing 1 ml BIO-ANF-2 (Biological industries) culture medium. Explants were transferred to the cell culture flask with a needle, following which 3 ml of BIO-ANF-2 culture medium. The flask is sealed and placed in a 37°C oven to grow the fibroblasts. Culture medium was changed every 3 days. When cells from the explants achieved adequate growth, fibroblasts were detached from the bottom of the flask with a trypsin-EDTA solution (LONZA) and transferred to two new sterile flasks to which 3 ml of BIO-ANF-2 culture medium. This procedure continued until enough confluent flasks are obtained for collecting or freezing in cell bank.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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