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SOD1, ANG, VAPB, TARDBP, and FUS mutations in familial Amyotrophic Lateral Sclerosis: genotype-phenotype correlations

Stéphanie Millecamps¹, François Salachas², Cécile Cazeneuve³, Paul Gordon², Bernard Bricka³, Agnès Camuzat¹, Léna Guillot-Noël¹, Odile Russaouen³, Gaëlle Bruneteau², Pierre-François Pradat², Nadine Le Forestier², Nadia Vandenberghe⁴, Véronique Danel-Brunaud⁵, Nathalie Guy⁶, Christel Thauvin-Robinet⁷, Lucette Lacomblez^{2,8}, Philippe Couratier⁹, Didier Hannequin¹⁰, Danielle Seilhean^{1,11}, Isabelle Le Ber¹, Philippe Corcia¹², William Camu¹³, Alexis Brice^{1,3}, Guy Rouleau¹⁴, Eric LeGuern^{1,3} and Vincent Meininger²

¹Centre de Recherche de l'Institut du Cerveau et de la Moelle, INSERM UMRS975, CNRS UMR7225, Université Pierre et Marie Curie-Paris 06, Hôpital Pitié-Salpêtrière, Paris, France.

²Assistance Publique Hôpitaux de Paris (AP-HP), Fédération des Maladies du Système Nerveux, Centre de référence maladies rares SLA, Hôpital Pitié-Salpêtrière, Paris, France.

³AP-HP, Département de Génétique et Cytogénétique, Unité Fonctionnelle de neurogénétique moléculaire et cellulaire, Hôpital Pitié-Salpêtrière, Paris, France.

⁴Hospices Civils de Lyon, Centre SLA de Lyon, Hôpital Neurologique Pierre Wertheimer, Lyon, France

⁵Centre SLA-MMN, Service de Neurologie et Pathologie du Mouvement, Hôpital Roger Salengro, CHRU Lille, France.

⁶Service de neurologie du CHU Montpied, INSERM U929, Université Clermont 1, Clermont-Ferrand, France.

⁷Centre de Génétique, Hôpital d'Enfants, Dijon, France

⁸INSERM UMRS 678, Université Pierre et Marie Curie-Paris 06, Paris, France.

⁹Université de Limoges, IFR 145 GEIST, Institut d'Epidémiologie Neurologique et de Neurologie Tropicale, Limoges, France.

¹⁰Service de Neurologie et CMRR, INSERM U614, Centre Hospitalier Universitaire, Rouen, France

¹¹Département de Neuropathologie, AP-HP, Université Pierre et Marie Curie-Paris 06, Hôpital Pitié-Salpêtrière, Paris, France.

¹²Centre SLA, CHU de Tours, Université François Rabelais, Tours, France

¹³Centre SLA, CHU de Montpellier, Université Montpellier 1, France

¹⁴Center of Excellence in Neuromics, Centre Hospitalier de l'Université de Montreal, and Department of Medicine, University of Montreal, Montreal, Quebec, Canada.

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Correspondence to:

Dr Stéphanie Millecamps: stephanie.millecamps@upmc.fr

Hôpital Pitié-Salpêtrière, CRICM UMR975, Bât Pharmacie - 4^{ème} étage, 47 bd de l'Hôpital, 75651 Paris cedex 13

Tel: 33(1)42-17-75-59

Fax: 33(1)42-17-75-33

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Abstract

Background. Mutations in *SOD1*, *ANG*, *VAPB*, *TARDBP* and *FUS* genes have been identified in amyotrophic lateral sclerosis (ALS).

Methods. We estimated the relative contributions of the different mutations to ALS by systematically screening a cohort of 162 families enrolled in France and 500 controls (1000 chromosomes) using molecular analysis techniques and performing phenotype-genotype correlations.

Results. We found 31 pathogenic missense mutations in 36 patients (20 *SOD1*, 1 *ANG*, 1 *VAPB*, 7 *TARDBP* and 7 *FUS*). Surprisingly two *FUS* mutation carriers also harbored *ANG* variants. We identified one family of Japanese origin with the P56S *VAPB* mutation. Seven novel mutations (3 in *SOD1*, 2 in *TARDBP*, 2 in *FUS*) were found. None of them was detected in controls. Segregation of detected mutations with the disease was confirmed in 11 families including 5 pedigrees carrying the novel mutations. Clinical comparison of *SOD1*, *TARDBP*, *FUS* and other FALS patients (with no mutation in the screened genes) revealed differences in site of onset (predominantly lower limbs for *SOD1* and upper limbs for *TARDBP* mutations), age of onset (younger with *FUS* mutations) and in life span (shorter for *FUS* carriers). One third of *SOD1* patients survived more than 7 years: these patients had earlier disease-onset than those presenting with more typical course. Differences were also observed among *FUS* mutations, with the R521H *FUS* mutation being associated with longer disease duration.

Conclusions. This study identifies new genetic associations with ALS and provides phenotype-genotype correlations with both previously reported and novel mutations.

Key words: ALS, family study, genetics, phenotype, mutation

Introduction.

Recent genetic advances have brought to five the number of genes involved in typical amyotrophic lateral sclerosis (ALS). The first identified was the *SOD1* gene, which encodes copper/zinc superoxide dismutase.[1] More than 130 mutations, which account for 12-23% of familial ALS (FALS), have now been reported (<http://alsod.iop.kcl.ac.uk/Als/Index.aspx>). A single mutation, c.166C>T/Pro56Ser (P56S), in the gene encoding VAMP (vesicle associated membrane protein) associated protein B (*VAPB*), was subsequently identified in Brazilian kindreds who shared a common founder from the time of the Portuguese colonization of Brazil.[2, 3] The recent discovery of mutations in the *ANG* (angiogenin) [4], *TARDBP* (TAR DNA-binding protein 43) [5, 6, 7, 8, 9, 10, 11] and *FUS* (fused in sarcoma) [12, 13] genes, all encoding proteins probably involved in RNA metabolism, have opened new areas of study into possible molecular mechanisms underlying ALS.

In the present study, we establish the frequencies of mutations in these five genes by systematically screening a cohort of 162 French patients with FALS, and compare the phenotypes related to the different mutations.

Methods.

Patients. The population comprised 162 index cases of unrelated families with probable or definite ALS[14], mainly of Caucasian origin (except three patients of Asian and one Sephardic Jewish heritage). DNA samples were collected over the past 15 years at the ALS National Reference Center of Pitié-Salpêtrière Hospital (Paris). During the same period, the number of ALS patients followed up by the center was 6602. Relatives of some families for which we identified a mutation were collected by other French ALS Centers belonging to the French ALS study group. One hundred patients were male and 62 were female (M:F ratio=1.61:1). Control samples were obtained from 500 healthy Caucasian individuals matched to the sex and age of the patients. Protocols were approved by the “Comité d’Ethique de la Pitié-Salpêtrière” and by the Medical Research Ethics Committee of “Assistance Publique-Hôpitaux de Paris”. All participants signed a consent form for the research.

Molecular analysis. Coding regions and exon-intron boundaries of *SOD1* (5 exons), *ANG* (exon 1 and 2), *VAPB* (5 exons), *TARDBP* (5 exons), and *FUS* (15 exons) genes were amplified from genomic DNA in 5, 3, 5, 6 and 13 fragments, respectively. Sequence of the primers and amplification conditions are available upon request.

Clinical features. The following clinical data were collected: age at onset (first symptom of weakness), site of onset (upper limb, lower limb, bulbar), presence of cognitive impairment[15] and survival. For survival we used two variables bringing complementary information for mutation carriers: the disease duration (interval from onset to death or censoring) and life span (age at death or censoring), defined by the delay between birth and death. This parameter appeared informative in FALS patients as it could provide an insight on the duration of the whole disease process. Features recorded at the first clinical evaluation (initial signs) and at the last evaluation (final signs) were: predominance of lower (LMN) or upper motor neuron (UMN) involvement (according to El Escorial criteria)[14] and bulbar function (using the modified Norris bulbar scale).[16] To assess the degree of deterioration in bulbar function during the course of the disease, we calculated a bulbar score (difference between initial and final Norris bulbar scale scores).

Statistical analysis. Mean and median age of onset, disease duration and life span were determined using maximum likelihood estimates including censored data. Data were censored at the last date of analysis (01/02/2010) if the patient was alive. The Cox proportional hazards regression model examined the relationship between potential predictors and the outcome of interest (either age at onset, disease duration or life span). Mutation status (*SOD1*, *TARDBP*, *FUS*, other FALS) was treated as a categorized variable. If the difference was significant ($P < 0.05$), a Log Rank analysis compared groups by pairs. A similar analysis compared disease duration in *FUS* patients carrying either the R521H or other mutations. Proportions of patients classified according to gender, onset site, disease duration and presence/absence of cognitive impairment were compared by pairs between *SOD1*, *TARDBP*, *FUS* and other FALS groups using Fisher's exact tests. A Mann Whitney test compared mean age at onset between two subgroups of *SOD1* patients (with disease duration >7 or ≤ 7 years) and mean disease duration in patients carrying the R521H or other *FUS* mutation. One way analysis of variance (ANOVA) tests compared mean bulbar scores in *SOD1*, *TARDBP* and *FUS* patients. If the difference was significant ($P < 0.05$), pairwise comparisons were made using a Tukey-Kramer post test. Statistical analyses were performed using the SPSS 11.0 data analysis software (SPSS Inc).

Results.

Genetic data.

We sequenced the *SOD1*, *ANG*, *VAPB*, *TARDBP* and *FUS* genes simultaneously in all patients.

We found 18 different *SOD1* missense mutations in 20 out of the 162 FALS patients, corresponding to a mutation frequency of 12.3% (sppl. table 1). Table 1 summarizes the mutations we found and indicates the correspondence between the numbering of *SOD1* mutations according to Human Genome Variation Society guidelines (<http://www.hgvs.org/>) and the ALS Online Database (<http://alsod.iop.kcl.ac.uk/Als/index.aspx>). Three patients harbored novel *SOD1* mutations, that were not detected in controls and that affected a highly conserved residue until *xenopus*: c.139C>G/p.His47Asp (H46D), c.200C>G/p.Pro67Arg (P66R) and c.251A>G/p.Asp84Gly (D83G). The affected relatives were deceased so the segregation of H46D and P66R mutations could not be established. The D83G mutation was present in an affected brother of the index case, but not in the healthy sister (sppl. fig 1A).

Next, we identified the same c.122A>T/p.Lys41Ile (K17I) *ANG* mutation in two families with dominant inheritance. The K17I mutation has been reported in 3 apparently healthy people,[4, 17] but was not detected in our 500 controls. We also found the c.232A>G/p.Lys78Glu (K54E) and the c.434G>A/p.Arg145His (R121H) variants in two FALS, which were previously reported as mutations in sporadic ALS (SALS).[17, 18] In the present study however, these substitutions were not detected in the other affected relatives. One of the K17I carrier was later found to carry a R521C *FUS* mutation and the K54E carrier was found to carry a new *FUS* mutation (R521S), which segregated with the disease (sppl. fig 1H).

We also identified the c.166C>T/p.Pro56Ser (P56S) *VAPB* mutation in one family, giving a mutation frequency of 0.6%. The P56S carrier was of Japanese descent, and represents the first non-Brazilian patient carrying this mutation. Three other members of his family had motor neuron disease, suggesting autosomal dominant transmission. We did not detect any other variant of this gene in FALS. The finding of a P56S *VAPB* mutation in a patient of Japanese origin presumably reflects the Portuguese trading connection with the Far East and Brazil in the mid 16th century.

Six different *TARDBP* missense mutations, all located in exon 6, were detected in 7 families (table 1), corresponding to a mutation frequency of 4.3%. Pedigrees of the G295S, the A382T and the A315T substitutions have been described elsewhere.[6, 19] An additional family carrying the G348C mutation was identified. This mutation was

detected in the mother of the index case, an obligate carrier who has not developed the disease at age 65 (sppl. fig 1G). We also identified two novel mutations: the c.1150G>C/p.Gly384Arg (G384R) mutation in an affected woman, her affected sister, and in an obligate carrier aged 73 years who has not developed the disease but who has transmitted it to her affected son (sppl. fig 1E); and the c.1153T>G/p.Trp385Gly (W385G) mutation in two affected brothers of a separate family (sppl. fig 1F). These two novel mutations seemed to be pathogenic as they co-segregated with the disease, they were not detected in 500 controls and the amino acids targeted, Gly384 and Trp385, were conserved until *xenopus*.

We identified five different missense mutations in *FUS* in seven families. Two of these mutations were novel (table 1). The frequency of *FUS* mutations was 4.3% of our FALS patients. All *FUS* mutations were located in exon 15. No mutation was found in controls. Four mutations (R521C, R521H, R521S, R521L) targeted the same arginine residue that is conserved until *xenopus*. The first novel *FUS* mutation was a c.1561C>A/p.Arg521Ser (R521S) substitution. The mutation was absent in the healthy sister of the index case but present in a far cousin who died from a bulbar ALS (sppl. fig 1H). The second novel mutation was a c.1562G>T/p.Arg521Leu (R521L) mutation. DNA samples of three healthy relatives (father and two aunts) did not bear the mutation, confirming that the disease was transmitted to the index case by her affected mother (sppl. fig 1I). In both families, the penetrance was incomplete as the disease was transmitted by obligate carriers who lived until the age of 73 and 77 without developing ALS. The already reported c.1561C>T/p.Arg521Cys (R521C), c.1562G>A/p.Arg521His (R521H) and c.1542G>T/p.Arg514Ser (R514S) mutations were also detected. We confirmed the segregation of the R521H mutation (sppl. fig 1J) and showed, for the first time, the segregation of the R514S mutation (sppl. fig 1K). Surprisingly a *FUS* mutation was identified in two patients who also carried K17I or K54E *ANG* variant (table 1).

Clinical findings.

Clinical data could be recovered for 100 relatives belonging to the 36 families with mutations and is summarized for 81 patients, from whom the information was the most complete, in supplementary table 2.

Age at onset for the *SOD1*, *TARDBP*, *FUS* and other FALS (with no mutation in the screened genes) groups was statistically different (table 2, Fig 1C). *FUS* patients had younger onset than *SOD1* (Log Rank: p=0.0111) and other FALS (Log Rank: p<0.0001) patients.

Life span for the *SOD1*, *TARDBP*, *FUS* and other FALS patient groups was significantly different (table 2, fig 1D). *FUS* patients had a shorter life span than *SOD1* (Log Rank: $p=0.0002$), *TARDBP* (Log Rank: $p=0.0028$) or other FALS (Log rank: $p<0.0001$) patients. The *SOD1* and *TARDBP* groups were similar.

Disease duration of the *SOD1*, *TARDBP*, *FUS* and other FALS groups was different (table 2, fig 1E). The disease was more rapid in *FUS* than in *SOD1* (Log Rank: $p=0.0082$), *TARDBP* (Log Rank: $p=0.0019$) or other FALS patients (Log rank: $p=0.0425$). To further examine disease duration, we separated the mutated patients into three groups: “rapid course” (disease duration <3 years), “medium course” (3-7 years) and “slow course” (>7 years). Our *SOD1* patients had heterogeneous disease durations with bimodal distribution; more than half (53%, $n=18$) had a rapid course as in some patients carrying the novel H46D and D83G mutations. One third of *SOD1* patients (32%, $n=11$) had slow disease progression (fig 1B). The average age at onset was lower in this group (41.2 ± 3.3 , $n=11$) than in patients with more rapid course (56.4 ± 2.3 , $n=23$, Mann Whitney test: $p=0.0017$). Slow progression was notably observed in some patients with the novel P66R (>27 years) and D83G (>12 years) mutations and with the poorly documented N139D mutation. These patients typically had young onset and prominent LMN signs resembling progressive muscular atrophy. Long disease duration was also observed in the family with a *VAPB* mutation, who, as a whole, had leg onset in the sixth decade (sppl. table 2). Among the 15 patients with *TARDBP* mutations, only one presented with a slow course; two thirds ($n=9$) had a medium course and one third ($n=5$) developed rapid disease (fig 1B). No *FUS* patient had slow disease progression. More than 85% ($n=18$) died after a rapid disease (fig 1B). Three patients with R521H mutations had a “medium course” leading to a disease duration longer for this *FUS* mutation than for the others (sppl. table 2, Log Rank: $p=0.0213$, fig 1F). The percentage of patients in each disease duration subgroup (<3 , 3-7, >7 years) was compared between *SOD1*, *TARDBP*, *FUS* and other FALS patients using Fisher’s exact test analyses. *FUS* patient distribution was different from that of *SOD1* (Fisher’s test: $p=0.006$), *TARDBP* (Fisher’s test: $p=0.002$) and other FALS (Fisher’s test: $p=0.044$). *SOD1* patients distribution was also different from that of *TARDBP* (Fisher’s test: $p=0.0063$) and other FALS (Fisher’s test: $p=0.0005$).

The site of onset in *FUS* patients was in the arms (43%, $n=9$), legs (38%, $n=8$) or bulbar muscles (19%, $n=4$) which is similar to the other FALS groups. Site of onset (fig 1A) was mainly in the lower limbs for *SOD1* (85%, $n=28/33$, Fischer’s exact test for the difference with FALS group: $p<0.0001$) and in the upper limbs for *TARDBP* patients (76.4%, $n=13/17$, Fisher’s exact test for the difference with FALS group: $p=0.04$). Initial and final predominance of UMN or LMN signs in the limbs was also evaluated (sppl. table 2). For *SOD1* patients, LMN signs usually

predominated. Most of the *TARDBP* patients initially presented with both UMN and LMN signs; with disease progression, LMN signs became predominant. One patient carrying the novel W385G mutation (exhibiting the longest survival time) developed final predominance of limb spasticity. *FUS* patients showed continuous predominance of LMN involvement except for the R521H patients with prolonged survival who developed marked UMN signs at onset. ANOVA tests used to compare mean bulbar scores between *SOD1*, *TARDBP*, *FUS* and other FALS groups showed a significant difference (table 2). Worsening of bulbar function with time tends to be less severe for the *SOD1* and *FUS* groups compared to both *TARDBP* and other FALS patients (table 2).

No clinically-apparent frontotemporal dysfunction occurred in *SOD1* patients (table 2). Two out of 10 patients with *TARDBP* mutations demonstrated cognitive impairment that met criteria for frontotemporal dementia (FTD). One with the G295S mutation had FTD two years before developing bulbar motor signs.[19] The other (with the novel G384R *TARDBP* mutation) developed FTD one year after the onset of motor weakness. A single patient carrying the R521H *FUS* mutation presented with cognitive impairment five months after the onset of ALS. The proportion of patients with cognitive impairment was not statistically different between the *SOD1*, *TARDBP*, *FUS* and other FALS patients (table 2).

Discussion.

This study represents the first large scale investigation of FALS among the French population and the first report comparing the phenotypes of the recently identified ALS genes. A mutation was identified in 36 out of the 162 tested families (22.2%). The overall percentages of *SOD1* (12.5%), *ANG* (0.6%), *VAPB* (0.6%), *TARDBP* (4.3%) and *FUS* (4.3%) mutations were relatively close to those previously reported in European populations (suppl. table 1).

All of the 31 different mutations reported here affected evolutionarily conserved residues in the corresponding proteins and were absent from 500 controls (1000 chromosomes). The segregation with the disease could be established in 11 families (4 *SOD1*, 3 *TARDBP* and 4 *FUS*). The co-segregation of 11 *TARDBP* mutations [5, 6, 8, 9, 10, 19, 20, 21, 22, 23] and 6 *FUS* mutations [12, 13, 24, 25] with ALS has been previously demonstrated. In this report, we extend these lists to include two new *TARDBP* mutations (G384R and W385G), two new *FUS* mutations (R521S and R521L) as well as the R514S *FUS* mutation. The extension of our pedigrees should allow confirming the segregation of the disease with these mutations. Indeed labeling a variant as pathogenic is a danger. We found two index cases harboring *ANG* variants (K54E and R121H) that did not segregate with disease and we concluded that

they were not the cause of the disease in these FALS. Moreover we found a *FUS* mutation in two index cases harboring *ANG* variants (K54E or K17I). These results suggest that, as for others,[26] the association of some *ANG* variants and ALS is questionable.

Some of the mutations we identified demonstrated variable penetrance. For example, we confirmed the presence of *TARDBP* mutations in 2 obligate female carriers who transmitted the disease to their children but who were asymptomatic at age 65 and 73.

The number of patients allowed comparison of phenotypes in relation to the involved gene. Although these findings have to be confirmed in larger populations, we could find statistical differences for some clinical traits between the different mutation groups. Patients with *SOD1* mutations usually first developed lower limb weakness whereas patients with *TARDBP* mutations had onset predominantly in the upper limbs. Patients harboring *FUS* mutations had various onset sites in the arms, legs or bulbar muscles, which more resembles to the typical ALS.

Evolution of the disease was categorized as rapid (< 3 years), medium (3-7 years), or long (> 7 years) for each gene. *FUS* mutations seemed to lead to the most aggressive disease, with a young onset and a rapid course for most of the patients, an observation that is consistent with previous reports.[12, 13] Analyses also showed that life span is shortened for *FUS* patients. Among patients with *FUS* mutations, three with the R521H mutation had a disease course that was approximately 2-fold longer than the others (sppl. table 2). Our results also showed that bulbar deterioration and cognitive impairment tended to be less frequent in patients harboring *SOD1* and *FUS* mutations.

Considerable intra-familial phenotypic differences were observed in some families carrying various mutations in the *SOD1*, *TARDBP* and *FUS* genes. Age and site of onset varied between members of a family. The disease duration could also differ, as shown in families carrying the D83G *SOD1* mutation (6-151 months in 2 brothers with similar age at onset), the W385G *TARDBP* mutation, and the R521H *FUS* mutation. These data support the hypothesis that a mutation is not the only factor that determines the clinical course of the disease. Other factors must also contribute to phenotype, and it is not yet possible to predict the evolution of patients based solely on presence of the mutation or rate of progression in other family members.

Altogether our results show that one determinant of ALS phenotype is the underlying causative mutation. However heterogeneity between and among families implies that other environmental and genetic influences contribute to not only the rate of evolution and which signs predominate, but also whether the disease will appear at all during life. Despite recent advances in the field of ALS genetics, most of the genes involved in FALS are still unknown. Considerable work lies ahead in determining the genetic and environmental factors that most contribute to ALS.

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Figure Legends

figure 1. Distribution of each group of patients by age of onset, site of onset, disease duration and life span.

A. Histograms showing the distribution of patients with *SOD1*, *TARDBP* or *FUS* mutations and other FALS patients according to bulbar (black), upper limb (dark-grey) or lower limb (light grey) onset. B. Histograms illustrating the distribution of *SOD1*, *TARDBP*, *FUS* or other FALS patients by disease duration (in months): Black: <36 months, dark-grey: 36-84 months, light grey: >84 months. Cox regression curves of (C) cumulative probability of disease onset according to the age of patients, (D) cumulative survival probability according to the age of patients and (E) cumulative survival probability from time of disease onset. C-E. The four patient groups were *SOD1* (grey thin line), *TARDBP* (grey bold line), *FUS* (black bold line) and other FALS (black thin line). The graph was truncated at 200 months in (E) to improve visualization at short time points. F. Kaplan-Meier plots of cumulative survival from time of disease onset of patients carrying different *FUS* mutations: either R521H (bold line) or other mutations combined (dotted line).

supplementary figure 1. *SOD1*, *TARDBP* and *FUS* mutations segregating with the disease. Pedigree of the F8S, F15S, F17S and F20S families with the D83G (A), G93V (B), N139D (C) and I151T (D) *SOD1* mutation. Pedigree of the F28T, F29T and F25T families with the G384R (E), W385G (F) and G348C (G) *TARDBP* mutations. Pedigree of the F31F, F36F, F35F and F30F families with the R521S (H), R521L (I), R521H (J) and R514S (K) *FUS* mutations. Arrows indicate index patients. When available, the age at death and the disease duration (in brackets) are indicated above the symbol representing the patients. The genotypes are indicated using a single letter amino acid code below the index case and some relatives for whom DNA was available.

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Genes	Family number	Nucleotide change*	Amino acid change** (HGVS)	Amino acid change***	Exon	First description of the mutation
<i>SOD1</i>	F1S	c.65A>G	p.Glu22Gly	E21G	1	[25]
	F2S	c.112G>A	p.Gly38Arg	G37R	2	[1]
	F3S	c.116T>G	p.Leu39Arg	L38R	2	[25]
	F4S	c.124G>A	p.Gly42Ser	G41S	2	[1]
	F5S	c.124G>A	p.Gly42Ser	G41S	2	[1]
	F6S	c.139C>G	p.His47Asp	H46D	2	<i>this report</i>
	F7S	c.200C>G	p.Pro67Arg	P66R	3	<i>this report</i>
	F8S	c.251A>G	p.Asp84Gly	D83G	4	<i>this report</i>
	F9S	c.255G>C	p.Leu85Phe	L84F	4	[25, 26]
	F10S	c.253T>G	p.Leu85Val	L84V	4	[27]
	F11S	c.260A>G	p.Asn87Ser	N86S	4	[28]
	F12S	c.280G>T	p.Gly94Cys	G93C	4	[1]
	F13S	c.281G>C	p.Gly94Ala	G93A	4	[1]
	F14S	c.281G>A	p.Gly94Asp	G93D	4	[29]
	F15S	c.281G>T	p.Gly94Val	G93V	4	[30]
	F16S	c.355G>C	p.Val119Leu	V118L	4	[31]
	F17S	c.418A>G	p.Asn140Asp	N139D	5	[32]
	F18S	c.443G>A	p.Gly148Asp	G147D	5	[32]
	F19S	c.443G>A	p.Gly148Asp	G147D	5	[32]
	F20S	c.455T>C	p.Ile152Thr	I151T	5	[33]
<i>ANG</i>	F21A	c.122A>T	p.Lys41Ile	K17I	1	[4]
<i>VAPB</i>	F22V	c.166C>T	p.Pro56Ser	P56S	2	[2]
<i>TARDBP</i>	F23T	c.883G>A	p.Gly295Ser	G295S	6	[19, 21]
	F24T	c.943G>A	p.Ala315Thr	A315T	6	[6]
	F25T	c.1042G>T	p.Gly348Cys	G348C	6	[6]
	F26T	c.1144G>A	p.Ala382Thr	A382T	6	[6]
	F27T	c.1144G>A	p.Ala382Thr	A382T	6	[6]
	F28T	c.1150G>C	p.Gly384Arg	G384R	6	<i>this report</i>
	F29T	c.1153T>G	p.Trp385Gly	W385G	6	<i>this report</i>
<i>FUS</i>	F30F	c.1542G>T	p.Arg514Ser	R514S	15	[12]
	F31F ⁽¹⁾	c.1561C>A	p.Arg521Ser	R521S	15	<i>this report</i>
	F32F	c.1561C>T	p.Arg521Cys	R521C	15	[12, 13]
	F33F ⁽²⁾	c.1561C>T	p.Arg521Cys	R521C	15	[12, 13]
	F34F	c.1561C>T	p.Arg521Cys	R521C	15	[12, 13]
	F35F	c.1562G>A	p.Arg521His	R521H	15	[12, 13]
	F36F	c.1562G>T	p.Arg521Leu	R521L	15	<i>this report</i>

table 1: The 31 identified mutations in the 162 FALS index cases.

*: cDNA numbering is according to the following transcripts: *SOD1* (NM_000454.4), *ANG* (NM_001145.4 or NM_001097577.2), *VAPB* (NM_004738.3), *TARDBP* (NM_800737.3), *FUS* (NM_004960.2). **: amino acid numbering according to Human Genome Variation Society guidelines (<http://www.hgvs.org/>). ***: amino acid numbering according to the ALS Online Database (<http://alsod.iop.kcl.ac.uk/Als/index.aspx>), which has been used in the previous published reports on *SOD1* and *ANG* mutations. These numberings lack the first methionine codon for *SOD1* or the signal peptide sequence (24 amino acids in length including the initiator methionine) for *ANG*. Index cases of F31F and F33F families (with *FUS* mutation) also carry K54E⁽¹⁾ or K171⁽²⁾ *ANG* variants.

Patient groups	<i>SOD1</i>	<i>TARDBP</i>	<i>FUS</i>	other FALS
M:F ratio	1.2:1 (n=55)	1:1.87 (n=23)	1.23:1 (n=29)	2:1 (n=126)
Age at Onset (years)				
Mean (SE)	55 (SE 2, n=38)	54 (SE 4, n=17)	45 (SE 3, n=23)	58 (SE 1, n=115)
Median (SE)	55 (SE 2, n=38)	58 (SE 5, n=17)	47 (SE 7, n=23)	58 (SE 2, n=115)
Cox regression test [*]	p=0.004			
Disease duration (months) ^{**}				
Mean (SE)	79 (SE 14, n=38)	58 (SE 9, n=17)	30 (SE 4, n=23)	45 (SE 5, n=100)
Median (SE)	36 (SE 10, n=38)	51 (SE 9, n=17)	26 (SE 3, n=23)	32 (SE 2, n=100)
Cox regression test [*]	p=0.005			
Life span (years) ^{**}				
Mean (SE)	59 (SE 2, n=45)	59 (SE 4, n=21)	46 (SE 2, n=29)	61 (SE 1, n=101)
Median (SE)	59 (SE 2, n=45)	60 (SE 5, n=21)	45 (SE 6, n=29)	59 (SE 2, n=101)
Cox regression test [*]	p<0.0001			
Mean bulbar score ^{***}	3.7 (SE 1, n=13)	11.7 (SE 3.2, n=7)	3.6 (SE 0.7, n=9)	9.3 (SE 1.4, n=50)
ANOVA	p=0.0454			
Cognitive impairments ^{****}	0/20	2/10	1/13	10/62

table 2. Disease characteristics by mutated gene.

SE= standard error. n= number of patients for whom the information was available. NA=data not available. *: equality of survival distribution between *SOD1*, *TARDBP* and *FUS* groups was analyzed using a Log Rank test. If the difference was significant $p<0.05$, the groups were compared by pairs using a Log rank test. **: disease duration and age at death data included censored data (8 for *SOD1*, 2 for *TARDBP* and 1 for *FUS*) ***: The difference between initial (at onset) and final (at the end of the disease) bulbar Norris scale (13 items) was recorded for each patient. Means were compared using one way ANOVA. ****: the fraction represents the number of patients with cognitive impairment / the total number of patients who were examined for cognitive deficits.

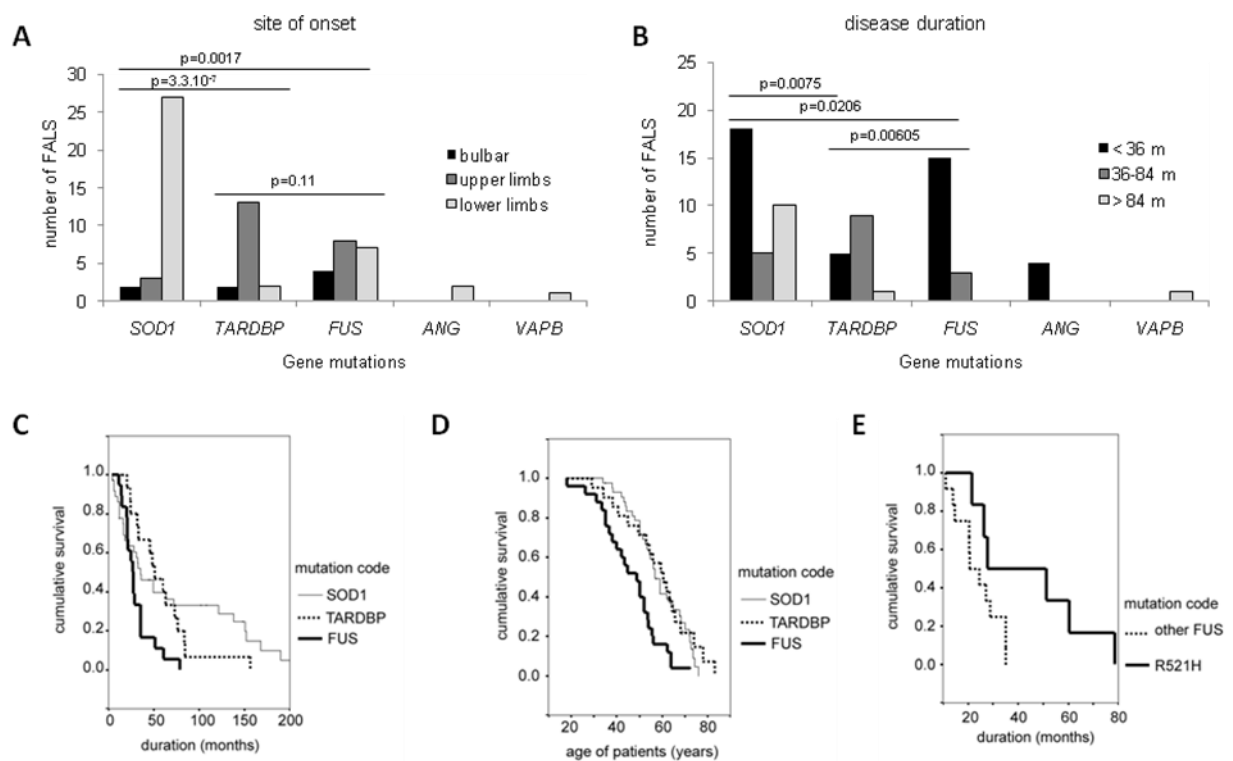


figure 1. Distribution of each group of mutated patients by site of onset, disease duration and life span. A. Histograms showing the distribution of patients with *SOD1*, *TARDBP*, *FUS*, *ANG* or *VAPB* mutations according to bulbar (black), upper limb (dark-gray) or lower limb (light gray) onset. B. Histograms illustrating the distribution of *SOD1*, *TARDBP*, *FUS*, *ANG* or *VAPB* mutated patients by disease duration (in months): Black: <3 years, dark-gray: 3-7 years, light gray: >7 years. C. Kaplan-Meier plots of cumulative survival from time of disease onset for *SOD1* (thin line), *TARDBP* (dotted line) and *FUS* (bold line) patients. The graph was truncated at 200 months to improve visualization at short time points. D. Survival probability for *SOD1* (thin line), *TARDBP* (dotted line) and *FUS* (bold line) mutated patients according to the age of patients (life span). E. Survival probability of patients carrying different *FUS* mutations: either R521H (bold line) or other mutations combined (dotted line). A Fisher's exact test compared group distributions by pairs (A-B).

Gene	mutational frequency (%)	nb mutated /nb studied	population tested for:				origin of population	references
			<i>SOD1</i>	<i>ANG</i>	<i>VAPB</i>	<i>TARDBP</i>		
<i>SOD1</i>	71.4	5/7	-	-	-	-	Japan	[34]
	63.6	7/11	-	-	-	-	Belgium	[35]
	50.0	5/10	-	-	-	-	Scotland	[36]
	23.5	12/51	-	-	-	-	Scandinavia	[37]
	23.4	68/290	-	-	-	-	USA	[38]
	21.1	8/38	-	-	-	-	England	[26]
	19.7	14/71	-	-	-	-	England	[39]
	18.2	2/11	-	-	-	-	Spain	[40]
	17.9	7/39	-	-	-	-	Italy	[41]
	16.7	5/30	-	-	-	-	Catalonia	[42]
	14.7	5/34	-	-	-	-	Italy	[43]
	14.3	10/70	-	-	-	-	France	[25]
	12.3	20/162					France	This series
	12.0	9/75	-	-	-	-	Germany	[44]
Total	19.7	177/897	-	-	-	-		
<i>ANG</i>	2.6	1/39	+	-	-	-	Netherlands	[45]
	2.3	3/132	+	-	-	-	Italy	[46]
	1.7	3/176	+	-	-	-	Ireland, Scotland, Sweden, England	[4]
	0.7	1/142	+	-	-	-	France	This series
	0.0	0/83	+	-	-	-	USA	[4]
	0.0	0/12	+	-	-	-	Italy	[24]
	0.0	0/8	-	-	-	-	Italy	[47]
Total	1.5	10/589	+	-	-	-		
<i>VAPB</i>	1.3	1/80	-	-	-	-	USA(Brazilian)	[48]
	0.7	1/141	+	+	-	-	France(Japanese)	This series
	0.0	0/23	+	-	-	-	England	[49]
Total	0.8	2/252	+	-	-	-		
<i>TARDBP</i>	18.8	3/16	+	+	+	-	Italy	[22]
	12.5	1/8	+	-	-	-	Europe	[5]
	6.5	2/31	+	-	-	-	Germany	[11]
	6.3	1/16	+	-	-	-	Japan	[10]
	5.0	7/140	+	+	+	-	France	This series
	5.0	4/80	+	+	+	-	France Québec	[6]
	4.8	2/42	+	-	-	-	Northern England	[20]
	4.8	6/125	+	+	-	-	Italy	[21]
	3.3	3/92	-	-	-	-	USA	[7]
	3.3	1/30	-	-	-	-	Japan	[50]
	3.1	2/65	+	-	-	-	USA	[9]
	0.6	1/154	+	+	+	-	England	[8]
Total :	4.1	33/796	+	+	+	-		
<i>FUS</i>	5.8	17/292	-	-	-	-	USA	[12]
	5.3	7/133	+	+	+	+	France	This series
	4.5	1/22	+	+	+	+	Belgium	[51]
	4.0	8/199	+	+	+	+	England	[13]
	3.8	2/52	+	-	-	+	Italy	[52]
	3.2	4/124	+	+	+	+	Australia	[53]
	2.5	2/80	+	+	+	+	France Québec	[54]
	2.4	1/41	-	-	-	-	Germany	[55]
Total :	4.2	40/940	+	+	+	+		

Supplementary table 1. Frequency of *SOD1*, *ANG*, *VAPB*, *TARDBP* and *FUS* mutations in FALS of various geographic origins. Data from this study is on grey background. To compare our data to other studies, frequencies we

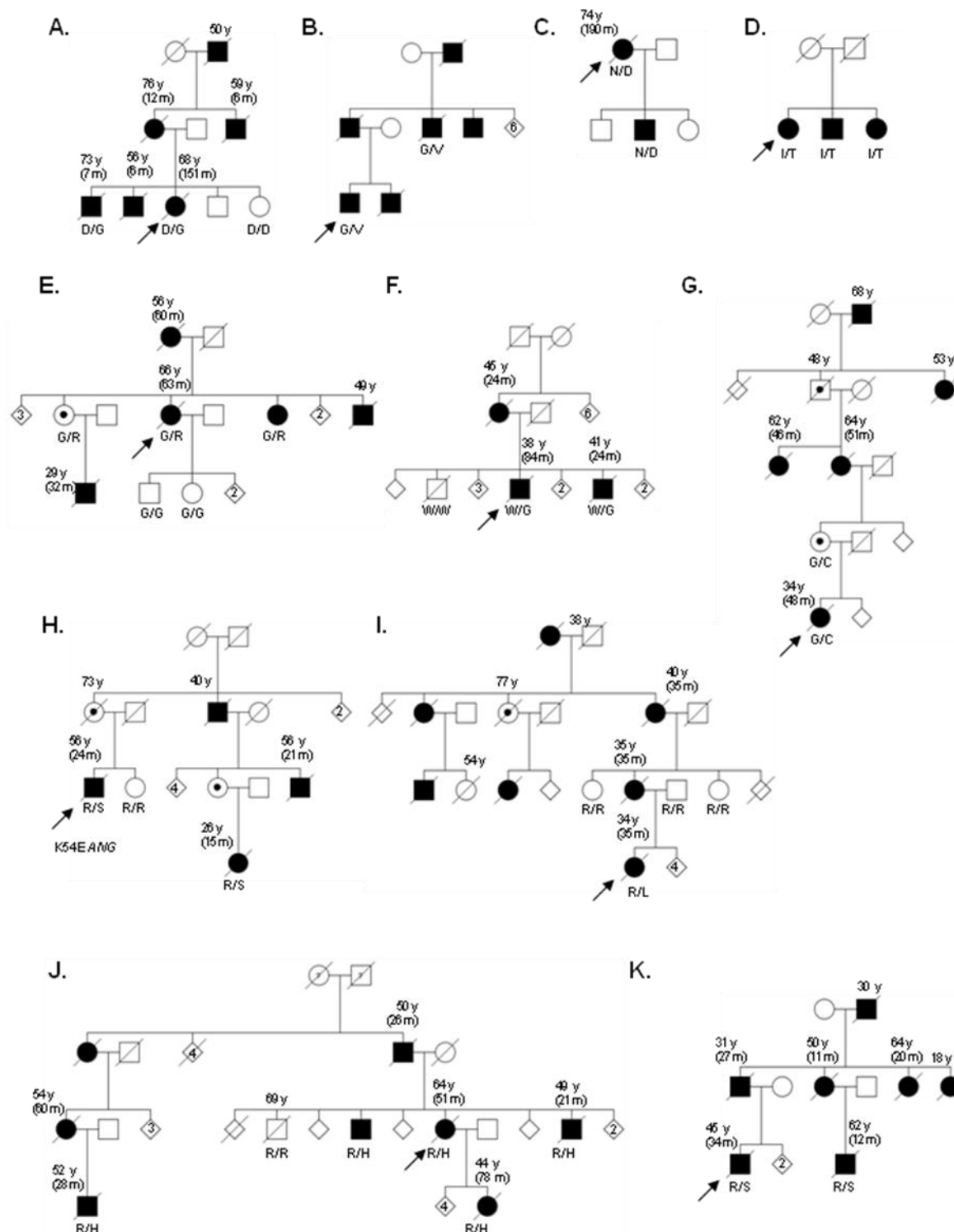
found were expressed in populations negative for previously tested genes. +: the corresponding gene has been analyzed in the population (mutated patients have been excluded from this sample). -: the corresponding gene has not been screened in the population or information is not available in the paper.

Supplementary references for the sppl. table 1:

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R521L	F36F	01	F	31	UL (prox)	L>U	L=U	L>U	L=U	1	no	35	33		
		02	F	32	LL	NA	NA	NA	NA	NA	NA	35	35		
		03	F	37	LL	NA	NA	NA	NA	NA	NA	35	40		
													33 (+/- 2)	35 (+/- 0)	

Supplementary table 2. Disease characteristics by patient. M=male. F=female. UL=upper limbs. LL=lower limbs. B=bulbar. L=lower motor neuron signs. U=upper motor neuron signs. NA=information not available. FTD= frontotemporal dementia. SE=standard error. *: the bulbar score is the difference between initial (at onset) and final (at the last evaluation) bulbar Norris scale. **: For patients who are still alive (with a disease onset that occurred more than 7 years ago), the duration was expressed as "> the present disease evolution".



supplementary figure 1. *SOD1*, *TARDBP* and *FUS* mutations segregating with the disease. Pedigree of the F8S, F15S, F17S and F20S families with the D83G (A), G93V (B), N139D (C) and I151T (D) *SOD1* mutation. Pedigree of the F28T, F29T and F25T families with the G384R (E), W385G (F) and G348C (G) *TARDBP* mutations. Pedigree of the F31F, F36F, F35F and F30F families with the R521S (H), R521L (I), R521H (J) and R514S (K) *FUS* mutations. Arrows indicate index patients. When available, the age at death and the disease duration (in brackets) are indicated above the symbol representing the patients. The genotypes are indicated using a single letter amino acid code below the index case and some relatives for whom DNA was available.