Contribution of major amyotrophic lateral sclerosis genes to the etiology of sporadic disease

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ABSTRACT

Objectives: To quantify the overall contribution of mutations in the currently known amyotrophic lateral sclerosis (ALS) genes in a large cohort of sporadic patients and to make genotype-phenotype correlations.

Methods: Screening for SOD1, TARDBP, FUS, ANG, ATXN2, OPTN, and C9ORF72 was carried out in 480 consecutive patients with sporadic ALS (SALS) and in 48 familial ALS (FALS) index patients admitted to a single Italian referral center.

Results: Mutations were detected in 53 patients, with a cumulative frequency of 11%. Seven of them were novel. The highest frequencies of positive cases were obtained in TARDBP (2.7%), C9ORF72 (2.5%), and SOD1 (2.1%). The overall group of mutated patients was indistinguishable from that without mutations as no significant differences were observed with regard to age and site of onset, frequency of clinical phenotypes, and survival. However, by separately evaluating genotype-phenotype correlation in single genes, clinical differences were observed among different genes. Duration of disease was significantly shorter in patients harboring the C9ORF72 expansion and longer in the SOD1 group. A high frequency of predominant upper motor neuron phenotype was observed among patients with TARDBP mutations. Two patients, 1 with C9ORF72 and 1 with SOD1 mutation, had concurrent ANG mutations. Mutations were detected in 43.7% of patients with FALS.

Conclusions: A considerable proportion of patients with SALS harbored mutations in major ALS genes. This result has relevant implications in clinical practice, namely in genetic counseling. The detection of double mutations in 2 patients raises the hypothesis that multiple mutations model may explain genetic architecture of SALS.

Neurology® 2012;79:66–72

GLOSSARY

ALS = amyotrophic lateral sclerosis; CI = confidence interval; FALS = familial ALS; FTD = frontotemporal dementia; JALS = juvenile ALS; LMN = lower motor neuron; SALS = sporadic ALS; SCA2 = spinocerebellar ataxia type 2; UMN-D = upper motor neuron dominant.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder involving upper and lower motor neurons (LMN). Familial cases of ALS (FALS) account for about 5% of total cases, and to date mutations in specific genes have been identified in about 50%–60% of FALS cases. Apart from the first studied SOD1 gene, in the last few years the pathogenic role of genes such as ANG, TARDBP, FUS, OPTN, ATXN2, VCP, UBQLN-2, and C9ORF72 has emerged.

Genetic factors may play a relevant pathogenetic role also in the sporadic form of ALS (SALS). According to a polygenic threshold model, simplex cases of ALS may be the result of cumulative effects of a large number of variants, each conferring a small increase of disease risk. Environmental factors may summate to genetic alterations to exceed a critical threshold of liability. Conversely, it is well-established that mutations in large-effect genes associated with FALS may be detected in apparently sporadic forms of ALS, but the contribution of known genes to the etiology of SALS has never been assessed systematically.

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Study funding: Supported in part by I.CO.M.M. onlus and FIGC (Federazione Italiana Giuoco Calcio).

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In the present study, we searched for mutations of SOD1, TARDBP, FUS, ANG, ATXN2, OPTN, and C9ORF72 in 480 consecutive patients with SALS and 48 patients with FALS admitted to a single Italian referral center and looked for genotype–phenotype correlations.

METHODS From 1987 to October 2011, 935 patients presenting with ALS were admitted to our Neurological Institute, which is a referral ALS Clinic Center for Lazio Region. All patients were Italian and were from the central or southern regions of Italy.

Fifty-three index patients (5.7%) were diagnosed with FALS and 882 were diagnosed with SALS. DNA samples were collected systematically from 480 consecutive patients with SALS and from 48 familial index patients.

Genomic DNA was extracted from leukocytes using 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). A subject with a 22/22 homozygous genotype was used as control. A repeat-primed PCR length analysis was performed on an ABI3130 Genetic Analyzer (Applied Biosystems), Carlsbad, CA) according to standard protocols. Sequence analysis was performed using DNA Sequencing Analysis Software v.5.1 and SeqScape Software v.2.5 (Applied Biosystems).

Multiple sequence alignment of the human proteins and their orthologues were generated using ClustalW program (http://www.ebi.ac.uk/clustalw).

ATXN-2 (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 analyzed using GeneMapper 4.0 software (Applied Biosystems). A subject with a 22/22 homozygous genotype was used as control. A repeat-primed PCR was used to identify patient carriers of the hexanucleotide GGGGCC expansion in the first intron of C9ORF72 gene (MIM: 614260), as described.8

All patients with ALS met El Escorial diagnostic criteria for definite, probable, or laboratory-supported probable ALS.9 Genealogies were actively investigated in all cases and patients with ascertained or suspected familiarity for ALS or frontotemporal dementia (FTD) were included in the FALS group. Patients were divided into 4 phenotypes: classic, upper motor neuron dominant (UMN-D), flail arm, and pure LMN. UMN-D ALS was characterized by predominant pyramidal signs, mainly sensitive sparing of lower limbs. Survival was defined as the period from disease onset to last follow-up or death or tracheostomy and it was analyzed using Kaplan-Meier method; survival curves were compared with log-rank test. A p value <0.05 was considered significant. Statistical analysis was performed using SPSS software.

RESULTS Genetic and clinical data. Demographic data of the cohort. One patient had juvenile ALS (JALS) with onset at 11 years; in the remaining 479 patients the mean age at onset was 59.9 years (range 22–86). A total of 282 patients were male and 198 female; 135 patients (28.1%) had bulbar, 341 (71%) spinal, and 4 (0.9%) respiratory onset, respectively. A total of 122 patients (25.4%) showed the UMN-D phenotype, 301 (62.7%) had the classic form, 24 (5%) the flail arm subtype, and 33 (6.9%) had apparent pure LMN involvement.

Overall results. Among patients with SALS, 53 had mutations in the analyzed genes, with a cumulative frequency of 11%.

Mutated patients were 27 women (51%) and 26 men (49%), compared to 171 women (40%) and 256 men (60%) in the group of patients without mutations (p = 0.16). The mean age at onset in the mutated group was 58 years (range 27–86), excluding the patient with JALS, and 60.1 years (range 22–84) in patients without detectable mutations (p = 0.24). No difference was observed between mutated and not mutated patients in the percentage of patients with bulbar onset (20.7% vs 29%; p = 0.26) and proportion of UMN-D phenotype (28.3% vs 25%; p = 0.7) (table 1 and table e-1 on the Neurology® Web site at www.neurology.org).

Median survival was 53 months (95% confidence interval [CI] 31.99–74.0) in mutated patients and 40 months (95% CI 33.26–46.73) in nonmutated ones; this difference was not significant (p = 0.31).

Clinic-genetic data. SOD1. Ten patients carried 7 heterozygous variants in SOD1 gene (2.1%). Six variants have been already reported, while we are describing for the first time the missense variant c.63 C>G responsible for the replacement of a phenylalanine with a leucine at position 20 (p.F20L). All patients harbored missense mutations with the exception of 1 case, in which an in-frame 3-nucleotide heterozygous deletion causing loss of glutamic acid at position 133 (p.E133del) was detected. In all patients the disease started in spinal regions. The age at onset ranged from 36 to 71 years (mean 56.3). Two patients, both harboring the p.D90A mutation, had an UMN-D phenotype. Median survival was 101 months (95% CI 30.73–171.26).

TARDBP. Six different heterozygous missense variants were detected in 13 patients (2.7%). Five of these variants have been previously described, whereas the p.Q303H is novel. An even gender distribution was observed (7 M/6 F), with average age at onset of 53.2 years (range 27–75), and median survival of 66 months (95% CI 4.13–127.86). The majority of patients (54%) had the UMN-D phenotype.
No patient developed cognitive impairment. Two patients with the same p.N390S variant also had a 30 CAG repeat expansion of \textit{ATXN-2}. \textit{C9ORF72}. Twelve patients (2.5%), 7 male and 5 female, harbored the large hexanucleotide (GGGGCC) repeat expansion in the first intron of \textit{C9ORF72}. The mean age at onset was 59.2 years (range 38–75) and median survival was 38 months (95% CI 15.91–60.08). Six patients had a classic form, an additional patient had classic ALS with associated cognitive/behavioral symptoms typical of FTD, 3 disclosed the UMN-D phenotype, and 2 the LMN form. \textit{FUS}. A total of 3 mutations (0.6%) were identified. They consisted of the already reported p.R521L and p.P525L variants in 2 patients. The patient with R521L mutation had a flail arm phenotype with duration of disease of 24 months. The patient with P525L mutation had a juvenile onset with rapid progression.\textsuperscript{11}

In 1 patient we detected a previously unreported 3-nucleotide heterozygous deletion (661_663delAGT) in exon 6 of \textit{FUS}, causing loss of serine at codon 221 (p.Ser221del). \textit{OPTN}. Three mutations, all novel, were detected in 3 different patients (0.6%). They were c.7 C>T, changing histidine into tyrosine (p.H3Y), c.46 C>G, causing replacement of proline with alanine (p.P16A), and c.1703 T>C, leading to substitution of leucine with serine (p.L568S). \textit{ANG}. Four different heterozygous mutations were detected in 6 patients (1.2%). One patient had a novel nonsense mutation c.338G>A, creating a premature stop codon at position 113 (p.W89X). Three missense variants were identified in the remaining 5 patients: c.208 A>G, leading to valine-isoleucine replacement at codon 70 (p.I46V), c.232 A>G, causing the substitution of lysine with glutamic acid at codon 78 (p.K54E), and c.433 C>T, consisting of the replacement of the arginine at aminoacidic position 145 with a cysteine (p.R121C) (table e-1). The patient with the p.I46V variant had also a \textit{C9ORF72} expansion, while the patient harboring the p.R121C \textit{ANG} variant had a concomitant p.G93D \textit{SOD1} mutation, as previously reported.\textsuperscript{12}

The mean age at onset was 60 years (range 38–86). Three patients had a UMN-D phenotype and 3 a classic form. \textit{ATXN-2}. Eight patients (1.7%) had an intermediate length of 31–33 CAG repeats. The mean age at onset was 57.5 years (range 42–76). Seven of them had classic ALS and 1 had the flail arm form. Eight additional patients, who had 27–30 CAG repeats, were not included in this analysis. Two of these last patients had mutations of \textit{TARDBP} (table e-1).

**Table 1** Clinical and demographic findings

<table>
<thead>
<tr>
<th>Patients with mutations</th>
<th>SOD1</th>
<th>TARDBP</th>
<th>FUS</th>
<th>ANG</th>
<th>OPTN</th>
<th>ATXN-2</th>
<th>C9ORF72</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) of patients</td>
<td>10 (2.1)</td>
<td>13 (2.7)</td>
<td>3 (0.6)</td>
<td>6 (1.2)*</td>
<td>3 (0.6)</td>
<td>8 (1.7)</td>
<td>12 (2.5)</td>
<td>53 (11)</td>
</tr>
<tr>
<td>Age at onset, y (range)</td>
<td>56.3 (36–71)</td>
<td>53.2 (27–75)</td>
<td>37 (11–58)</td>
<td>60 (38–88)</td>
<td>70 (67–76)</td>
<td>57.5 (42–76)</td>
<td>58.8 (38–75)</td>
<td>57.9 (27–86)</td>
</tr>
<tr>
<td>No. (%) of men</td>
<td>2 (20)</td>
<td>7 (53.8)</td>
<td>2 (66.7)</td>
<td>1 (16.6)</td>
<td>1 (33.3)</td>
<td>6 (75)</td>
<td>7 (58.3)</td>
<td>26 (49)</td>
</tr>
<tr>
<td>Site of onset, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bulbar</td>
<td>0</td>
<td>3 (23.1)</td>
<td>1 (33.3)</td>
<td>1 (16.6)</td>
<td>1 (33.3)</td>
<td>2 (25)</td>
<td>3 (25)</td>
<td>11 (20.7)</td>
</tr>
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<td>Spinal</td>
<td>10 (100)</td>
<td>10 (76.9)</td>
<td>2 (66.7)</td>
<td>5 (83.4)</td>
<td>2 (66.7)</td>
<td>6 (75)</td>
<td>9 (75)</td>
<td>42 (79.3)</td>
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<td>Phenotype, n (%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td>5</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>7 (1)*</td>
<td>30 (1)*</td>
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<tr>
<td>UMN-D</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>3</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
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<tr>
<td>LMN</td>
<td>2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Median survival, mo</td>
<td>101</td>
<td>66</td>
<td>24</td>
<td>33</td>
<td>16</td>
<td>54</td>
<td>53</td>
<td>40</td>
</tr>
</tbody>
</table>

Abbreviations: LMN = lower motor neuron; UMN-D = upper motor neuron dominant.

* Two of the 6 patients are also included in \textit{SOD1} and \textit{C9ORF72} groups.

* The patient with juvenile amyotrophic lateral sclerosis was excluded.

* In brackets are reported the number of cases with associated FTD.
disease. Also the p.L67P SOD1 mutation was detected in the healthy 63-year-old father of the patient, as described. The p.D11Y variant of SOD1 was found in the 58-year-old sister of one patient, who was normal at clinical examination. Genetic analysis of relatives of the patient with the p.G294V TARDBP mutation revealed the same mutation in her healthy father, aged 90 years, and in her 2 brothers and 1 sister, aged 56, 66, and 57 years, respectively, who had normal neurologic examination (figure 1).

The 78-year-old mother of one patient (SALS535) had C9ORF72 hexanucleotide expansion and the mother, aged 64, harbored the p.I46V ANG mutation.

**DISCUSSION**

The distinction between familial and apparently sporadic ALS appears to be less clear than previously assumed. By using a mathematical model of familiarity of disease, with parameters for penetrance, mutation frequency, and family size, it has been suggested that monogenic, high-penetrance variants can account for a large proportion of cases with no apparent family history. Accordingly, recent discoveries of new genes involved in FALS have been invariably followed by the identification of mutations in the same genes also in patients with apparent sporadic disease.

FALS. Mutations were detected in 21 of 48 patients with FALS (43.7%). C9ORF72 expansion was found in 9 patients (18.7%), SOD1 mutations in 7 (14.6%), TARDBP mutations in 1 (2.1%), FUS mutations in 1 (2.1%), and intermediate-length (>30) ATXN2 repeats were detected in 3 (6.2%). No mutations were detected in OPTN and ANG.

The highest frequency of positive cases was obtained in TARDBP (2.7%), C9ORF72 (2.5%), and SOD1 (2.1%). Five of the TDP43 variants have been previously described: p.N267S, p.G294V, p.A382T, p.I383V, and p.N390S. Another identified TDP43 mutation is novel, consisting of p.Q303H. Supporting its pathogenicity, glutamine residue at codon 303 is highly conserved throughout evolution (figure 2), the variant has never been reported in the SNP database nor in 1000 Genomes Project database, and finally it is absent in 330 healthy Italian individuals already screened in other studies.

Sequencing SOD1 revealed previously described missense variants in 9 patients, including p.D90A, p.G93D, p.D11Y, and p.G85S. The reported p.F20L mutation is novel. This mutation is not reported in the SNP database nor in 1000 Genomes Project database. Of note, one different mutation was reported in the same codon (p.F20C) which
is highly conserved among vertebrates and invertebrates (figure 2).

Screening for ANG revealed 1 novel p.W89X nonsense mutation in 1 patient and 3 previously reported missense variants in 5 patients. The p.K54E and p.R121C mutations are absent in the SNP database and in about 1,800 healthy Italian individuals already screened in other studies. The p.I46V, which was identified in 3 different patients, has been frequently found in several reported ALS series, but has been also detected in controls. Interestingly, 2 patients with ANG variants had concurrent mutations in SOD1 and C9ORF72, respectively. The patient with p.G93D SOD1 and p.R121C ANG mutations was previously described: he had a more severe clinical course of the disease with respect to that usually reported in patients with G93D SOD1 variant.12,32 The patient with C9orf72 expansion and p.I46V ANG variant had disease onset at 38 years and thus was the youngest patient in the group with C9orf72 mutations (table e-1). Though the role of ANG variants in the etiology of ALS has been questioned, these findings raise the hypothesis that ANG may act as a modifier gene influencing phenotypic variability or penetrance of other genes.33 The observed frequency of double mutations in our patients was 2/480 = 0.4%. If it can be assumed that mutations in the 2 different genes are independent events and that all the events obtained as the intersection of 2 mutations are disjoint, the theoretical frequency of individuals harboring a double mutation is given by the sum of the probabilities of all the pairs, that is: \( f = f(SOD1 \cdot ANG) + f(C9ORF72 \cdot ANG) = 0.055\%. \) Thus, the frequency of double mutation found in patients was approximately 7 times greater than would be expected by chance.

As far as FUS and OPTN are concerned, we confirm that mutations in these genes are rare in SALS. The p.Ser221del FUS mutation is not reported in the SNP database and it has not been found in 793 Italian controls already analyzed.34 All the 3 OPTN mutations have never been reported in the SNP database, are absent in about 280 healthy Italian individuals already screened, and involve highly conserved amino acidic residues (figure 2).35

Intermediate length of a 27–33 CAG repeat in ATXN-2, the causative gene of spinocerebellar ataxia type 2 (SCA2), has been recently proposed as a risk factor for sporadic ALS.36 The association is mainly driven by the longer (31–33) polyQ repeats, which have been found in 1.8%–3.7% of different series of patients with ALS, compared to 0%–0.2% of control individuals.36–38 Differently from other genes, ATXN2 does not act as a Mendelian gene in ALS; however, a length of repeat expansions >30 represents a robust genetic risk factor for SALS. In our series, 8 patients (1.6%) had expansions of 31–33 CAG repeats, a frequency consistent with previously reported series.36,37 Nine additional patients had expansions of 27–30 repeats. Although they were not evaluated in this study, it is worth noting that a meta-analysis of literature data showed that a cutoff of \( \geq 29 \) appeared optimal to discriminate ALS from controls.39 Although the significance of shorter size repeats is unclear, the observation that 2 of our patients with 30 repeats had coexistent TARDBP mutations (table e-1) is of interest, because ATXN2 is a potent modifier of TDP-43 toxicity in animal and cellular models.36

Genetic data from unaffected relatives of mutation carriers were available in only 7 families. The same mutations found in patients were detected in most relatives, including very old individuals, suggesting that they act mainly as low penetrance alleles, whereas de novo mutations are rare. Interestingly, the patient harboring the double C9orf72 hexanucleotide expansion and p.I46V ANG variant was shown to have inherited the former from her father and the latter from her mother. Taken together these findings are consistent with the hypothesis of a polygenic cause of the disease, with identified variants representing only one contributor in a cascade of events.

Overall, clinical manifestations of patients with mutations were indistinguishable from those of patients without detectable mutations, as no significant differences were observed with regard to age and site of onset, frequency of clinical phenotypes, and survival. However, by evaluating individual geno-
type–phenotype correlations, we observed a high frequency of UMN-D phenotype among patients with *TARDBP* mutations (7/13; 54%) with a significant difference with respect to nonmutated patients (25% *p* = 0.04). Comparison of survival was carried out in patients with *TARDBP*, *C9ORF72*, and *SOD1* mutations, for which sufficient data were available (figure 3). Duration of disease was significantly shorter in patients harboring the *C9ORF72* expansion (median 38 months; *p* = 0.01). In the *SOD1* group, the long duration of disease (101 months) can be partially explained by the fact that 3 patients had the p.D11Y mutation, which is associated with a relatively benign course. Conversely, the patient with the p.G85S SOD1 variant had an aggressive course, similar to that described in one familial case with the same mutation.

Considering that only half of the patients with FALS have mutations in currently available genes, the discovery of new genes in the future is supposed to considerably increase the proportion of SALS with proven genetic etiology.

Our results show that recent discoveries in the genetics of FALS are changing the scenario of sporadic ALS, with relevant implications in clinical practice and in a research setting. Though mutation frequency may differ between different populations, countries, and regions, there is evidence that the same genes may act as either Mendelian genes in FALS or low-penetrance risk alleles in SALS. In a clinical setting, this observation suggests that systematic genetic screening should be performed in patients with SALS. Conversely, genetic counseling in families of patients with SALS with mutations is difficult because knowledge of factors influencing penetrance is poor. Further studies will elucidate if SALS arises from summation of the effects of a series of low-frequency dominantly and independently acting mutations of a variety of different genes.

**AUTHOR CONTRIBUTIONS**

S. Lattante: acquisition of data, analysis or interpretation of data, study concept or design, drafting/revising the manuscript. A. Conte: acquisition of data, analysis or interpretation of data, study concept or design, drafting/revising the manuscript. M. Zollino: acquisition of data, analysis or interpretation of data, study concept or design, drafting/revising the manuscript. M. Del Grande: acquisition of data, analysis or interpretation of data, study concept or design, drafting/revising the manuscript. M. Luigetti: acquisition of data, analysis or interpretation of data, study concept or design, drafting/revising the manuscript, statistical analysis. M. Sabatelli: acquisition of data, analysis or interpretation of data, study concept or design, drafting/revising the manuscript, obtaining funding.

**DISCLOSURE**

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

Received December 19, 2011. Accepted in final form February 23, 2012.

**REFERENCES**

AAN Publishes Guideline Update on Infantile Spasms

The AAN has published evidence-based recommendations for the treatment of infantile spasms that update a 2004 guideline. “Evidence-based Guideline Update: Medical Treatment of Infantile Spasms,” published in the June 12, 2012, issue of *Neurology*®, suggests that the therapy adrenocorticotropic hormone, also known as ACTH, and the antiepileptic drug vigabatrin (VGB) may be effective in the treatment of infantile spasms in children.

To read the guideline and access PDF summaries for clinicians and patients, a slide presentation, and a clinical example, visit www.aan.com/go/practice/guidelines. For more information, contact Julie Cox at jcox@aan.com or (612) 928-6069.