Extensive genetics of ALS

A population-based study in Italy

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ABSTRACT

Objective: To assess the frequency and clinical characteristics of patients with mutations of major amyotrophic lateral sclerosis (ALS) genes in a prospectively ascertained, population-based epidemiologic series of cases.

Methods: The study population includes all ALS cases diagnosed in Piemonte, Italy, from January 2007 to June 2011. Mutations of *SOD1, TARDBP, ANG, FUS, OPTN,* and *C9ORF72* have been assessed.

Results: Out of the 475 patients included in the study, 51 (10.7%) carried a mutation of an ALS-related gene (*C9ORF72*, 32; *SOD1*, 10; *TARDBP*, 7; *FUS*, 1; *OPTN*, 1; *ANG*, none). A positive family history for ALS or frontotemporal dementia (FTD) was found in 46 (9.7%) patients. Thirty-one (67.4%) of the 46 familial cases and 20 (4.7%) of the 429 sporadic cases had a genetic mutation. According to logistic regression modeling, besides a positive family history for ALS or FTD, the chance to carry a genetic mutation was related to the presence of comorbid FTD (odds ratio 3.5; $p = 0.001$), and age at onset ≤54 years (odds ratio 1.79; $p = 0.012$).

Conclusions: We have found that ~11% of patients with ALS carry a genetic mutation, with *C9ORF72* being the commonest genetic alteration. Comorbid FTD or a young age at onset are strong indicators of a possible genetic origin of the disease. *Neurology*® **2012;79:1983–1989**

GLOSSARY

ALS = amyotrophic lateral sclerosis; **bvFTD** = behavioral variant frontotemporal dementia; **CI** = confidence interval; **fALS** = familial ALS; **FTD** = frontotemporal dementia; **OR** = odds ratio; **PARALS** = Piemonte and Valle d'Aosta register for ALS; **sALS** = sporadic ALS.

Amyotrophic lateral sclerosis (ALS) is a degenerative disorder of adult age involving the motor system, with a progressive and invariably fatal course. In 5% of cases it is considered to be genetically transmitted (familial ALS [fALS])[1,2] while in the remaining cases it occurs sporadically in the population (sporadic ALS [sALS]). Mutations in several genes have been found to cause ALS, namely *superoxide dismutase 1* (*SOD1*),[3] *TAR DNA binding protein* (*TARDBP*),[4] *angiogenin* (*ANG*),[5] *fused in sarcoma* (*FUS*),[6,7] *optineurin* (*OPTN*),[8] and the recently described *chromosome 9 open reading frame 72* (*C9ORF72*).[9,10]

Several studies have assessed the frequency of mutations of single genes,[11] but few studies have compared relative frequencies of *SOD1, TARDBP, ANG,* and *FUS*[11–15]; all these studies were based on tertiary center series of cases, and were consequently skewed toward a younger population of patients with a larger number of fALS[16]; moreover, only one of them reported data on *C9ORF72*, which is now

Supplemental data at www.neurology.org

Supplemental Data

recognized as the major contributor to this form of neurodegeneration. Therefore there are no data on the frequency of mutations of ALS-related genes in a population-based setting that would be representative of the general ALS population, with the exception of our previous article on *SOD1* mutation in a province of Torino, Italy, in the period 2000–2005.[16]

The aim of this study is to assess the frequency of mutations of major ALS genes in a prospectively ascertained, population-based epidemiologic series of cases identified through the Piemonte and Valle d'Aosta register for ALS (PARALS) and to compare the clinical characteristics of patients with and without genetic mutations.

METHODS The study population includes all ALS cases diagnosed in Piemonte and Valle d'Aosta, Italy, during the 4.5-year period January 1, 2007, to June 30, 2011. The cases were recruited through the PARALS, a prospective epidemiologic register involving all the neurologic departments of the 2 regions of Northern Italy. The register was established in 1995 and is still in operation. Epidemiologic data regarding the 1995–2004 period have been published elsewhere.[17]

Case collection. The main sources of cases were the neurology departments of the 2 regions. Investigators used an ad hoc questionnaire to collect patients' demographic data, disease history, neurologic and laboratory findings, and treatments. Diagnostic EMG examination was performed in all patients according to standard procedures. The secondary sources for case collection were the Piemonte and Valle d'Aosta Central Regional Archives and mortality coding from the Italian Bureau of Statistics. Clinical records of cases found through secondary sources were obtained, and relevant clinical information for each case was analyzed in order to verify whether the patient met the eligibility criteria for the diagnosis of ALS; all living patients were contacted by phone and visited by one of the neurologists involved in the study.

Diagnostic criteria. The diagnosis of ALS was based on El Escorial revised criteria.[18] Patients with definite, probable, and probable laboratory-supported ALS were included in the register.

Definition of fALS. Currently there is not a consensus on the diagnostic criteria for fALS,[19] and the recent identification of *C9ORF72* gene hexanucleotide expansions as a major cause of both ALS and frontotemporal dementia (FTD) co-occurring in the same pedigrees[20–22] has further complicated the analysis of ALS pedigrees. For the present article, we used 2 different definitions of fALS: first, a narrower definition requiring the presence of at least 1 first- or second-degree relative affected by ALS in the pedigree of the index case[23]; second, a broader definition, considering as fALS also the patients with 1 first-degree relative with FTD, when the index case carried a GGGGCC hexanucleotide expansion in the first intron of *C9ORF72* gene.

Genetic analysis. All the coding exons and 50 bp of the flanking intron-exon boundaries of *SOD1*, of exon 6 of *TARDBP*, and of exons 14 and 15 of *FUS* and exons 5, 9, 12, and 14 of *OPTN* and the only exon of *ANG* have been PCR amplified, sequenced using the Big-Dye Terminator v3.1 sequencing kit (Applied Biosystems Inc.), and run on an ABIPrism 3130 genetic analyzer. These exons were selected as the vast majority of known pathogenic variants are known to lie within these mutational hotspots. A repeat-primed PCR assay was used to screen for the presence of the GGGGCC hexanucleotide expansion in the first intron of *C9ORF72*.[10]

Cognitive assessment. From January 2007 patients' neurobehavioral dysfunction was assessed with the Frontal Systems Behavior Scale.[24] From June 2009, >95% of patients also underwent a complete cognitive battery according to the consensus criteria for the diagnosis of frontotemporal cognitive and behavioral syndromes in ALS.[25] Patients were classified in 2 categories based on neurobehavioral and cognitive testing: subjects with normal cognition and subjects with FTD. Patients with FTD were further subclassified as having behavioral variant frontotemporal dementia (bvFTD), progressive aphasia, or semantic dementia, according to the proposed criteria for the classification of dementia in ALS.[25]

Controls. Controls were neurologically healthy subjects, resident in the area of the study, and age- and gender-matched to cases. *SOD1* was sequenced in 130 controls, *C9ORF72* in 245, *TARDBP* in 196, *ANG* in 140, *FUS* in 280, and *OPTN* in 96.

Statistical methods. Comparisons between means were made with analysis of variance. Cochran-Armitage test for trend was used to test the frequency of mutated cases in different age groups. Logistic regression (stepwise forward) was used to identify factors independently related to presence of a genetic mutation. Variables included in the logistic regression were age ($\leq$54, 55–69, $\geq$70 years), gender, type of onset (bulbar vs spinal), comorbid FTD, and family history positive for 1 first- or second-degree relative with ALS or FTD. A *p* value <0.05 was considered significant. Data were processed using SPSS statistical package version 18 (IBM Corporation, Chicago, IL). No patients were lost to follow-up. The follow-up included a total of 1,148.5 person/years.

Ethical approval. The study has been approved by the ethical committees of the involved centers. All patients and controls signed a written informed consent. Databases were treated according to the Italian regulations for privacy.

RESULTS During the period of the study a total of 601 patients were diagnosed with ALS in the study area. Of these, 40 did not give their consent for genetic analysis and 42 died before blood sampling. Forty-four patients were found only through secondary sources and were therefore not tested for DNA (figure). Therefore, a total of 475 patients (79.0%) were included in the mutational analysis study. The patients not included in the genetic analysis had an older mean age at onset than those included in the study (68.7 [SD 10.7] vs 65.5 [SD 10.6] years, *p* = 0.02), but were similar in terms of site of onset and clinical phenotype (data not shown).

Frequency of familial ALS. Thirty-six patients (7.6%) belonging to 34 families met the narrower criteria for fALS. A total of 46 (9.7%) patients, belonging to 44

Figure Flow chart showing capture rate and the sequence of participant selection
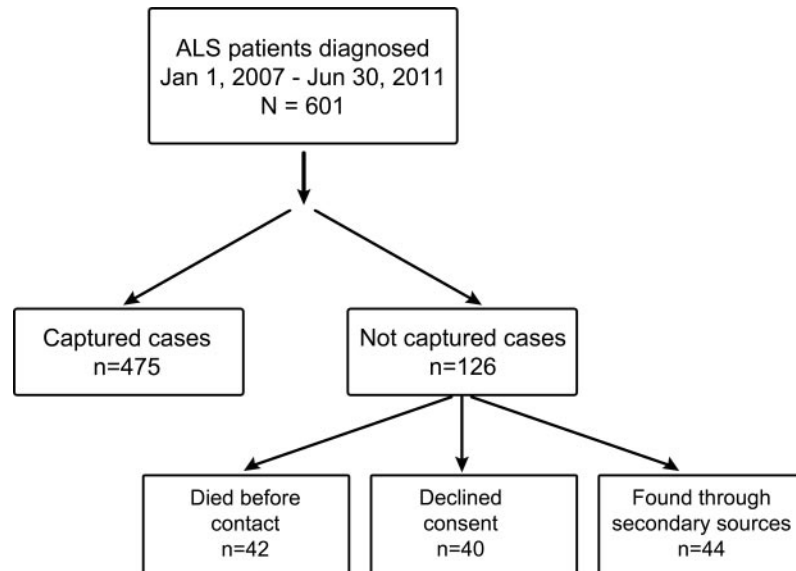
ALS = amyotrophic lateral sclerosis.

apparently unrelated families, met the broader criteria for fALS.

Frequency of genetic mutations. Thirty-one (67.4%) out of the 46 patients with fALS carried a mutation in one of the tested ALS genes. In contrast, only 20 (4.7%) out of the 429 apparently sALS cases had a genetic mutation. Overall, 51 patients (10.7% of the whole series) carried a mutation of an ALS-related gene. Younger patients were nearly 3 times more likely to carry a mutation in one of the known ALS genes compared to older patients (Cochran-Armitage test for trend, $p = 0.03$, table 1).

Clinical factors related to the risk of carrying a pathogenic mutation. According to logistic regression modeling, the chance of a patient having a genetic mutation was most strongly related to a positive family history for ALS or FTD in a first- or second-degree relative (odds ratio [OR] 22.2, 95% confidence interval [CI] 9.6–52.6; $p = 0.0001$). The presence of comorbid FTD (OR 3.5, 95% CI 1.7–7.4; $p = 0.001$) and age at onset ≤54 years (OR

1.79; 95% CI 1.1–3.6; $p = 0.012$) were also significant independent factors influencing the likelihood of carrying a genetic mutation.

The clinical characteristics of patients carrying or not carrying gene mutations are reported in table 2. The different genetic mutations are characterized by rather different phenotypes. No pathogenetic mutations of *ANG* were found, and none of the patients in this series carried mutations of 2 different genes.

***C9ORF72* hexanucleotide repeat expansions.** Thirty-two (6.7%) ALS cases carried a hexanucleotide repeat expansion of *C9ORF72* gene, making it the commonest mutation found in our series, representing 62.7% of all mutated cases. Among these cases, 24 (75.0%) had a family history for ALS, FTD, or both. The mean age at onset of ALS in patients with *C9ORF72* hexanucleotide repeat expansion was 61.6 years (SD 8.5) (range 43–71; median 62.6, interquartile range 55.7–67.3). Fourteen patients (45.2%) had a bulbar onset and 10 (31.3%) were female. More than half of the patients with *C9ORF72* hexanucleotide repeat expansions had a florid FTD, significantly more than patients with other mutations and those without mutation. Symptomatology was consistent with bvFTD in all our cases carrying the pathogenic expansion. Three patients had psychotic-like symptoms. Comorbid FTD was significantly more frequent among patients with a family history positive for ALS, FTD, or both ($p = 0.008$) (table 3).

***SOD1* mutations.** *SOD1* mutations were the second most common mutations, representing 2.1% of all

Table 1	Frequency of mutated patients (all tested genes) according to age[a]			
	≤54 y, n (%)	55–69 y, n (%)	≥70 y, n (%)	Total, n (%)
Mutated	12 (16.0)	29 (12.3)	10 (5.6)	51 (10.7)
Wild-type	62 (85.1)	207 (87.9)	155 (94.4)	424 (89.3)
Overall	74	236	165	475

[a] Cochran-Armitage test for trend, $p = 0.03$.

Table 2	Main clinical characteristics of patients with different mutations and without known mutations					
	No. of cases	% of cases[a]	Gender (female), n (%)	Median age at onset, y (IQR)	Site at onset (bulbar), n (%)	FTD, n (%)
C9ORF72	32	6.7	10 (31.3)	62.6 (55.7–67.3)	14 (43.8)	17 (53.1)
SOD1	10	2.1	8 (80.0)	57.0 (46.7–70.7)	1 (10.0)	0
TARDBP	7	1.5	3 (42.9)	66.8 (60.0–68.2)	3 (42.9)	2 (28.6)
Wild type, fALS	15	3.2	7 (46.7)	61.3 (55.1–68.0)	5 (33.3)	2 (13.3)
Wild type, sALS	409	86.1	187 (45.7)	66.6 (59.5–73.3)	126 (30.8)	66 (16.1)
Overall	475[b]	—	216 (45.7)	66.3 (59.1–73.0)	149 (31.4)	87 (18.3)
p			NS	0.0001	0.31	0.0001

Abbreviations: fALS = familial amyotrophic lateral sclerosis; FTD = frontotemporal dementia; IQR = interquartile range; NS = not significant; sALS = sporadic amyotrophic lateral sclerosis.
[a] Calculated on 475 cases.
[b] The 2 cases with *FUS* and *OPTN* missense mutations are not indicated in the table; both patients had a spinal phenotype and were cognitively normal.

ALS cases and 19.6% of all mutations. The following missense mutations were identified: c59A>G (p.N19S) (1 sALS), c.115C>G (p.L38V) (1 fALS), c.142G>T (p.V47F) (1 fALS), c.271G>A (p.D90N) (1 sALS, in heterozygosis), c.272A>C (p.D90A, in heterozygosis) (1 sALS), c.281G>A (p.G93D) (2 fALS), c.328 G>T (p.D109Y) (2 unrelated sALS cases), and c.435G>C (p.L144F) (1 sALS). Patients with *SOD1* mutations had a mean age at onset of 54.4 years (SD 20.3) (range 16–85; median 57.0, interquartile range 44.7–70.7). Only 1 patient had a bulbar onset. No patients with *SOD1* mutations had cognitive impairment.

***TARDBP* mutations.** Seven patients carried a missense mutation of the *TARDBP* gene, accounting for 1.5% of all ALS cases and 13.7% of all mutations. The most common *TARDBP* mutation was the c.1144G>A (p.A382T) missense mutation (1 fALS and 3 sALS). Other identified mutations were c.1009A>G (p.M337V) (1 fALS), c.1102G>A (p.G368S) (1 sALS), and c.1169 A>G (p.N390S) (1 sALS). Patients with *TARDBP* mutations had a mean age at onset of 64.4 years (SD 6.1) (range 52–70, interquartile range, 60.0–68.2). Three out of the

7 patients (42.9%) had a bulbar onset and 3 (42.9%) presented with bvFTD.

***FUS* mutations.** Only 1 patient had the c.1542G>C (p.R514S) missense mutation of the *FUS* gene. A detailed description of this patient's clinical picture and pedigree has been reported elsewhere.[26]

***OPTN* mutations.** We detected 1 patient with fALS carrying the never previously described c.1499T>C (p.L500P) missense mutation in the exon 14 of the *OPTN* gene. This mutation was predicted to be pathogenic by 2 different programs, Polyphen2 (http://genetics.bwh.harvard.edu/pph2) and PMut (http://mmb.pcb.ub.es/PMut/).

fALS with no identified mutations. Fifteen patients had a positive family history for ALS, but did not carry mutations of one of the major ALS genes. These patients had a mean age of 62.1 years (SD 9.2; range 47–80; median 61.3, interquartile range 55.1–68.0). Only 2 patients (13.3%) had bvFTD, and 3 (33.3%) had a bulbar onset.

Pedigrees of apparently sporadic patients carrying genetic mutations. In the pedigrees of the 20 apparently sporadic patients with *C9ORF72*, *SOD1*, and *TARDBP* mutations, no relatives with dementia, Parkinson disease, or other neurodegenerative disorders were detected.

DISCUSSION In our study, we analyzed the genetics etiology of a large epidemiologic series of patients with ALS. The patients were prospectively identified through the Piemonte and Valle d'Aosta register for ALS during a period of 4.5 years and ~80% of all identified cases underwent genetic testing. More than 10% of the tested patients carried a mutation of one of the major ALS genes, with *C9ORF72*

Table 3	Frequency of FTD among patients with *C9ORF72* hexanucleotide repeat expansion according to the presence of a family history for ALS, FTD, or both (*p* = 0.008)		
	FTD, n (%)	Normal cognition, n (%)	Overall, n (%)
fALS	16 (94.1)	8 (53.3)	24 (75)
sALS	1 (5.9)	7 (46.7)	8 (25)
fALS and sALS	17	15	32

Abbreviations: ALS = amyotrophic lateral sclerosis; fALS = familial ALS; FTD = frontotemporal dementia; sALS = sporadic ALS.

hexanucleotide expansion being the most commonly identified genetic mutation.

Only one population-based study on ALS genetics has been performed to date in one of the provinces of Piemonte in the period 2000–2005.[16] In that article, 5.7% of the 386 patients with ALS had a positive family history for ALS and 5 patients (1.3%) (2 of whom were apparently sporadic) carried a *SOD1* mutation. In the clinical-based series that have compared the frequencies of various ALS genes,[11–15] the most frequent mutations have been found in the *SOD1* gene (5% to 12% of cases) followed by *TARDBP* and *FUS* (2% to 4% of cases). In the only study having also assessed *C9ORF72*, this was the commonest mutated gene.[15] All but one of these studies included only patients with fALS. The higher mutational rates observed in these studies compared to ours most likely arose from their use of patients referred to ALS clinics. Such referral cohorts are known to be biased toward the inclusion of younger patients who are more likely to carry a genetic mutation.[16]

The frequency of fALS in the Piemonte population is unexpectedly higher than that reported by previous epidemiologic studies,[1,2] with the exception of certain genetically conserved populations, such as Finnish,[10,27] Northern Swedish and Northern Norwegians,[28] and Sardinians.[29] Using the restrictive criteria for fALS (i.e., the presence of a first- or second-degree relative with ALS),[23] the frequency of fALS in our series was 7.6%, higher than the 5.1% reported in a meta-analysis of published studies.[2] This difference is probably related to the systematic collection of patients' pedigrees in the Piemonte register after 2007 as well as to the existence of a historical archive of ALS cases diagnosed in the Piemonte region going back to the 1950s which allows us to search for surnames recurring in patients' pedigrees and to identify cases according to the communities of origin.

The recent identification of *C9ORF72* as a major cause of both ALS and FTD and the frequent identification of apparently sALS carrying genetic mutations challenges the classic definition of fALS.[2,20] Based on this, we broadened the criteria for fALS to include patients with *C9ORF72* hexanucleotide repeat expansions and one first- or second-degree relative with FTD. With these broader criteria, the frequency of fALS increased to 9.7% of the whole series. We believe that this rate represents a more realistic estimation of familial disease within the ALS population. The fact that two-thirds of patients meeting these criteria for fALS had a gene detected strongly supports our use of more inclusive criteria than those previously proposed.[23]

C9ORF72 hexanucleotide repeat expansions have been found in 6.7% of all ALS cases and represent the commonest mutations in this epidemiologic population of Italian ancestry. About two-thirds of these cases have a positive family history for ALS or FTD. In keeping with the first clinical descriptions of *C9ORF72*,[21,22,30–32] patients carrying this mutation are younger than those without genetic mutations and about half of them have a bvFTD. The high frequency of positive family history for FTD in these cases agrees with the findings of 2 recent articles.[20,30] It is still unclear whether PCR is sufficient for diagnosis of *C9ORF72* mutations in the clinical setting. Moreover, accurate measurements of the repeat expansion size by Southern blot analysis are necessary to making genotype-phenotype correlations in patients with this mutation.[22]

The frequency of *SOD1* mutations, 19.6% of all mutations and 2.1% of all fALS cases, is in keeping with the frequency observed in the same area in the 2000–2005 period.[16] About half of the patients carrying *SOD1* mutations were apparently sporadic and none of the cases with *SOD1* mutations had FTD.[33] The pathogenicity of some *SOD1* missense mutations has been questioned.[34] However, most mutations in our series are recognized pathogenic mutations and the others are likely to be mutations with a reduced penetrance.[35]

The third most common gene was *TARDBP*, identified in 1.5% of all ALS cases. The commonest *TARDBP* mutation was p.A382T, identified in 3 cases of Sardinian ancestry and 1 case of Sicilian ancestry. We have previously published that this particular founder mutation is highly prevalent on Sardinia.[29] About half of the cases carrying *TARDBP* mutations had a bvFTD, a relatively common feature in association with *TARDBP* mutations.[29]

Not surprisingly, we found no putatively pathogenetic mutations of *ANG*. In fact, a recent meta-analysis reported that *ANG* mutations have been detected in only ~0.5% of patients with ALS of European ancestry[36] and the frequency was even lower in Italian patients.[37–40] Although it remains possible that our cohort was not sufficiently powered to detect *ANG* mutations, our data confirm that mutations in this gene are not a frequent cause of motor neuron degeneration.

Overall, ALS cases carrying genetic mutations had some clinical peculiarities. First, the median age at onset of patients with genetic mutations was significantly lower than that of patients without genetic mutation. As a consequence, the probability of carrying a genetic mutation is inversely related with the age at onset of patients: the younger the age at onset, the higher the likelihood of having a genetic muta-

tion. Second, 19 (21.8%) out of the 87 of patients with a comorbid FTD carried a genetic mutation (either *C9ORF72* or *TARDBP*) compared to only 31 (8.0%) out of the 357 patients with normal cognition. Logistic regression analysis also demonstrated that comorbid FTD increased the chance of having a mutation by 3.5 and an age at onset <55 by 1.6. Therefore, our data, if confirmed with other studies, might suggest to offer the testing also to patients with comorbid FTD and those younger than 55, in addition to patients with a clear positive family history for ALS or FTD, who are obvious candidates for genetic testing.

A limitation of this study is that ~20% of cases did not undergo genetic analysis. However, cases who were not captured did not differ on any relevant clinical or demographic characteristic from those who were included in the study.

This epidemiologic study has found that at least 10% of patients with ALS carry a genetic mutation of one of the major ALS genes, with *C9ORF72* being the commonest genetic alteration in this Italian mainland population. We found that the presence of comorbid FTD or a young age at onset are strong indicators of a possible genetic origin of the disease, with possible implication both for future genetic studies and for genetic counseling in ALS.

AUTHOR CONTRIBUTIONS

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DISCLOSURE

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