

Phenotypic Heterogeneity in a *SOD1* G93D Italian ALS Family: An Example of Human Model to Study a Complex Disease

Silvana Penco · Christian Lunetta · Lorena Mosca · Eleonora Maestri ·
Francesca Avemaria · Claudia Tarlarini · Maria Cristina Patrosso ·
Alessandro Marocchi · Massimo Corbo

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Abstract We report different clinical expression in seven members of a large family with amyotrophic lateral sclerosis (ALS) and the G93D mutation in exon 4 of the Cu/Zn superoxide dismutase (*SOD1*) gene. The ALS clinical course in the proband showed an unusually fast progression of the disease compared to the paucisymptomatic presentation associated to this mutation in the two previously Italian families described. The remaining mutation carriers did not show the aggressive clinical course displayed by the proband. We selected few genes known to be ALS modifiers searching for genetic variants that could explain the wide phenotypic diversity within the family. Exclusion of causative genes such as TDP43, FUS, PGRN and VAPB was performed too. We believe that this kind of family with contrasting phenotypes of ALS may be considered an excellent human model to study the relationship between a wider genetic profile, including modifier genes, and the clinical expression of the disease. Therefore, the novelty of our approach is also represented by the study of a single family to reproduce a composite structure in which search for possible modifier genes/genetic variants linked to *SOD1* mutated.

Keywords Complex disease · FALS · Genetic variations · Human model · *SOD1* mutation

Introduction

Amyotrophic lateral sclerosis (ALS), the most common form among motoneuron diseases, is a progressive neurodegenerative disorder involving motor neurons in motor cortex, brain stem, and spinal cord. Ninety percent of patients are sporadic (SALS), and almost 10% are familial (FALS), with multiple autosomal dominant and recessive forms. Currently, three major ALS genes and five additional loci have been identified (Valdmanis and Rouleau 2008). The first ALS gene (ALS1) associated with adult-onset autosomal dominant form of the disease was mapped to the chromosome 21q22.1–22.2 and encodes for the cytoplasmic Cu/Zn superoxide dismutase (*SOD1*) (Rosen et al. 1993). Recently, mutations in TAR-DNA binding protein 43 (*TARDBP*) located on chromosome 1p36.22 have been reported to be associated to familial cases as well as sporadic cases (Kabashi et al. 2008; Sreedharan et al. 2008). In addition, mutations in the *FUS/TSN* gene on chromosome 16 are present in familial ALS (Kwiatkowski et al. 2009; Vance et al. 2009) as well as in sporadic cases (Corrado et al. 2010). Nevertheless, *SOD1* still remains the major gene involved in the disease since approximately 20% of FALS cases and 2% of overall ALS cases have identifiable mutations. At the present time, more than 120 different mutations located in all five exons of the gene have been identified worldwide in ALS patients (<http://www.alsod.org/>). All *SOD1* mutations are autosomal dominantly inherited except D90A and D96N. The latter was found in compound heterozygosity with D90A in a

S. Penco (✉) · L. Mosca · F. Avemaria · C. Tarlarini ·
M. C. Patrosso · A. Marocchi
Department of Laboratory Medicine, Medical Genetics,
Niguarda Ca' Granda Hospital,
Milan, Italy
e-mail: silvana.penco@ospedaleniguarda.it

C. Lunetta · E. Maestri · M. Corbo
NEuroMuscular Omnicentre, Fondazione Serena Onlus,
Niguarda Ca' Granda Hospital,
Milan, Italy

recessive FALS (Andersen et al. 1995; Robberecht et al. 1996; Hand et al. 2001).

Although some genotypes are associated with a consistent phenotype, considerable phenotypic heterogeneity, especially in terms of age at onset, duration of the disease, and severity, exists among family members carrying the same mutation (Kim et al. 2007), and it could be related to the influence of undetermined modifier genes. Recently, two Italian families have been described carrying the G93D *SOD1* mutation. Both the described families presented a slowly progressive disease with a long lasting paucisymptomatic phase (Luigetti et al. 2008; Restagno et al. 2008).

We describe a family with G93D *SOD1* gene mutation characterized by wide variability of disease expression among family members and with the proband showing a fast progressing motor neuron disease compared to the G93D carriers within her family and the two previously described families. In order to search for possible modifier genes explaining the clinical phenotype observed in the proband, we performed an analysis of angiogenin gene (*ANG*) and genetic variants (paraoxonase 1, *PON1*; paraoxonase 2, *PON2*; vascular endothelial growth factor, *VEGF*; methylenetetrahydrofolate reductase, *MTHFR*; apolipoprotein E, *APOE*; hemochromatosis, *HFE*) reported to be associated to ALS (Figlewicz and Orrell 2003). Other genes known to be causative of ALS such as TDP43, *FUS*, progranulin (*PGRN*), and vesicle-associated membrane protein-associated protein B and C (*VAPB*) were also analyzed to exclude their possible role (Millecamps et al. 2010).

Patients and Methods

Pedigree of the family is shown in Fig. 1. We studied ten family members from a two-generation pedigree with three members affected by FALS: a 60-year-old female (III:10), her uncle (II:3 died and referred to be affected) and her niece (IV:7), respectively. ALS diagnosis related to (II:3), for whom clinical records were not available, was obtained from his descendent.

Genetic Analysis

DNA was extracted from peripheral blood leukocyte (Miller et al. 1988). Written informed consent was obtained from all the participants. Genetic analysis was performed with two different approaches: direct sequencing and single nucleotide polymorphisms (SNPs) analysis.

DNA Sequencing SOD1, ANG, TDP43, FUS, PGRN and VAPB genes were amplified by PCR with a specific subset of primers described elsewhere (Leverenz et al. 2007; Millecamps et al. 2010). Direct DNA sequencing included coding regions as well as splice site regions; the 600-bp promoter region analysis was included for the *ANG* gene (Corrado et al. 2007). The analysis was performed with an ABI 3730 automated sequencer (Applied Biosystems, UK).

Genetic Variants Analysis The following genetic variants were analyzed through specific PCR amplifications followed by enzymatic restriction analysis: *PON1* L55M (rs854560) and Q192R (rs662); *PON2* S311C (rs6954345); and *VEGF*

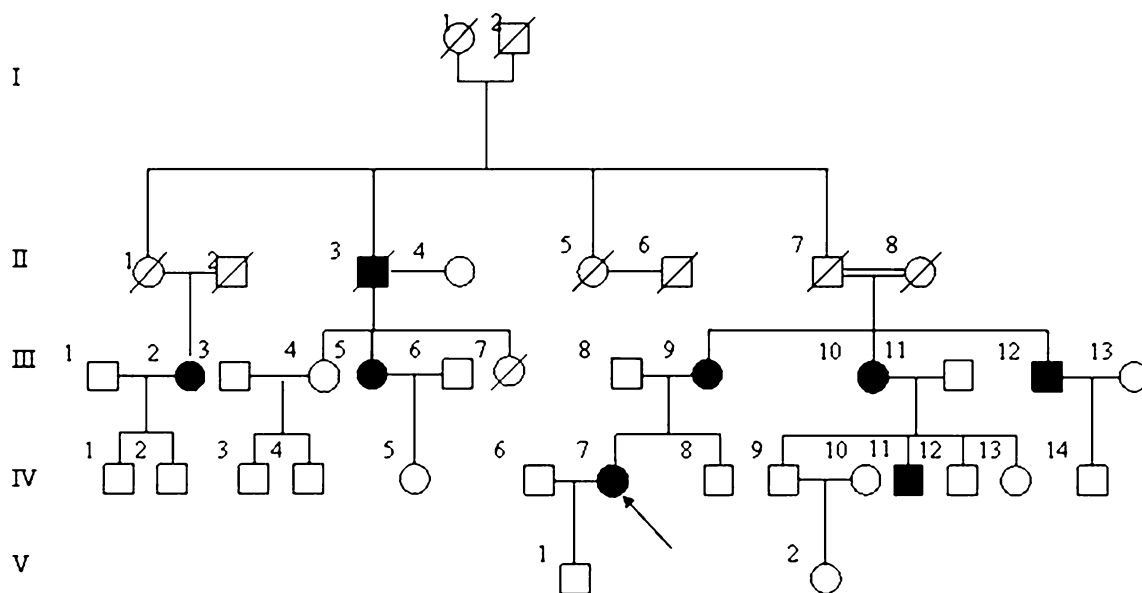


Fig. 1 Pedigree of the *SOD1* G93D Italian ALS family. All individuals carrying the mutation, with or without phenotype, are filled in. Arrow indicates index patient; diagonal line, individual deceased; horizontal double line, consanguineous marriage

promoter variants -2578C/A (rs699947), -1154A/G (rs1570360), and -634C/G (rs2010963) (Ricci et al. 2010). We applied the LightCycler technology (Roche Molecular Biochemicals, Milan) for the typing of the following genetic variants according to PCR protocol already set out: C677T (rs1801133) mutation in the *MTHFR*; combinations of the codons 112 Cys/Arg (rs429358) and 158 Cys/Arg (rs7412) in the *APOE* gene determining the haplotype E2/E3/E4; and genetic variants C282Y, H63D, and S65C in the *HFE* gene.

Results

Molecular Analysis

The obtained results are summarized in Tables 1 and 2. We identified seven G93D (included the index patient) and three G93G (equal to wild type) subjects within the family. The whole *SOD1* gene, screened in all the tested subjects, identified the IVS3 + 34 A/C in both the mutated and not mutated cases (IV:11 and IV:9, respectively): this variant has been already reported not associated to ALS (Battistini et al. 2005). The *ANG* gene analysis revealed the presence of three variants, the G110G (rs11701) in the coding region, and the IVS1+27C/T plus -127T/C in the regulatory regions. The IVS+27C/T variant was found in the index patient and in her father (IV:7 and III:8, respectively; see Table 1). Allele frequency of *ANG* variations has been previously determined in a healthy local population (Corrado et al. 2007). *TDP43*, *FUS*, *PGRN* and *VAPB* genes were negative for mutation screening, as expected as causative genes.

The other genetic variants analyzed (*PONs*, *VEGF* promoter, *HFE*, *MTHFR*, and *APOE*) showed a wide distribution among the family member tested (see Table 2).

Combination of single genotypes revealed the presence of a unique haplotype in the index patient (Table 1).

Clinical Characteristics of Symptomatic G93D *SOD1* Mutation Carriers

Case IV:7 A previously healthy 33-year-old pregnant woman, unipara, presented with subacute, severe, left lower limb pain the day after the normal delivery, followed by relentless progressive weakness and wasting of the left leg. At birth, the newborn was healthy. Later, she developed a progressive worsening of the weakness with involvement of her four limbs and bulbar district, and complained a severe respiratory failure after 2 years. After 5 years, the patient is still alive, wheelchair-bound, in ventilatory assistance by tracheostomy and in enteral nutrition by gastrostomy. Her family, which included her 55-year-old mother (III:9), 60-year-old father (III:8), and a 26-year-old brother (IV:8), had positive history for motoneuron disease (III:10 and II:3); no clinical information were available for the proband’s grandfather (II:7). On June 2008, the patient was admitted at NEMO and her neurologic examination revealed severe weakness (ranging from grade 1/5 to 2/5 at the Medical Research Council (MRC) scale) of both upper and lower limb muscles; all deep tendon reflexes were reduced, and plantar reflexes were flexor bilaterally. Hoffmann sign was negative bilaterally, and jaw jerk reflexes were normal. MRI of the spine was negative. Needle electromyography (EMG) showed increased insertional activity, spontaneous activity (fibrillation potentials and positive sharp waves), increased amplitude, and long duration motor unit action potentials, associated with reduced recruitment in all limbs. Fasciculation potentials were diffusely present. The study was interpreted as consistent with lower motor neuronopathy. Comprehensive laboratory studies were normal, except

Table 1 Results of molecular analysis in all the tested family members (affected and non-affected) for *SOD1* (amino acid nomenclature) and *ANG* (nucleotide nomenclature) genes analysis by direct sequencing. Negative results of *TDP43*, *FUS*, *PGRN*, and *VAPB* are not shown

Subjects	<i>SOD1</i>	<i>ANG</i>		
		-127T/C	IVS1+27C/T	rs11701
IV:7	G93D	T/C	C/T	T/G
IV:11	G93D, IVS3+34 A/C	T/T	C/C	T/T
III:10	G93D	T/T	C/C	T/T
III:12	G93D	T/C	C/C	T/G
III:9	G93D	T/C	C/C	T/G
III:2	G93D	T/C	C/C	G/G
III:5	G93D	T/T	C/C	T/T
III:4	G93G	T/T	C/C	T/T
IV:8	G93G	T/T	C/C	T/T
IV:9	G93G, IVS3+34 A/C	T/T	C/C	T/G
III:8	G93G	T/T	C/T	T/T

Table 2 Results of molecular analysis in all the tested family members for *PON1*, *PON2*, and *VEGF* by enzymatic restriction procedures and for *HFE*, *MTHFR*, and *APOE* genetic variants analysis by LightCycler technology

Subjects	<i>PON1</i>		<i>PON2</i>	<i>VEGF</i>			<i>HFE</i>		<i>MTHFR</i>	<i>APOE</i>
	L55M	Q192R	C311S	-2578C/A	-1154G/A	-634G/C	C282Y	H63D	C677T	E2/E3/E4
IV:7	L55M	Q192R	S311S	C/A	G/G	C/G	C282C	H63H	C/C	E3/E3
IV:11	L55M	Q192Q	S311S	A/A	G/G	G/G	C282C	H63H	C/T	E3/E3
III:10	L55L	Q192Q	S311S	C/A	G/G	G/G	C282C	H63H	C/T	E3/E3
III:12	L55L	Q192Q	S311S	C/A	G/G	G/G	C282C	H63H	C/T	E3/E3
III:9	L55M	Q192R	S311S	C/A	G/G	C/G	C282C	H63H	C/T	E3/E3
III:2	L55M	Q192R	S311S	C/A	G/G	G/G	C282C	H63D	C/T	E3/E3
III:5	L55M	Q192R	C311S	A/A	G/A	G/G	C282C	H63H	T/T	E3/E4
III:4	L55M	Q192Q	S311S	A/A	G/A	G/G	C282C	H63H	T/T	E3/E3
IV:8	L55L	Q192R	S311S	C/A	G/G	G/C	C282C	H63H	C/T	E3/E3
IV:9	L55L	Q192R	S311S	A/A	G/G	G/G	C282C	H63H	C/T	E3/E3
III:8	L55M	Q192R	S311S	C/A	G/G	C/G	C282C	H63H	C/C	E3/E3

Amino acid nomenclature for *PON1*, *PON2*, and *HFE* and nucleotide nomenclature for *VEGF* and *MTHFR* are reported; for *APOE*, the haplotype is shown

mild creatine kinase (CK) level increment (175 U/L; normal value, 30–150 U/L). ALS Functional Rating Scale-revised (ALSFRS-r) score was 11.

Case III:10 A 60-year-old female was admitted to our center for a 4-year history of fasciculations in all four limbs and bilateral hand weakness. Neurological examination of the patient revealed diffuse fasciculations in both upper and lower limbs with bilateral muscular weakness (grade 3/5 on the MRC scale) and atrophy of both hands; tendon reflexes in upper and lower limbs were reduced. Hoffmann, Babinski, or Chaddock signs were not present. Laboratory tests revealed mild CK level increment (160 U/L). EMG of tibialis anterior, biceps brachii, and first dorsal interosseous bilaterally showed a neurogenic pattern: high frequency large motor unit potentials, sporadic denervation (fibrilla-

tion potentials, positive waves), and fasciculations. Transcranial magnetic stimulation of the motor cortex revealed normal central conduction time. Forced vital capacity as percent predicted was 96%. ALSFRS-r score was 45.

Clinical characteristics of all G93D *SOD1* mutation carriers are summarized in Table 3.

Discussion

We report a detailed profile of clinical and genetics findings of members of an Italian family carrying the G93D *SOD1* mutation with different phenotypic expression. In particular, to explore the phenotypic heterogeneity within the family, we investigated the possible relationship between

Table 3 Clinical characteristics of G93D *SOD1* mutation carriers

Subjects	Clinical characteristics
III:2	58 year-old; female; asymptomatic; normal neurological examination; mild increment of CK serum level (160 U/L; normal value, 30–150 U/L)
III:5	45 year-old; female; cramps and fasciculations; normal neurological examination; normal CK serum level
III:9	58 year-old; female; cramps and fasciculations; normal neurological examination; mild increment of CK serum level (158 U/L)
III:10	60 year-old; female; hand weakness and hypotrophy; mild increment of CK serum level (162 U/L)
III:12	51 year-old; male; asymptomatic; normal neurological examination; normal CK serum level
IV:7 (proband)	38 year-old; female; tetraplegic, in ventilatory assistance by tracheostomy and in enteral nutrition by gastrostomy; mild increment of CK serum level (175 U/L)
IV:11	27 year-old; male; asymptomatic; normal neurological examination; normal CK serum level

the *SOD1* mutation and some modifier genes reported to be involved in ALS pathogenesis. Various genes/genetic variants could have been tested; however, we decided to focus on a few of them. Since the involvement of angiogenic factors in ALS, albeit controversial, is still attractive (Lambrechts et al. 2006), we selected *ANG* and *VEGF* SNPs for this study. Considering the important role of oxidative stress in motor neuron degeneration (Barber and Shaw 2010), we looked for *HFE* gene variants, in particular for H63D allele found associated with ALS (Restagno et al. 2007; Sutedja et al. 2007). *PON* genes have been studied as susceptibility factor to sporadic ALS (Slowik et al. 2006). However, association studies between *PON* genetic variants and the risk for SALS in different populations reported conflicting results (Wills et al. 2009). We have recently studied *PON* polymorphisms in an Italian population negative for *SOD1* mutations and no significant association was observed between the examined SNPs and the risk of ALS (Ricci et al. 2010). Despite that, point mutations in *PON* genes have been identified in FALS and SALS (Ticozzi et al. 2010). *MTHFR* C677T variant was selected because linked to an increased level of homocysteine (Hcy) and, in preliminary studies, ALS subjects showed higher median Hcy levels compared to age- and sex-matched controls (Valentino et al. 2010); higher Hcy levels were also correlated with a possible marker of disease progression (Zoccolella et al. 2010). Finally, alleles E2, E3, and E4 of *APOE* gene were included in our analysis because of their association with some neurodegenerative disorders, such as ALS (Zetterberg et al. 2008).

Results of our study did not disclose haplotypes common to G93D mutation carriers or to wild-type subjects. As unique characteristic of the proband (IV:7), we identified the *ANG* IVS1+27 variant in heterozygous state C/T. This genetic variation has been previously described, although no association with ALS has been reported in a *SOD1* negative population (Corrado et al. 2007). To date, little information about the role of *ANG* variants in a *SOD1* mutated genetic context are available. In the present family, the proband (IV:7) manifested the disease after the delivery that physiologically could determine a hypoxic condition: *ANG*, like *VEGF*, is expressed in response to hypoxia (Campo et al. 2005; Nakamura et al. 2006) and plays a neuroprotective role in motoneurons (Subramanian et al. 2008). Thus, we might speculate that the patient's genetic framework, including the G93D mutation and the IVS1+27 variation, together with the hypoxic status linked to the delivery facilitated the anticipation of onset age and contributed to the aggressive clinical course observed.

The G93D *SOD1* mutation has been previously described in two Italian families (Luigetti et al. 2008; Restagno et al. 2008) and associated with a slowly

developing motor neuron disease; this mutation was reported to confer a high propensity of protein aggregation too (Stathopoulos et al. 2006). On the other hand, the G93A *SOD1* mutation has been shown to cause the protein exclusion from the nucleus with consequent reduction of DNA protection from oxidative damage, thus underlying the important role of the amino acidic position at 93 in the protein (Sau et al. 2007).

The innovation of our approach consists in studying a family as a model to search for possible modifier genes/genetic variants linked to *SOD1* mutations. A similar approach was introduced by Broom WJ et al. (Broom et al. 2006), which looked for variants in candidate ALS modifier genes linked to Cu/Zn superoxide dismutase explaining divergent survival phenotypes. DNA of four genes within the haplotypic region shared in A4V and D90A ALS patients was sequenced, and 15 novel variants were identified, but none resulted specifically associated with *SOD1* D90A/D90A or *SOD1* A4V ALS.

In conclusion, the current study describes ten family members, seven of which with G93D *SOD1* mutation, and represents an attempt at using the whole family group as human model to identify putative modifier genes responsible for different intrafamilial phenotypic expression.

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