Genetics of amyotrophic lateral sclerosis

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Received June 19, 1996

Amyotrophic lateral sclerosis (ALS) is a paralytic disorder caused by degeneration of motor neurons in the brain and spinal cord. Identification of mutations in the gene for Cu,Zn superoxide dismutase (SOD1) in a subset of ALS families made it possible to develop a transgenic mouse model of ALS and to investigate its pathogenesis. These investigations suggest that mutant SOD1 acts through a toxic gain of function which may involve generation of free radicals. Conformational change in the mutant SOD1 protein, especially the distortion of the 'rim' of the electrostatic guidance channel may be central to this toxic gain of function and to the pathogenesis of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also called motor neuron disease (MND) is an age-dependent and fatal paralytic disorder, caused by degeneration of motor neurons in the motor cortex, brainstem and spinal cord. ALS usually begins asymmetrically with involvement of the muscles of one or more limbs, speech or deglutition in middle adult life. The involvement appears to spread in an anatomically contiguous manner and eventually becomes bilateral and symmetrical, progressing to paralysis and death. Mean survival is 3 years after the onset of symptoms (1).

ALS occurs as an apparently sporadic disease (SALS) in 90% of cases; however, in 10% of ALS patients, a family history of the disease can be documented and this subset of patients is classified as familial ALS (FALS). Multigenerational familial cases are usually genetic, but complex environmental and genetic interaction cannot be ruled out in duplex clusters. Genetically inherited FALS is transmitted either as an autosomal dominant (DFALS) or an autosomal recessive (RFALS) trait (2,3).

DFALS is the most common form of FALS and is clinically indistinguishable from SALS. The mean age at onset of symptoms is 46 years which is about 10 years earlier than SALS (4,5). Studies of ALS families suggest that the penetrance of DFALS is age-dependent: 50% of the gene carriers develop disease by the age of 46 years and 90% by the age of 70 years (5). Although the age at onset of symptoms within DFALS families is commonly variable, no apparent genetic anticipation has been observed (6).

Current ALS research is focused on chromosome 21-linked FALS because of the identification of mutations in the gene for Cu,Zn superoxide dismutase (*SOD1*) (7,8). In this review we will summarize the most recent developments in FALS research which may give insight into the possible mechanisms of pathogenesis underlying this disease.

FALS AND SUPEROXIDE DISMUTASE

Chromosomal mapping of a locus for FALS on chromosome 21q22.1 was an important development as it directly led to the

identification of mutations in the gene for Cu/Zn superoxide dismutase (*SOD1*) as a hitherto undiscovered cause of ALS (9). The power of linkage analysis is demonstrated by the fact that even though SOD1 is a well studied and characterized ubiquitous enzyme, the connection between ALS and SOD1 had not been made until genetic linkage was established (9). The other two isoforms of SOD are MnSOD (SOD2), which maps to 6q25 and is primarily located in mitochondria and extracellular SOD (SOD3), which was mapped to 4p15.2 (H-X. Deng and T. Siddique, unpublished result) and is primarily present in extracellular fluid. These two genes are not linked to FALS.

SUPEROXIDE AND CU/ZN SUPEROXIDE DISMUTASE

Superoxide (O_2^{-}) is an unstable and highly active molecule which causes oxidation of cell constituents either directly or through toxic and stable derivatives. O_2 - is produced during normal aerobic metabolism. The major superoxide dismutase activity in cytoplasm is from SOD1, which consists of 153 highly conserved amino acids with a molecular weight of 16 kDa. SOD1 is a homodimer and within each monomer there is a cave-like active site containing one atom each of copper and zinc. The major known function of this enzyme is to detoxify O_2 - to form O₂ and hydrogen peroxide. H₂O₂, in turn, is detoxified to form water by either glutathione peroxidase or catalase. The dimer interface of SOD1 is strongly hydrophobic resulting in a stable dimer formation; dimerization doubles the dismutase activity of SOD1. O_2 ⁻ is guided to the Cu²⁺ containing active site through a positively charged electrostatic guidance channel, the positive charges being provided by the amino acids Lys122, Lys136 and Arg143. The latter allows the docking of the negatively charged O_2 ⁻ in close proximity to the copper atom. Twenty-one highly conserved amino acid residues coded by exons 3 and 5 contribute to the active channel. The channel narrows down in a stepwise fashion from a large shallow depression about 24 Å across to a deeper well about 10 Å wide and 5 Å deep, which further narrows to less than 4 Å above the copper atom (10,11). The dismutase reaction proceeds at a very rapid rate of 2×10^9 M/s and is most

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likely only limited by substrate (12). The electrostatic guidance channel thus provides optimum access to an otherwise small surface area constituting the active site. Access to the active site is limited by size and charge which favors the negatively charged O_2 ⁻ and excludes molecules of larger size. The two steps dismutase reaction proceeds as follows:

$$O_2^{\cdot -} + Enz - Cu^+ + H^+ \rightarrow O_2 + Enz - Cu^+$$
(i)

 $O_2`^+ Enz-Cu^+ + H^+ \rightarrow H_2O_2 + Enz-Cu^{2+}$ (ii)

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD1}} H_2O_2 + O_2$$
(iii)

Beside the dismutase activity, SOD1 has a marginal peroxidase activity (13-15) and a role in Cu and Zn homeostasis has also been attributed to SOD1. H₂O₂, one of the products of the dismutase reaction is thought to inactivate SOD1 (16). The inactivation of SOD1 by its own H₂O₂ production and peroxidase activity is as follows:

 $H_2O_2 + Enz-Cu^{2+} \leftrightarrow O_2^{-} + Enz-Cu^{+} + 2H^{+}$ (iv)

$$Enz-Cu^{+} + H_2O_2 \leftrightarrow Enz-Cu^{2+} \cdot OH + OH^{-}$$
(v)

 $Enz-Cu^{2+}-OH + ImHis \rightarrow Enz-Cu^{2+} + - ImHis + H_2O$ (vi)

Enz is the SOD1 enzyme and ImH is the imidazole moiety of histidine residue at the active site of the SOD1 polypeptide. *In vivo*, the peroxidase activity could be significant in the presence of an excess production of H_2O_2 which may result from a burst of O_2 ⁻ molecules (15).

SOD1 AND CHROMOSOME 21 LINKED FALS

Forty-nine mutations in SOD1 gene have been identified in FALS families linked to chromosome 21q (Table 1). These mutations segregate with the disease (8). Among these 49 mutations, there are 45 missense mutations, a stop codon mutation (H-X. Deng and T. Siddique, unpublished data), a deletion mutation and two intronic mutations in intron 4 which alter the splicing site. One of the intronic mutation changes an A to G, 11 bp upstream of the splicing site (H-X. Deng and T. Siddique, unpublished data). The other intronic mutation changes a T to G, 10 bp upstream of the splicing site (17). All of the reported mutations are in exons 1, 2, 4 and 5, but not in exon 3, which contributes to the rim of the electrostatic guidance channel and the Cu and Zn binding sites.

When the initial mutations identified in FALS were mapped to the crystallographic structure of SOD1, they were found to be clustered in the loops joining the β barrel (8). The mutated side chains cluster near the two Greek key connections closing off the ends of the β barrel, in the dimeric interface and at the base of the active side loop. These side chains, which are structurally conserved in the wild-type SOD1 structure from different species, appear to be critical for the structural integrity of this dimeric enzyme. Some of the side chain mutations, such as A4V, L38V, L106V and V148A, would also be expected to destabilize the subunit folding or the dimer contact. Three mutations, H46R, L84V and D90A, occur outside of the β strand and the loop forming the β barrel. The mutations H46R and H48Q directly affects the ligation of Cu atom in the protein, as H46 and H48 are two of the four histidines that bind to Cu in SOD1. The mutation L84V occurs at the end of the Zn binding loop where it joins the β barrel, while D90A is expected to have only local effects and cause few changes in the protein structure (8,18,19). Most mutations in *SOD1* were predicted to destabilize the protein, resulting in a less active, rather than an inactive SOD1 protein. D90A is expected to be relatively stable without apparent decrease in enzyme activity (8,18,19).

The analysis of the SOD1 enzyme activity supports the structural prediction of loss of activity in most instances due to structural instability. The cytosolic superoxide dismutase activity assayed in red blood cells, transformed lymphoblastoid cells and brain tissues from FALS patients with mutations in SOD1 gene, was found to be between 30 and 70% of normal activity (8,20,21). FALS patients homozygous for D90A mutation had a near normal SOD1 activity (19,22). The intrinsic activity of mutated SOD polypeptide expressed in COS-1 cells and in yeast, was between 0 and 70% of the normal polypeptide (23-25). These observations support the hypothesis that a decrease in SOD activity in FALS patients may result from instability of the mutant and wild-type heterodimers and mutant homodimers. The random dimerization of mutant and wild-type monodimers would predict that 25% of the SOD are wild-type homodimers, 25% are mutant homodimers and 50% are mutant/wild-type heterodimers. However, the mutant SOD1 monomers were not found to have a dominant negative effect on the dismutase activity of normal monodimers. A contrary result was shown in mutant SOD1 from Drosophila (26). The effect of a mutation may differ from mutation to mutation. Some mutations may have dominant negative effects but not all.

Another mechanism for the reduction in SOD1 activity in the red blood cells of FALS patients is due to the destabilized SOD1 protein, resulting in a shorter half life of mutant SOD1 protein. For example, in a COS 1 cell culture system, the half life for A4V is about 7 h, while the normal SOD1 protein is about 30 h (23). Quantitative analysis of SOD1 protein in FALS patients showed a reduction in SOD amount by 35–75% of the normal value (Siddique T *et al.*, unpublished data); no decrease in SOD1 protein was observed in non-chromosome 21 FALS patients.

Different mutations in *SOD1* do not seem to have significant influence on the age at onset of symptoms. Fifty percent of FALS patients develop symptoms by 46 years of age. However, different mutations do have different effects on the progression of the disease. The mean duration for FALS patients with A4V mutation is 1.2 years which is significantly shorter than the mean duration observed in patients with E100G mutation which is that of 4.7 years (27,28). Duration for most of the FALS patients with SOD1 mutations range from 3 to 5 years; while H46R and G37R have the longest duration of about 18–20 years (18,27).

Two mutations are worth mentioning due to their specificity. D90A mutation was previously described as a polymorphism with a relatively high frequency of 2.5% in the northern Swedish/Finnish population; ALS is not significantly high in this population (29). It has been recently observed that homozygotes for the D90A mutation develop ALS (19,22). The penetrance is estimated to be 38%, suggesting that D90A is a recessive mutation for ALS (22). Another interesting mutation, I113T, has been reported in several apparently sporadic cases. We identified two apparently sporadic ALS cases with the I113T mutation. A detailed family history and analysis of family DNA indicated that this mutation was actually inherited. The D90A and I113T mutations suggest that some cases of SALS are genetic due to a low penetrance of the inherited gene, strongly arguing for modifying factors.

Table 1. SOD1 mutations in FALS

Amino acid substitution	Mutation symbol	Nucleotide substitution	Reference
Exon 1			
Ala4→Val	A4V	GCC→GTC	8
Ala4→Thr	A4T	GCC→ACC	56
Val7→Glu	V7E	GTG→GAG	53
Leu8→Gln	L8Q	CTG→CAG	57
Val14→Met	V14M	GTG→ATG	18
Gly16→Ser	G16S	GGC→AGC	58
Glu21→Lys	E21K	GAG→AAG	59
Glu21→Gly	E21G	GAG→GGG	60
Exon 2			
Gly37→Arg	G37R	GGA→AGA	7
Leu38→Val	L38V	CTG→GTG	7
Gly41→Ser	G41S	GGC→AGC	7
Glv41→Asp	G41D	GGC→GAC	7
His43→Arg	H43R	CAT→CGT	7
His46→Arg	H46R	CAT→CGT	61
His48→Gln	H480	CAT→CAG	62
Exon 3	NONE		02
Exon 4	NONE		
LAURA Mal	1.841	TTG \GTG	18 40
$Clu85 \rightarrow Var$	C95P		7
$Oiyo \rightarrow Aig$	DOOA		10.22
$Asp90 \rightarrow Ala$	D90A	GAC→GCC	19,22
Gly93→Ala	G93A	GGT→GCI	7
Gly93→Cys	G93C	GGT→IGT	/
Gly93→Arg	G93R	GGT→CGT	55
Gly93→Asp	G93D	GGT→GAT	63
Gly93→Ser	G93S	GGT→AGT	58
Gly93→Val	G93V	GGT→GTT	68
Glu100→Gly	E100G	GAA→GGA	7
Glu100→Lys	E100K	GAA→AAA	#
Asp101→Asn	D101N	GAT→AAT	51
Asp101→Gly	D101G	GAT→GGT	62
Ile104→Phe	I104F	ATC→TTC	47
Leu106→Val	L106V	CTC→GTC	7
Gly108→Val	G108V	GGA→GTA	68
Ile112→Thr	I112T	ATC→ACC	63
Ile113→Thr	I113	ATT→ACT	7
Arg115→Gly	R115G	CGC→GGC	64
Exon 5			
Asp124→Val	D124V	GAT→GTT	*
Asp125→His	D125H	GAC→CAC	62
Leu126→stop	L126Z	TTG→TAG	#
Ser134→Asn	S134N	AGT→AAT	58
Asn139→Lys	N139K	AAC→AAA	65
Leu144→Phe	L144F	TTG→TTC	8
Leu144→Ser	L144S	TTG→TCG	17
Ala145 \rightarrow Thr	A145T	GCT→ACT	17
Cvs146→Arg	C146R	TGT→CGT	58
Val148→>Glv	V148G	GTA→GGA	8
Val148→Ile	V148I	GTA→ATA	48
$Val140 \rightarrow Thc$	V 1401 11/0T		40 65
Deletion	11471	AIT	05
Lon	GAC T*T*CCC12	CAC CCC	66
(with stop order)	of Lys 130 22 as delates		00
(with stop couon a	a Lys130,23 aa deleted	1)	
De Los Char	C 10hn hefers area	5	17
+Pre-Leu-Glu $T \rightarrow G$. 10bp before exon 5			1/
+rne-rne-inr-Giy-Pro-stop. $A \rightarrow G$ 11bp before exon 5 (all exon 5 deleted)			tf
exon 5 delettu)			

*N. Laing et al., unpublished observation.

H-X. Deng and T. Siddique, unpublished observation.

OTHER FORMS OF FALS

FALS is a disorder with genetic heterogeneity (5,30). Both DFALS and RFALS have more than one loci in the genome. RFALS is rare and observed in relatively high prevalence in Tunisia (31). RFALS families of Pakistani origin and other ethnicities have also been identified (T. Siddique, unpublished data). The penetrance of RFALS is 100% by the age of 20. The mean age at onset of symptoms in RFALS is 12.03, ranging 3 to 23 years and the duration of the disease ranging from 15 to 20 years. RFALS is a juvenile form of ALS, but not the only juvenile form because symptoms can occur early in some patients with sporadic ALS or with DFALS (T. Siddique *et al.* unpublished data). In most cases, the symptoms of SALS and DFALS do not occur before the age of 10 years and rarely before the age of 20.

Three clinical variants of RFALS are known (31). The more common, RFALS type 1, is characterized by the relative predominance of lower motor neuron involvement early in the disease, accompanied by atrophy and weakness in hand and feet muscles. The tongue is affected in later stages and bulbar involvement is usually slowly progressive. The involvement of upper motor neurons is moderate in early stages and becomes more apparent as the disease progresses. In RFALS type 3, the symptoms of upper motor neuron degeneration predominate. Patients with RFALS type 3 show spasticity of limb and facial muscles, spastic and slurred speech usually leading to anarthria and uncontrolled weeping and laughter. Lower motor neuron involvement occurs later in the disease and affects both hands and feet. In RFALS type 2, symptoms are confined to the lower limbs which is most likely a form of autosomal recessive familial spastic paraplegia.

A large inbred Tunisian family with RFALS type 3 (genetic nomenclature symbol ALS2) has been linked to chromosome 2q33–35 (30). The ALS2 locus was subsequently refined to 2q33 (A.Hentati, H-X. Deng and T. Siddique, unpublished observation). The genetic analysis of families with RFALS type 1 did not show linkage to markers on either chromosome 2q33 or chromosome 21q, demonstrating genetic locus heterogeneity in RFALS (30). The locus for RFALS type 1 (ALS4) has not been identified.

Some cases of ALS with dementia can also be familial; several pedigrees with dominant inheritance have been described (2). We studied three multigenerational families in which either frontal lobe dementia or ALS was prominent or coexisted in the same individual. In three individuals in one family, we observed pathological changes that were identical to those seen in ALS. In addition a spongiform change with neuronal loss and gliosis in the superficial layer of the cortex was also noted. Positive LOD scores (logarithm of the odds of linkage) in the range of 1 were obtained with chromosome 17q markers, but linkage could not be established (T. Siddique *et al.*, unpublished observation).

Disinhibition–dementia–Parkinsonism–amyotrophy complex (DDPAC) is a condition characterized by association frontal lobe dementia, Parkinsonism and amyotrophy (32). The variability of clinical symptoms between patients is a striking feature of DDPAC. The most common features are disinhibition and frontal lobe dementia. Amyotrophy is absent in some patients but is the most prominent clinical feature in other patients. The pathological hallmark of DDPAC is spongiform change and neuronal loss with gliosis affecting mostly the superficial layer of the brain. There is also a marked loss of motor neurons in the spinal cord. The gene locus for DDPAC is on chromosome 17q21–22 (32).

TRANSGENIC MOUSE MODEL FOR FALS

Three transgenic mouse lines were initially made immediately after SOD1 mutations in FALS were identified: two lines expressing mutant SOD1 (A4V and G93A) and the other line expressing wild-type SOD1(33). The mice overexpressing G93A mutation developed symptoms similar to human ALS at 3-4 months of age, while mice overexpressing wild-type human SOD1 at a comparable age were not affected (33). Similar results have been reported in transgenic mice overexpressing the human SOD1 gene with G37R mutation and mouse SOD1 gene with G86R mutation that corresponds to G85R mutation in humans (34,35). A clear dosage effect of the mutant gene has been observed in the mice where the higher the copy number of the mutant gene, the earlier the onset of the disease (33,35). The two lines of mice with low copy number of the A4V mutation, the most frequent mutation identified in FALS, developed disease phenotype >700 days of age (T. Siddique et al., unpublished observation), this is at least 200-500 days later than the mice with higher expression of mutant SOD1.

The observations from the various lines of mice expressing different mutations suggest that it is unlikely that disease phenotype is caused by a position effect of the integration of the mutant SOD1 gene (36). Rather, it is the toxic effect of mutant SOD1 on cells leading to motor neuron death. This mechanism may also underlie FALS in humans. One major question regarding these transgenic mice with extraordinary overexpression of mutant SOD1 is whether these mice are ideal models for FALS in humans? This question arises due to the following differences between transgenic mice and humans. (i) Genetic background-FALS patients have one copy for each of the normal and mutant SOD1 genes, while the mice have two copies of endogenous SOD1 and several copies of mutant SOD1. The copy number or expression level significantly affects the phenotype of the mice (33,35). (ii) Pathology-it has been observed that mice with different mutations and varying levels of expression have different pathology from each other and also different from that of FALS patients (35,37,38). (iii) Age at onset-the disease phenotype in high copy number transgenic mice is about 3-4 months of age which is earlier than that of FALS patients (33-35). Additionally, pathological changes have also been identified in the nervous system of the transgenic mice overexpressing wild-type SOD1 (37-39). However, the pathology in the mice with lower-overexpression of mutant SOD1 is more similar to that in ALS patients (37,38). Thus, to date this mouse model is the most proximate to human ALS.

POSSIBLE MOLECULAR MECHANISMS FOR ALS

The theory of mechanisms by which mutant SOD causes motor neuron degeneration has focused on the exaggerated or disturbed known function of SOD1. The known functions of SOD1 are: (i) dismutase; (ii) peroxidase; and (iii) possibly metal binding.

Loss of dismutase function

SOD1 is an antioxidant defense which catalyzes the conversion of superoxide free radical anion (O_2^{-}) to hydrogen peroxide (H_2O_2) and oxygen (O_2) . However, the biological effects of the conversion of O_2^{-} to H_2O_2 by SOD1 on the viability of the cells may be double-edged. Hydrogen peroxide and its derivatives are toxic to the cell. Mapping of the mutation sites to the crystallographic structure of wild-type SOD1 predicted that these mutations destabilize the protein structure, leading to a less active enzyme rather than to a completely inactive or a more active SOD1 in FALS (8). This prediction has been confirmed by enzymatic assay of SOD1 activity in red blood cells, lymphoblastoid cells and brain tissues from FALS patients (8,20,21). These observations excluded the possibility that a more active SOD1 is involved in FALS and supported the hypothesis that the decrease of SOD1 activity, therefore an increase of superoxide free radical may be responsible for the disease. This hypothesis is supported by in vitro study; reduction of SOD1 activity by chronic application of SOD1 antisense oligonucleotides triggers neuronal death. This death process can be averted by agents which enhance intracellular antioxidant defense (25). However, this hypothesis is not defensible because of the following two reasons: (i) although most mutations in SOD1 gene cause decrease in steady state of cytosolic SOD1 activity, there are at least two exceptions, G37R and D90A, which have no significant decrease in SOD1 activity; and (ii) transgenic mice overexpressing FALS-linked mutations in SOD1 gene on normal mouse background developed disease similar to ALS in humans, while those overexpressing normal SOD1 remained phenotypically unaffected (33-35). These observations from transgenic mouse models and SOD1 knockout mice strongly disagree with 'loss of function' but support a 'gain of toxic function' hypotheses (33–35,40). Firm conclusions from these two hypotheses are not possible yet, although 'gain of a toxic function' appears more likely.

SOD1 as a free radical generator

It is known that SOD1 has some peroxidase activity in addition to the major superoxide dismutase activity (13, 14). Our studies with lymphoblastoid cell lines have shown an increased detection of H₂O₂ in FALS but not in normal controls (S.Ahmed, W.Y.Hung and T. Siddique, unpublished results). It has also been reported that FALS-associated mutant SOD1 enzyme catalyzes the reduction of H₂O₂, therefore acting as a peroxidase; this process occurs more rapidly with mutant than wild-type SOD1 (41). These results, although derived from in vitro studies, support the gain of a toxic function mechanism and indicate that increased peroxidase activity of mutant SOD1 may play a critical role in the pathogenesis of SOD1-linked FALS. Recently, screening of potential therapeutics in our transgenic mouse model expressing G93A mutation indicated that dietary supplementation with vitamin E delayed onset of clinical disease and slowed progression, although did not prolong survival (42). This supports the hypothesis that the pathogenesis of SOD1-related FALS may be due to oxidative damage. The target of such damage would mainly be the lipids of the membranes of the cells.

SOD1 complexes

As most SOD1 mutations destabilize SOD1 protein (8), except for the D90A mutation which has only local interactions in the protein (19), it is possible that the mutant protein with altered conformation precipitate to form aggregates in motor neurons which, in turn, could disturb normal cell function and lead to cell death. These inclusions may be easily formed when SOD1 protein stability is decreased due to the fact that this protein exists in large amounts accounting for 0.5–1% of total cytosolic protein in neurons. Lewy body-like inclusions have been documented in one of our FALS families with an A4V mutation. These inclusions are positive with antibody against SOD1 (18,43).

'Rim' distortion hypothesis

The crystallographic structural mapping study predicts that mutations will lead to structural change of mutant SOD1 (8). If these changes distort the electrostatic guidance channel and allow the catalytic site to become exposed and more shallow, molecules normally excluded may gain the access to the catalytic reactive site. Beckman and colleagues proposed that mutant SOD1 may react with peroxynitrite (ONOO⁻) formed from superoxide and nitric oxide and facilitate nitration of tyrosine residues of critical cytosolic proteins thus injuring cells (44). This access would be in keeping with the model of a promiscuous accessibility of the Cu site. Our preliminary studies on protein structure using antibodies against SOD1 suggest that a common conformational change occurs in the mutants A4V, G37R and H46R that may affect the rim of the electrostatic guidance channel coded by exon 3. The 'rim' distortion hypothesis is also favored by our newly identified truncation mutations. These mutations result in a truncated SOD1 by deleting exon 5 encoded polypeptide which constitutes a part of the rim of the electrostatic channel (H-X. Deng and T. Siddique, unpublished results).

Mutant SOD1 possibly exhibit metal mediated cytotoxicities by disrupting the intracellular homeostasis of Cu and Zn. It is known that Cu and Zn are potential neurotoxins (45,46).

The present hypotheses for the pathogenesis of FALS are focused on free radical damage to motor neurons. Although many questions remain unanswered, elucidation of the pathogenesis of FALS may benefit patients with other forms of ALS or other neurodegenerative disorders that share similar mechanisms of disease.

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