Identification of Six Novel SOD1 Gene Mutations in Familial Amyotrophic Lateral Sclerosis

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ABSTRACT: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the premature death of motor neurons. In approximately 10% of the cases the disease is inherited as autosomal dominant trait (FALS). It has been found that mutations in the Cu/Zn superoxide dismutase gene (SOD1) are responsible for approximately 15% of FALS kindreds. We screened affected individuals from 70 unrelated FALS kindreds and identified 10 mutations, 6 of which are novel. Surprisingly, we have found a mutation in exon 3, which includes most of the active site loop and Zn^{2+} binding sites, a region where no previous SOD1 mutations have been found. Our data increase the number of different SOD1 mutations causing FALS to 55, a significant fraction of the 154 amino acids of this relatively small protein.

RÉSUMÉ: Identification de six nouvelles mutations dans le gène SOD1 de patients atteints de la Sclérose Laterale Amyotrophique. La Sclérose Laterale Amyotrophique (SLA) est une maladie neurodégénérative caractérisée par la mort prématurée des motoneurones. Dans 10% des cas, la SLA est hérédiataire et caractérisée par une transmission autosomique dominante (SLAF). Il a été prouvé que des mutations dans le gène codant pour la Cu/Zn superoxyde dismutase (SOD1) sont responsables de 15% des cas de SLAF. L'analyse des régions condantes du gène SOD1 de 70 patients non-apparentés atteints de SLAF nous a permis de découvrir 10 mutations dont six sont nouvelles. Fait inatendu, nous avons trouvé la première mutation dans l'exon 3 qui code pour la majeure partie du site actif et des sites de fixation du Zn^{2+}. De plus, nous avons identifé une deuxième mutation récessive. Cette étude porte le nombre de mutations SOD1 différentes à 55 impliquant 37 codons, une fraction significative de cette protéine relativement petite constituée de 154 acides aminés.

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder characterized by progressive degeneration of large motor neurons in the motor cortex, brainstem and spinal cord. The mean age at onset of the disease is 45 years with a mean survival of 3 years.[1,2] ALS occurs in two clinically indistinguishable forms, sporadic (SALS; 90%) and familial (FALS; 10%). FALS is usually inherited as autosomal dominant trait[3] though a few kindreds show autosomal recessive inheritance.[4,5]

Approximately 15% of all dominant FALS are caused by a defect in the cytosolic Cu/Zn superoxide dismutase gene (SOD1) localized to human chromosome 21q22.1.[6,7] To date forty-five different missense mutations affecting 34 codons, one nonsense mutation, one deletion mutation causing a frameshift and two intronic mutation in intron 4 have been described in the SOD1 gene.[8] All the SOD1 mutations are dominant except for the Asp90Ala mutation which is thought to be recessive.[5,9] In order to study the effect of these mutations and to gain insight into the mechanisms leading to motor neuron degeneration animal models of FALS have been generated using mutant SOD1 transgenes.[10,12]

We have screened genomic DNA from 70 unrelated patients

for mutations in the SOD1 gene by PCR-SSCP. Here, we report six new and unpublished SOD1 gene mutations found in six unrelated patients.

PATIENTS AND METHODS

Patients

Blood was collected from 70 unrelated patients with FALS. These FALS kindreds were of Canadian and French origins. The El

Escorial diagnostic criteria were used.[13] Genomic DNA was prepared from blood using standard procedures.

Primer design, SSCP analysis and direct sequencing

To improve the PCR amplification of SOD1 gene exons for SSCP screening, we designed new intronic primers for exons 1 to 3 (Table 1). PCR amplification of exons 4 and 5 was performed using primers as previously described.[14]

The 50 µl amplification reaction contained 100 ng of genomic DNA, 1X PCR buffer (Promega), 100 ng of each primer, 50 µM of dCTP, 50 µM of dGTP, 50 µM of dTTP, 25 µM of dATP, 12.5 µM of (α-^{35}S)-dATP (1,000 mCi/mmol, Amersham) and 1.5 units of *Taq* polymerase (Promega). All SOD1 gene exons were amplified using thirty cycles of amplification: 30 s at 94°C, 30 s at 59°C (62°C for exon 2) and 45 s at 72°C followed by a 5-min extension at 72°C.

The single-strand conformational polymorphism (SSCP) analysis[15] was performed as described by Michaud et al.[16] except for using an acrylamide concentration of 8% in the gels. In order to maximize the mutation detection, we also used the 0.5X Hydrolink MDE gel supplemented with or without 5% glycerol. Electrophoretic migration at 6V and autoradiology were performed for 16 and 18 hours, respectively. Any difference in migration between patient and control samples was noted as positive. To confirm the SSCP results, symmetric direct sequencing method[17,18] using modified T7 polymerase (*sequenase*, Amersham) was used as described by Brody et al.[19] All exons were sequenced at least once on each strand.

RESULTS

In our hands the new oligonucleotide primers designed to amplify exons 1-3 (Table 1) gave clearer and more intense bands on SSCP than those described in reference 14. Genomic DNA from 70 unrelated FALS affected patients and from 26 normal controls were screened for mutations in SOD1 gene in all 5 exons using primers from the flanking intron sequences. PCR-SSCP analysis of all DNA samples showed variant bands in 10 unrelated families (14.3%). We had previously screened 300 controls for mutations in these exons; none were found.

Direct sequencing of PCR-amplified DNA from the 10 FALS individuals with SSCP variants confirmed the presence of mutations in all cases. We identified 6 new mutations of which 5 were missense nucleotide substitutions and one 3 bp deletion in intron 4 (Table 2). Two other known mutations were found in four patients (Asp90Ala and Ile113Thr).[5,20] All affected patients with the SOD1 mutations are heterozygotes except for Leu84Phe and Asp90Ala amino acid substitutions where patients were homozygous. The 3 bp (CTT) deletion detected in intron 4 is 30 bp downstream of exon-intron splice junction.

DISCUSSION

All 5 exons of the SOD1 gene from 70 unrelated FALS affected patients were screened for mutations using SSCP. Using our SSCP protocol, which involves testing of DNA fragments smaller than 300 bp using many different gel conditions, we expect to have

Table 1: Amplified DNA length, annealing temperature and oligonucleotide primer sequences for PCR amplification of Human SOD1 gene exons.

Exon	DNA length (bp)	Annealing temperature (°C)	Oligonucleotide primers	
			Forward	Reverse
1	209	55	5' CGTCTGGGGTTTCCGT 3'	5' CGTCCATGCAAAGGGT 3'
2	260	62	5' GTCTGGCTGCTTTTTACTTCA 3'	5' GGGGCTACTCTACTGTTTACT 3'
3	342	59	5' CTTGTTTCTGTTCCCTTCT 3'	5' GGGAAACACGGAATTATCT 3'
4	242	59	5' CATCAGCCCTAATCCATCTGA 3'	5' TGGATCTTAGAATTCGCGAC 3'
5	249	59	5' GTAGTGATTACTTGACAGCCCA 3'	5' AACAGATGAGTTAAGGGGCCT 3'

Table 2: Novel SOD1 mutations in familial amyotrophic lateral sclerosis.

	Codon changes	Restriction site changes	Amino acid substitution	Structural location[a]	Mammalian amino acid identity[b]
Exon 2	GAG6 GGG	–Taq I	Glu 21 Gly	β strand	8/8
	CTG6 CGG		Leu 38 Arg	Greek key	8/8
	GAG6 AAG	–BspH I –Nla III	Glu 49 Lys	Cu²⁺ binding site or dimer contact	3/8
Exon 3	CTA6 CGA	+Taq I, +Mbo I +Sau3A I	Leu 67 Arg	Active site loop	6/8
Exon 4	TTG6 TTC		Leu 84 Phe	Zinc binding site	8/8
Intron 4	AAAACTTCTTCTAA6 AAAACTTCTAA				

[a] Structural location is predicted as reported by H.X. Deng et al.[21]
[b] Frequency in which amino acid is conserved in 8 different mammals.
+ and - indicate a gain and loss of the corresponding restriction site.

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consensus    A T - K A V C V L K G D G P V Q G T I H F E Q K A S G - P V V V K G S I T G L T E G L H G F H V H E   48

human        A T - K A V C V L K G D G P V Q G I I N F E Q K E S N G P V K V W G S I K G L T E G L H G F H V H E   49
rabbit       A T - K A V C V L K G D G P V E A T I H F E Q K G T G - P V V V K G R I T G L T E G L H E F H V H Q   48
horse        A L - K A V C V L K G D G P V H G V I H F E Q Q Q E G G P V V L K G F I E G L T K G D H G F H V H E   49
pig          A T - K A V C V L K G D G P V Q G I I H F E Q K A N G - P V V V K G R I T G L V E G K H G F H V H E   48
cow          A T - K A V C V L K G D G P V Q G T I H F E A K - - G D T V V V T G S I T G L T E G D H G F H V H Q   47
sheep        A T - K A V C V L K G D G P V Q G T I R F E A K - - G D K V V V T G S I T G L T E G D H G F H V H Q   47
mouse        A M - K A V C V L K G D G P V Q G T I H F E Q K F E Q Q Q E G P V V L S G Q I T G L T E G Q H G F H V H Q   49
rat          A M - K A V C V L K G D G P V Q G V I H F E Q K A S G E P V V V S G Q I T G L T E G E H G F H V H Q   49

chicken      A T L K A V C V M K G D G P V E G V I H F Q Q Q G S G - P V K V T G K I T G L S D G D H G F H V H E   49
swordfish    - V L K A V C V L R G A G E T T G T V Y F E Q E G H A N A V G K G I I L K G L T P G E H G F H V H G   49
shark        - - - K A V C V L K G T G E V T G T V L F E Q A A D G - P V T L K G S I T G L T P G K H G F H V H A   46
fruit fly    - V V K A V C V I N G D - - V K G T V H F E Q Q D A K S P V L V T G E V N G L A K G L H G F H V H E   47
D. Simulans  - V V K A V C V I N G D - - A K G T V F F E Q E S S G T P V K V S G E V C G L A K G L H G F H V H E   47
xenopus      - - V K A V C V L A G S G D V K G V V H F E Q Q D E G - A V S V E G K I E G L T D G L H G F H I H V   47
haemonchus   S N - R A V A V L R G D P G V T G T V W F S Q D K E S D P C V I K G E I K G L T P G L H G F H V H Q   49


consensus    F G D N T N G C T S A G P H F N P L S K K H G G P K D E E R H V G D L G N V T A G K D G V A D V S I   98

human        F G D N T A G C T S A G P H F N P L S R K H G G P K D E E R H V G D L G N V T A D K D G V A D V S I   99
rabbit       F G D N R Q G C T S A G P H F N P L S K K H G G P K D E E R H V G D L G N V T A G S N G V A D V L I   98
horse        F G D N T Q G C T T A G A H F N P L S K K H G G P K D E E R H V G D L G N V T A D E N G K A D V D M   99
pig          F G D N T Q G C T S A G P H F N P L S K K H G G P Q D E E R H V G D L G N V T A G A D G V A N V S I   98
cow          F G D N T Q G C T S A G P H F N P L S K K H G G P K D E E R H V G D L G N V T A D K N G V A I V D I   97
sheep        F G D N T Q G C T S A G P H F N P L S K K H G G P K D E E R H V G D L G N V K A D K N G V A I V D I   97
mouse        Y G D N T Q G C T S A G P H F N P H S K K H G G P A D E E R H V G D L G N V T A G K D G V A N V S I   99
rat          Y G D N T Q G C T T A G P H F N P H S K K H G G P A D E E R H V G D L G N V A A G K D G V A N V S I   99

chicken      F G D N T N G C T S A G A H F N P E G K Q H G G P K D A D R H V G D L G N V T A - K G G V A E V E I   98
swordfish    F G D N T N G C I S A G P H F N P A S K K H A G P K D E D R H V G D L G N V T A D A N G V A K I D I   99
shark        F G D N T N G C I S A G P H Y N P F S K N H G G P D D E E R H V G D L G N V E A N G N G V A E F E I   96
fruit fly    F G D N T N G C T S A G P H F N P Y G N S H G A P S D L N R H L G D L G N I E A S G D G A T K V E I   97
D. Simulans  F G D N T N G C M S S G P H F N P Y G K E H G A P V D E N R H L G D L G N I E A T G D C P T K V N I   97
xenopus      F G D N T N G C M S A G P H F N P E N K N H G A P G D T D R H V G D L G N V T A - E G G V A Q F K I   96
haemonchus   Y G D S T N G C T S A G P H F N P F N K T H G G P K D D V R H V G D L G N V E A G A D G V A H F E I   99


consensus    E D S V I S L S G E H S I I G R T M V V H E K A D D L G K G G - - - N E E S T K T G N A G S R L A C   145

human        E D S V I S L S G D H C I I G R T L V V H E K A D D L G K G G - - - N E E S T K T G N A G S R L A C   146
rabbit       E D S V I S L S G D M S V I G R T L V V H E K E D D L G K G G - - - N D E S T K T G N A G S R L A C   145
horse        K D S V I S L S G K H S I I G R T M V V H E K Q D D L G K G G - - - N E E S T K T G N A G S R L A C   146
pig          E D S L I S L S G A N S I I G R T M V V H E K P D D L G K G G - - - N E E S T K T G N A G S R L A C   145
cow          V D P L I S L S G E Y S I I G R T M V V H E K P D D L G R G G - - - N E E S T K T G N A G S R L A C   144
sheep        V D P L I S L S G E Y S I I G R T M V V H E K P D D L G R G G - - - N E E S T K T G N A G G R L A C   144
mouse        E D R V I S L S G E H S I I G R T M V V H E K Q D D L G K G G - - - N E E S T K T G N A G S R L A C   146
rat          E D R V I S L S G E H S I I G R T M V V H E K Q D D L G K G G - - - N E E S T K T G N A G S R L A C   146

chicken      E D S V I S L T G P H C I I G R T M V V H A K S D D L G R G G - - - D N E S K L T G N A G P R L A C   145
swordfish    T D K - I S L T G P Y S I I G R T M V I H E K A D D L G R G G - - - N E E S L K T G N A G S R L A C   145
shark        K D R Q L H L S G E R S I I G R T L V V H E K E D D L G K G G - - - D E E S L R T G N A G S R L A C   143
fruit fly    S D K L I T L F G E N S I V G R T I V V H A D P D D L G K G G - - - H E L S K T T G N A G A R L G C   144
D. Simulans  T D S K I T L F G A D S I I G R T V V V H A D A D D L G Q G G - - - H E L S K S T G N A G A R I G C   144
xenopus      T D S L I S L K G P N S I I G R T A V V H E K A D D L G K G G - - - N D E S L K T G N A G G R L A C   143
haemonchus   K D H L V K I H G E H T V V G R S L V V H A G T D D L G K G V G E K K E E S L K T G N R G A R V A C   149


consensus    G V I G I A - K -   152

human        G V I G I A - P     153
rabbit       G V I G I S - P     152
horse        G V I G I A - Q     153
pig          G V I G I A - Q     152
cow          G V I G I A - K     151
sheep        G V I G I A - P     151
mouse        G V I G I A - Q     153
rat          G V I G I A - Q     153

chicken      G V I G I A - K C   153
swordfish    G V I G T E         151
shark        G V I G I A - K D   151
fruit fly    G V I G I C - K I   152
D. Simulans  G V I G I A - K V   152
xenopus      G V I G Y S - P     150
haemonchus   G V I A T A A P Q   158
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Figure: *Amino acid sequence comparison of the SOD1 protein in 15 different species.*

All SOD1 protein sequences were obtained from GenBank. The protein sequence comparison was performed using DNASTAR package software (DNASTAR Inc., Wisconsin). The amino acids representing the novel mutations reported in this article are boxed. Hyphens indicate amino acid deletions.

detected over 90% of all mutations, suggesting that few, if any, were missed in our screen. Therefore, 14.3% of our FALS families showed a defect in the SOD1 gene, confirming the relatively low prevalence of SOD1 mutations found in previous studies.[8,22]

We found three new mutations in exon 2 replacing Glu 21 by Gly, Leu 38 by Arg and Glu 49 by Lys. At the position 21, a Glu21Lys mutation was previously reported in one sporadic case of ALS.[23] The SOD1 protein of all compared species in the Figure contain the Glu 21, except chicken and nematodes, showing that this amino acid is highly conserved. Based on the bovine and yeast crystallographic SOD1 protein structure[21] Glu 21 is part of a β strand. Thus the Glu21Gly mutation may destabilize the SOD1 structure. As predicted for Leu38Val mutation,[21] Leu38Arg would be expected to destabilize protein folding by altering a Greek Key. The bovine and yeast crystallographic SOD1 protein structure[21] show that Glu 49 is involved in dimer contact or Cu^{2+} binding. While the amino acid Glu 49 is not highly conserved between species, this substitution constitutes a significant amino acid change from a negatively charged (Glu) to a positively charged (Lys) amino acid.

In exon 4 we found another novel mutation, Leu84Phe, and two previously published mutations Asp90Ala and Ile113Thr in six unrelated families. One of our families carrying an Asp90Ala mutation contains two affected patients and two normal individuals who are homozygotes and heterozygotes, respectively for the mutation (B. Moulard and Y. Boukaftane, in preparation). This finding supports a recessive inheritance mechanism for the Asp90Ala mutation.[5,9] The Leu 84 amino acid is conserved in all known mammalian SOD1 genes (Figure), and it is a part of fourteen conserved amino acids region which would have an important role in SOD1 function. Surprisingly, the woman patient carrying the Leu84Phe mutation is homozygote. She died at the age of 43 years, 3 years after the onset of the disease. She has a 48-year-old normal sister found also to be homozygous for the same mutation.

We also found a deletion of three nucleotides 30 bp downstream of the exon-intron splice junction in intron 4. The consequence of this mutation is unknown. The patient and his parents have died and no cell line is available to test for splicing errors or other biological effects. However, the mutation is not seen in 600 control chromosomes.

We found one kindred showing a missense mutation (CTA to CGA; Leu67Arg) in exon 3 which encodes the Zn-binding loop of the active site. This mutation replaces a predicted Leu at position 67 by an Arg, introducing a voluminous amino acid with a positive charge, which constitutes a major structural change. The Zn^{2+} ion is known to be a potential neurotoxin.[24-26] Therefore, the alteration of SOD1 role in the binding of Zn^{2+} may affect its homeostasis leading to neurodegeneration. We have found two different mutations involving the Zn^{2+} binding site (Leu67Arg and Leu84Phe), in two unrelated FALS patients suggesting a possible role of Zn^{2+} ions in the development of ALS.

This study increases the number of known mutations causing FALS to 55 involving 37 different codons. The fact that we have found 5 new missense mutations suggest there are other as yet undiscovered SOD1 mutations in FALS. This represents a surprisingly large number of gain of function mutations for such a small protein.

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